

Pranlukast, a cysteinyl leukotriene receptor 1 antagonist, attenuates allergen-specific tumour necrosis factor alpha production and nuclear factor kappa B nuclear translocation in peripheral blood monocytes from atopic asthmatics

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Summary

Background The cysteinyl leukotriene receptor 1 (cysLTR1) antagonists are useful for oral treatment of bronchial asthma. The underlying mechanism of cysLTR1 antagonists on inhibition of inflammatory cytokine production is yet to be determined.

Objective The present study was designed to determine the effect of pranlukast, a cysLTR1 antagonist, on production of inflammatory cytokines by allergen-stimulated peripheral blood monocytes (PBM) from atopic asthmatics.

Methods PBM were obtained from normal control ($n = 10$) and *Dermatophagoides farinae* (Der f) allergen-sensitized atopic asthmatics ($n = 12$), and were cultured in the presence of Der f allergen. The production of TNF- α and nuclear-translocation of nuclear factor kappa B (NF- κ B) was determined. In atopic asthmatics, pranlukast, tacrolimus or dexamethasone was added before stimulation by Der f. The additive effect of pranlukast and dexamethasone was also determined.

Results PBM from atopic asthmatics cultured with Der f exhibited a significant increase in TNF- α production and nuclear translocation of NF- κ B compared with normal control ($P < 0.01$). Pranlukast, tacrolimus and dexamethasone significantly inhibited production of TNF- α and nuclear-translocation of NF- κ B in PBM of atopic asthmatics ($P < 0.01$). An additive effect of pranlukast on low-dose dexamethasone was also demonstrated. However, LTD₄ did not induce TNF- α production or NF- κ B nuclear translocation.

Conclusion Our results suggest that pranlukast may inhibit TNF- α production via suppression of NF- κ B activation through pathways distinct from cysLTR1 antagonism.

Keywords atopic asthma, leukotriene, NF- κ B, pranlukast, TNF- α

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Introduction

Bronchial asthma is characterized by reversible airflow limitation, airway hyper-responsiveness and chronic eosinophilic airway inflammation. Eosinophils, lymphocytes, mast cells and macrophage/monocytes play important roles in the pathophysiology of asthma [1, 2]; they are involved in airway inflammation via production of various molecules, such as cytokines, enzymes and adhesion molecules [1, 2]. Such molecules are regulated by transcription factors, including nuclear factor kappa B (NF- κ B) [3–7]. NF- κ B is a ubiquitously expressed transcription factor composed of homo- or heterodimers of DNA-binding proteins from the Rel family, which controls the transcription of genes encoding many inflammatory cytokines. In its inactive state, the NF- κ B dimer is present in the cytoplasm, where it is bound to an inhibitory protein, I- κ B. Activation of NF- κ B by several stimuli induces the release and degradation of I- κ B from the dimeric complex, followed by translocation of NF- κ B to the nucleus [8]. Recently, NF- κ B has

been shown to be involved in the pathophysiology of bronchial asthma. Two reports suggested that activation of NF- κ B is triggered by the low-affinity receptor for IgE (Fc ϵ RII, also called CD23) expressed in human monocytes [9, 10].

Because the primary purpose of asthma treatment includes inhibition of eosinophilic airway inflammation, inhaled corticosteroid is the first-choice therapeutic regimen in the National Asthma Education and Prevention Program [11] and updated Global Initiative for Asthma [12]. Recently, cysteinyl leukotriene receptor 1 (cysLTR1) antagonists [13–15], long-acting inhaled β_2 -agonists [16] and theophylline [17] have also become recommended regimens. Among these antiasthma drugs, steroids [18, 19] and cys-LTR1 antagonists [20, 21] inhibit production of various inflammatory cytokines. The anti-inflammatory mechanism of steroids and tacrolimus includes inhibition of NF- κ B activation [22, 23]. However, the underlying mechanism of cysLTR1 antagonists on inhibition of inflammatory cytokine production is yet to be determined.

We hypothesized that pranlukast, a cysLTR1 antagonist, inhibits production of inflammatory cytokines via regulation of NF- κ B. Peripheral blood monocytes (PBM) were obtained from *Dermatophagoides farinae* (Der f) allergen-sensitized

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atopic asthmatics. PBM were cultured in the presence of Der f allergen to determine the production of allergen-induced TNF- α and NF- κ B nuclear-translocation. In addition, the effects of dexamethasone, tacrolimus and pranlukast on these responses were determined.

Materials and methods

Subjects

Twelve mild or moderate atopic asthmatics (male : female 10 : 2, mean age 30.8 ± 3.2 years, $\pm$ SD) who visited the outpatient clinic of the Second Department of Internal Medicine, Nagasaki University School of Medicine, and 10 non-atopic healthy subjects (male : female 8 : 2, mean age 30.3 ± 3.9) were entered in this study. Bronchial asthma was diagnosed and the severity was evaluated according to criteria defined by updated Global Initiative for Asthma [12]. None of the patients had ever smoked or had other pulmonary or bronchial diseases for at least 2 weeks prior to the study. All asthmatics were treated regularly with inhaled steroid and/or a short-acting β_2 -agonist on demand, but none were treated with oral steroid, pranlukast, tacrolimus, long- or short-acting β_2 -agonist on a regular basis. One patient was taking sustained release theophylline. These drugs were stopped 24 h before the study. Serum total IgE (RIST) was measured by fluoroenzyme immunoassay (FEIA; CAP RIST FEIA, Pharmacia, Uppsala, Sweden) and the mean RIST of asthmatics was significantly higher than that of normal subjects (1109.3 ± 922.4 IU/mL vs. 90.1 ± 75.5 IU/mL, $P < 0.01$). Serum allergen-specific IgE (RAST) levels were measured using FEIA (CAP RAST FEIA, Pharmacia). The RAST score of Der f in asthmatic subjects was more than 4, and was 0 in control subjects. This study was approved by the Human Ethics Review Committee of Nagasaki University Hospital and informed consent was obtained from all patients.

Preparation of PBM

Heparinized venous blood, obtained from control and atopic asthmatics, was diluted with RPMI-1640 medium (Gibco-BRL Life Technologies, Inc., Grand Island, NY, USA)/10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μ g/mL streptomycin (hereafter referred to as cRPMI). Mononuclear cells were separated by Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. Cells were washed twice in cRPMI, placed in cell culture Petri dishes and incubated overnight at 37 °C in 5% CO₂. After removal of non-adherent cells by washing the dishes three times with warm RPMI-1640 medium, adherent cells were incubated with cold phosphate-buffered saline (PBS) on ice for 15 min. The adherent cells were subsequently recovered by vigorous pipetting, washed and resuspended in cRPMI at 10^6 cells/mL. The viability of adherent cell was assessed by the trypan blue exclusion test (more than 98% viable). Endotoxin in cRPMI was less than 50 pg/mL as assayed by the Toxicolor test (SPL Co., Tokyo, Japan). Surface CD14 expression was analysed in a flow cytometer (FACScan, Becton Dickinson, Franklin Lakes, NJ, USA) using phycoerythrin-conjugated anti-CD14 (BD PharMingen, San Diego, CA, USA). CD14 expression was detected on more than 90% of adherent cells.

Preparation of leukotriene D₄, pranlukast, tacrolimus and dexamethasone

Leukotriene (LT)D₄ (Sigma, St Louis, MO, USA) was dissolved in RPMI medium, pranlukast (ONO Pharmaceutical Co., Osaka, Japan) was dissolved in dimethylsulfoxide, tacrolimus (Fujisawa Pharmaceutical Co., Osaka, Japan) and dexamethasone (Sigma) were dissolved in 99% ethanol. Preliminary experiments showed that TNF- α production and translocation of NF- κ B were not stimulated by medium alone.

Cell culture

PBM cells (2×10^5 cells/200 μ L/well) were cultured with medium or Der f (1 μ g/mL; LSL Inc., Tokyo, Japan) in a 96-well plate for 6 h. PBM were also cultured with endotoxin lipopolysaccharide (LPS; 1 ng/mL) from *Escherichia coli* (Sigma) for 2 h. For the PBM from atopic asthma, pranlukast (10^{-5} , 10^{-6} or 10^{-7} M), tacrolimus (10^{-6} , 10^{-7} or 10^{-8} M) or dexamethasone (10^{-6} , 10^{-7} , 10^{-8} or 10^{-9} M) were added to other wells before the addition of Der f. In the present study, dexamethasone was considered to represent an anti-inflammatory drug for asthma and tacrolimus was a NF- κ B inhibitor [22, 23]. We selected the aforementioned concentrations based on the effective plasma concentrations in clinical dosage. To determine the impact of cysLT, LTD₄ (10^{-8} , 10^{-9} , 10^{-10} or 10^{-11} M) was also added to other wells before the addition of Der f. To determine the additive effects of pranlukast and dexamethasone, 10^{-6} M pranlukast was added to 10^{-7} , 10^{-8} or 10^{-9} M dexamethasone. Following culture, supernatants were collected and cells were resuspended in fresh RPMI medium. The supernatants were stored at -80 °C until measurement of TNF- α or LTC₄/D₄/E₄, and cells were used for immunohistochemistry and enzyme-linked immunosorbent assay (ELISA) for NF- κ B. Cell viability was measured by trypan blue staining before and after addition of drugs, and all cultures were found to be more than 98% viable. Thus, none of the drugs used in the present study exhibited cell toxicity.

Quantitative determination of TNF- α and cysLT

The cultured supernatants were subjected to quantitative determination of TNF- α using the sandwich ELISA method (Quantikine, R & D Systems, Minneapolis, MN, USA). Supernatants were also examined for cysLT (LTC₄/D₄/E₄) by the enzyme immunoassay (EIA) method (Amersham Pharmacia Biotech).

Immunocytochemistry for NF- κ B

Cycentrifuged preparations were fixed with cold acetone. Endogenous peroxidase activity was blocked by incubating slides in methanol containing 3% hydrogen peroxidase for 15 min, followed by washing in PBS. After blocking with 10% normal rabbit serum for 20 min, sections were incubated for 2 h at room temperature with a goat polyclonal anti-human NF- κ B p65 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at a dilution of 1 : 100 of a 200- μ g/mL solution. Following application of the primary antibody, we applied a biotinylated rabbit anti-goat antibody (Vector Laboratories, Inc., Burlingame, CA, USA) followed by the addition of peroxidase-conjugated avidin for 30 min at room temperature. Chromogen-fast diaminobenzidine with cobalt chloride and nickel chloride (metal DAB, Sigma) was applied for 5 min. Preparations were numbered, and they were examined by each

of three observers in a blinded fashion. Observed fields were randomly selected at low magnification; whether the nuclei were stained or not was unknown. At $\times 1000$ magnification, 500 cells were examined under an oil immersion lens. Cells with stained nuclei were regarded as positive. Data were expressed as the mean positive cell ratio (%) assessed by the three observers. Inter- and intraoperative variations were less than 10%.

NF- κ B subunit-specific ELISA

NF- κ B subunits RelA (p65) and NF- κ B1 (p50) were quantified using Trans-AMTM transcription factor assay kit (Active Motif, Carlsbad, CA, USA). The assay is essentially an ELISA in which the consensus NF- κ B binding site (5'-GGGACTTCC-3') is immobilized onto the 96-well plate. Nuclear cell extracts (3 μ g) were added to the wells and assayed for either RelA or p50 binding, using the protocol recommended by the manufacturer. Optical density was determined on a spectrophotometer at 450 nm. Western blot analysis or electrophoretic mobility shift assay has been used previously for assessment of translocation of NF- κ B; however, these methods are not primarily quantitative. NF- κ B subunit-specific ELISA is a simple method that does not require the use of radioisotope and yields quantitative results, as reported previously [24, 25].

Statistical analysis

Data are presented as mean $\pm$ SD. Differences between control and atopic asthmatics were compared using the Mann-Whitney test, and differences in the same patient were compared using the Wilcoxon signed rank test. A *P*-value less than 0.05 denoted the presence of a statistically significant difference.

Results

Production of TNF- α and activation of NF- κ B in PBM from asthmatics and normal controls

In control subjects, TNF- α production was comparable in Der f- and medium-stimulated PBM, whereas LPS stimulation

induced a significant increase in TNF- α production compared to medium. Baseline TNF- α production in asthmatic subjects was significantly higher than in control subjects, and both LPS and Der f stimulation resulted in a significant increase in TNF- α production compared to medium stimulation (Fig. 1).

Medium-stimulated PBM from atopic asthmatics exhibited a significant increase in positivity compared with PBM from control subjects. In control subjects, the ratio of positively stained cells following LPS, but not Der f, stimulation was significantly higher than that following medium stimulation. In contrast, in atopic asthmatics, the ratios of positively stained cells following both LPS and Der f stimulation were significantly higher than that following medium stimulation (Fig. 1). These results were compatible with those obtained using NF- κ B-specific ELISA (Table 1), and suggested that the baseline production of TNF- α and nuclear-translocation of NF- κ B in PBM from atopic asthmatics were significantly higher than the corresponding values in PBM from control subjects.

Effects of drugs on production of TNF- α and translocation of NF- κ B

In PBM from atopic asthmatics, pranlukast (as well as tacrolimus and dexamethasone) significantly inhibited Der f-induced TNF- α production in a dose-dependent manner (*P* < 0.01; Fig. 2). Pranlukast inhibited production of TNF- α at 10^{-5} and 10^{-6} M, but not at 10^{-7} M, whereas all concentrations of

Table 1. NF- κ B activity in peripheral blood monocytes of normal subjects and asthmatics

	Optical density	
	Normal control	Asthma patients
Medium	0.12 $\pm$ 0.06	0.28 $\pm$ 0.13†
LPS	0.45 $\pm$ 0.08*	0.60 $\pm$ 0.18*
Der f	0.14 $\pm$ 0.08	0.62 $\pm$ 0.21*†

Data are mean $\pm$ SD. **P* < 0.01 versus medium. †*P* < 0.01 versus control.

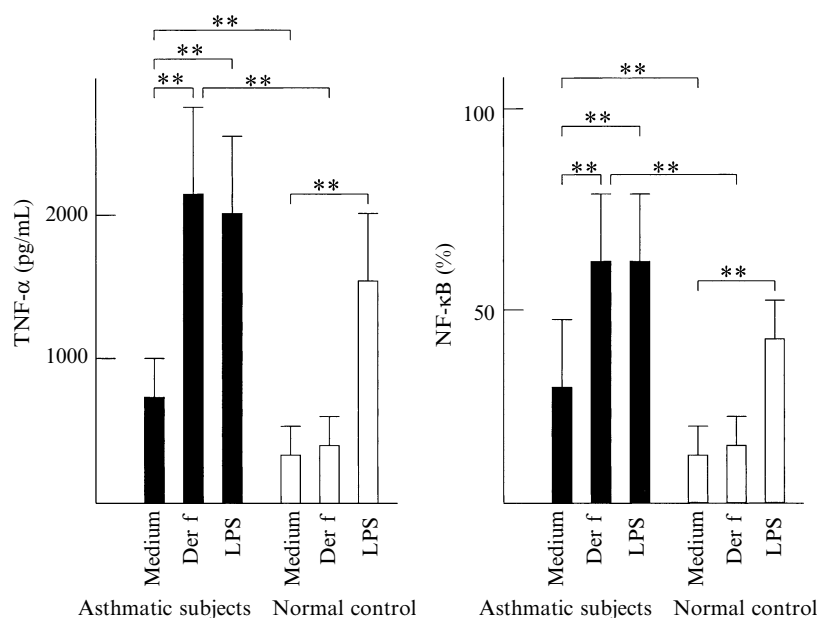


Fig. 1. Peripheral blood monocytes (PBM) were obtained from asthmatic subjects (■, *n* = 12) and normal control (□, *n* = 10), and were cultured in the absence (medium) or presence of *Dermatophagoides farinae* (Der f; 6 h) or lipopolysaccharide (LPS; 2 h). TNF- α production and NF- κ B translocation were determined by ELISA and immunocytochemistry, respectively. Data represent mean $\pm$ SD. ***P* < 0.01.

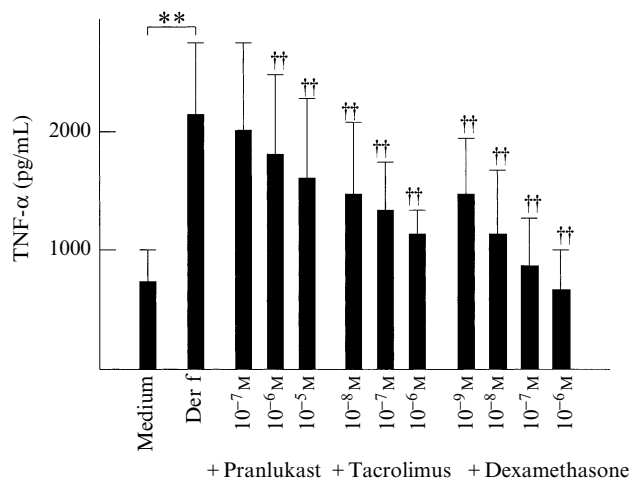


Fig. 2. Pranlukast, tacrolimus or dexamethasone was added to PBM from asthmatic subjects ($n = 12$) before 2-h stimulation with Der f allergen. TNF- α production was determined by ELISA. Data represent mean $\pm$ SD. ** $P < 0.01$ versus control, †† $P < 0.01$ versus Der f.

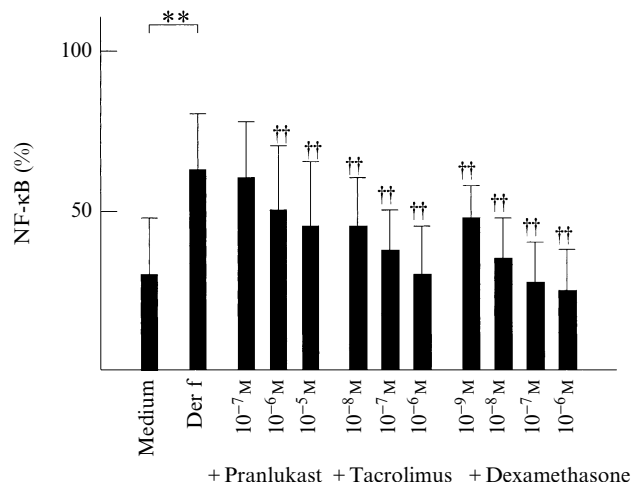


Fig. 3. Pranlukast, tacrolimus or dexamethasone was added to PBM from asthmatic subjects ($n = 12$) before 2-h stimulation with Der f allergen. NF- κ B nuclear-translocation was determined by immunocytochemistry. Data represent mean $\pm$ SD. ** $P < 0.01$ versus control, †† $P < 0.01$ versus Der f.

tacrolimus and dexamethasone used in the present study significantly inhibited TNF- α production. Pranlukast, tacrolimus and dexamethasone also significantly inhibited the Der f-induced nuclear-translocation of NF- κ B in a dose-dependent manner ($P < 0.01$; Fig. 3 and Table 2). In addition to TNF- α production, pranlukast (10^{-7} M) failed to inhibit Der f-induced nuclear-translocation of NF- κ B. Collectively, these results suggested that pranlukast inhibited allergen-specific TNF- α production via regulation of NF- κ B in a dose-dependent manner, and that this effect of pranlukast was less potent than that of tacrolimus and dexamethasone.

Der f-induced cysLT and CysLT-induced TNF- α production from PBM

It is possible that Der f allergen stimulates PBM to produce cysLT, which in turn stimulates PBM to produce TNF- α . Thus,

Table 2. Inhibition of NF- κ B activity in peripheral blood monocytes of asthmatics by various drugs

	Optical density
Medium	0.28 $\pm$ 0.14
Der f	0.62 $\pm$ 0.21*
Der f + pranlukast 10 ⁻⁵ M	0.45 $\pm$ 0.20†
Der f + pranlukast 10 ⁻⁶ M	0.50 $\pm$ 0.22†
Der f + pranlukast 10 ⁻⁷ M	0.59 $\pm$ 0.21
Der f + tacrolimus 10 ⁻⁶ M	0.32 $\pm$ 0.13†
Der f + tacrolimus 10 ⁻⁷ M	0.37 $\pm$ 0.14†
Der f + tacrolimus 10 ⁻⁸ M	0.43 $\pm$ 0.15†
Der f + dexamethasone 10 ⁻⁶ M	0.23 $\pm$ 0.12†
Der f + dexamethasone 10 ⁻⁷ M	0.30 $\pm$ 0.12†
Der f + dexamethasone 10 ⁻⁸ M	0.35 $\pm$ 0.10†
Der f + dexamethasone 10 ⁻⁹ M	0.44 $\pm$ 0.11†

Data are mean $\pm$ SD. * $P < 0.01$ versus medium. † $P < 0.01$ versus control.

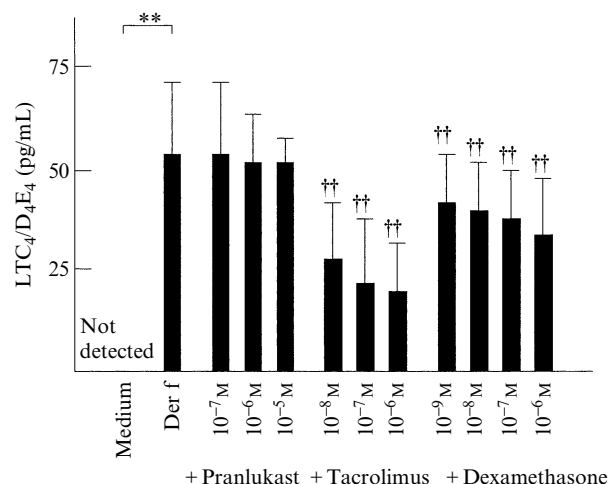


Fig. 4. Pranlukast, tacrolimus or dexamethasone was added to PBM from asthmatic subjects ($n = 12$) before 2 h stimulation with Der f allergen. The concentrations of LTC₄/D₄/E₄ in cultured supernatants were determined by EIA. Data represent mean $\pm$ SD. ** $P < 0.01$ versus control, †† $P < 0.01$ versus Der f.

pranlukast might inhibit TNF- α production via antagonism of Der f-induced cysLT production. To evaluate this hypothesis, Der f-induced cysLT production and cysLT-induced TNF- α production were determined.

CysLT levels in the supernatant after culture with Der f in atopic asthmatics were significantly higher than from supernatants after culture with medium alone ($P < 0.01$). Tacrolimus and dexamethasone significantly inhibited Der f-induced cysLT production in a dose-dependent manner, however, in contrast to TNF- α , tacrolimus showed a stronger effect than dexamethasone. Pranlukast failed to inhibit Der f-induced cysLT production (Fig. 4).

TNF- α production and NF- κ B nuclear-translocation were determined when PBM were stimulated by LTD₄ (10^{-8} , 10^{-9} , 10^{-10} , 10^{-11} , 10^{-12} , 10^{-13} and 10^{-14} M). However, these parameters did not change by stimulation with LTD₄ (data not shown). These results suggested that pranlukast inhibited allergen-induced TNF- α production via a pathway distinct from cysLTR1 antagonism.

Additive effect of pranlukast on dexamethasone

The additive effects of pranlukast and dexamethasone were examined in PBM from 12 atopic asthmatics. Then, 10^{-6} M of pranlukast, the lowest dose that significantly inhibited both TNF- α production and NF- κ B translocation, was combined with 10^{-7} , 10^{-8} or 10^{-9} M of dexamethasone. An additive effect of pranlukast on TNF- α production was observed at 10^{-8} and 10^{-9} M of dexamethasone (Fig. 5). However, the additional effect of pranlukast on NF- κ B translocation was observed only at 10^{-9} M of dexamethasone (Fig. 6 and Table 3). Thus, pranlukast, at the therapeutic concentration, exhibited an additive effect with low-dose dexamethasone treatment on TNF- α production and NF- κ B translocation.

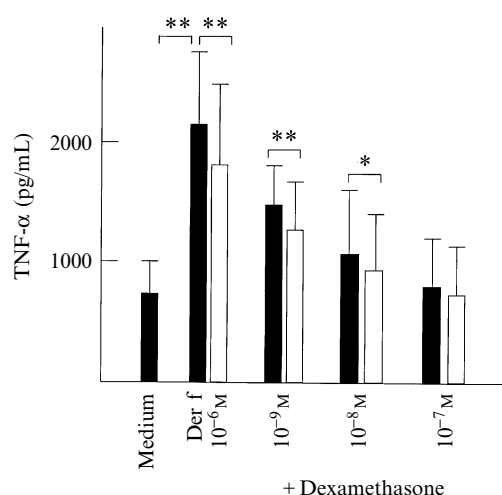


Fig. 5. TNF- α production from Der f stimulated PBM from asthmatic subjects ($n = 12$) was determined by ELISA in the presence of dexamethasone without (■) or with (□) 10^{-6} M of pranlukast. Data represent mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$.

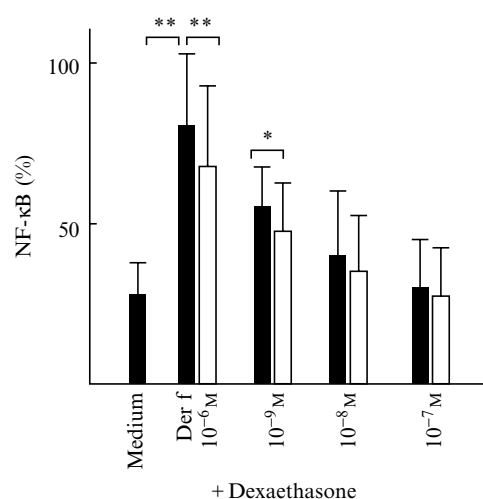


Fig. 6. NF- κ B nuclear translocation in Der f-stimulated PBM from asthmatic subjects ($n = 12$) was determined by immunocytochemistry in the presence of dexamethasone without (■) or with (□) 10^{-6} M of pranlukast. Data represent mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$.

Table 3. Combination effects of dexamethasone and pranlukast on NF- κ B activity

	Optical density	
	Without pranlukast	With pranlukast
Der f	0.58 $\pm$ 0.21	0.44 $\pm$ 0.20*
Der f + dexamethasone 10^{-7} M	0.28 $\pm$ 0.11†	0.27 $\pm$ 0.10
Der f + dexamethasone 10^{-8} M	0.34 $\pm$ 0.09†	0.32 $\pm$ 0.12
Der f + dexamethasone 10^{-9} M	0.42 $\pm$ 0.10†	0.36 $\pm$ 0.11*

Data are mean $\pm$ SD. * $P < 0.01$ versus without pranlukast. † $P < 0.01$ versus Der f.

Discussion

The major findings of the present study are as follows: (i) pranlukast, a cysLTR1 antagonist, inhibited allergen-induced TNF- α production by PBM from atopic asthmatics concomitant with downregulation of NF- κ B; (ii) the mechanism of these effects may be due to a pathway distinct from cysLTR1 antagonism; (iii) at therapeutic concentration, pranlukast exhibited an additive effect to dexamethasone on allergen-induced TNF- α production and NF- κ B activation.

Previous studies suggested that arachidonic acid metabolites, especially LTs, act as second messengers in TNF-induced NF- κ B activation, as TNF-induced NF- κ B activation is attenuated by LT synthesis inhibitors [26, 27]. To our knowledge, this study is the first to show the effect of a cysLTR1 antagonist, but not LT synthesis inhibitors, on activation of NF- κ B. Tacrolimus and dexamethasone are already known to inhibit translocation of NF- κ B [22, 23]. Pranlukast inhibited translocation of NF- κ B in the present study, however, the underlying mechanism remains unclear. Because LTD₄ *per se* failed to induce TNF- α production and translocation of NF- κ B, pranlukast might inhibit the NF- κ B activation by a mechanism other than cysLTR1 antagonism. Consistent with our present results, Menard et al. [28] reported that TNF was not induced in response to LT stimulation. They also reported that LT had a priming effect on LPS-induced cytokine production. LT might also have shown a priming effect on allergen-induced TNF- α production in the present study.

NF- κ B translocation in monocytes occurs through cross-linking of CD23 by IgE-allergen immunocomplexes. In a previous study, we demonstrated a significant increase in CD23 expression on PBM of atopic asthmatics compared with the control (unpublished data). Ten et al. [9, 10] demonstrated the process of CD23-dependent activation of NF- κ B, and indicated that pranlukast could act somewhere in this process. To date, it remains unclear whether these effects are specific for pranlukast or whether other cysLTR1 antagonists also have this effect. In this regard, both monterlukast and zafirlukast show similar trends, but the effects of pranlukast are much stronger than these cysLTR1 antagonists (our unpublished observations). Menard et al. [28] also failed to demonstrate inhibition of TNF production by verlukast, another cysLTR1 antagonist, suggesting that pranlukast might have a distinct effect, not shared by other cysLTR1 antagonists.

We used PBM in this study, although alveolar macrophages might be more suitable to examine the pathophysiology of

asthma. However, NF- κ B activation and TNF- α production are easily induced by non-specific stimuli, such as LPS. Therefore, stimulation and culture of alveolar macrophages with allergens must be performed in a clean environment. Bronchoalveolar lavage is the only method available for acquiring sufficient alveolar macrophages; however, NF- κ B activation and inflammatory cytokine production can be induced by the bronchoscopic procedure *per se*, which would inherently confound the experiment.

Clinically, a subset of patients does not respond to pranlukast [29, 30]. The present study included mild asthmatics. None of the subjects had ever used pranlukast and thus we do not know whether the present study included clinical non-responders to pranlukast. Although some investigators have reported involvement of the 5-lipoxygenase genotype [31], various clinical parameters or urine LT [32, 33], the mechanism of resistance to antileukotriene therapy in asthma remains unclear. Whether the present methods could predict clinical responders to pranlukast may be clearer when larger numbers of patients are investigated in the future.

Recently, cysLTR1 antagonists have been used for asthma treatment, usually in combination with inhaled corticosteroids [34–36]. The present study demonstrated an additive effect of pranlukast with low-dose, but not high-dose, dexamethasone, on NF- κ B activation and on TNF- α production. These findings are consistent with previous reports [37, 38], wherein pranlukast was effective in asthmatics who used only inhaled corticosteroids, but not in those taking oral corticosteroids. However, the additive response of pranlukast to dexamethasone on PBM was modest. The effect of pranlukast might be more significant in cell types with a greater capacity for cysLTs synthesis and cysLTR1 expression, such as mast cells and eosinophils.

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