

Leukotriene E₄-induced persistent eosinophilia and airway obstruction are reversed by zafirlukast in patients with asthma

Annika Laitinen, MD,^a Ari Lindqvist, MD,^b Maija Halme, MD,^b Alan Altraja, MD,^a and Lauri A. Laitinen, MD, PhD, FRCP^b Helsinki, Finland

Background: We have shown that inhalation of leukotriene (LT) E₄ contributes to specific recruitment of eosinophils to the airway mucosa in patients with asthma at the time of maximal decrease in airway-specific conductance.

Objective: We examined the ability of the cysteinyl LT₁ receptor antagonist, zafirlukast, to improve or prevent LT-mediated eosinophilia and airway obstruction in asthma.

Methods: Bronchial biopsies were taken and pulmonary function was measured before and 4 to 6 hours after the dose of inhaled LTE₄ causing a $\geq 15\%$ fall in FEV₁ at baseline both at week 0 and after 6-week randomly assigned treatment with a high dose of zafirlukast, 80 mg twice daily.

Results: Leukotriene E₄ inhalation at week 0 doubled the number of eosinophils in the airway mucosa in 21 of 25 patients with mild asthma, increased the numbers of neutrophils and lymphocytes, and decreased FEV₁ (-17%). Zafirlukast reduced both airway eosinophilia and obstruction in FEV₁, whereas with a double-blind placebo treatment, the effect of LTE₄ on both parameters persisted for 6 weeks. On repeat LTE₄ inhalation challenge after 6 weeks, zafirlukast treatment prevented further airway eosinophilia and decrease in FEV₁ seen in the placebo group.

Conclusion: Persistent LTE₄-induced airway eosinophilia may form the basis of an amplification mechanism for further eosinophil recruitment. Zafirlukast prevents LTE₄-induced eosinophilic airway inflammation in mild asthma. (J Allergy Clin Immunol 2005;115:259-65.)

Key words: Asthma, airways, biopsies, CysLT₁ receptor, LTE₄, inflammation, eosinophilia

The significance of cysteinyl leukotrienes (CysLTs) leukotriene (LT) C₄, LTD₄, and LTE₄ in the pathogenesis

Abbreviations used

CysLT: Cysteinyl leukotriene

5-LO: 5-Lipoxygenase

LT: Leukotriene

Mab: Mouse monoclonal antibodies

of asthma is widely recognized. Inhaled LTD₄ is a bronchoconstrictor 10,000 times more potent than histamine or methacholine.¹ In addition to potent bronchoconstrictive properties, CysLTs induce other pathophysiologic responses characteristic of asthma, including tissue edema, mucus secretion, and increased airway responsiveness to histamine.^{2,3} *In vivo*, CysLT levels are increased during spontaneous exacerbation of asthma⁴ and exposure to allergens.⁵

Direct evidence of the contribution of CysLTs to the inflammatory cell recruitment in asthma was obtained by showing that inhaled LTE₄, capable of inducing a significant airway obstruction (fall in specific airway conductance $>35\%$) elicited a significant increase in eosinophil numbers in the airway mucosa in bronchial biopsies, whereas a similar obstruction induced by methacholine was without a cellular effect.⁶

The discovery of the formation of leukotrienes from arachidonic acid by the 5-lipoxygenase (5-LO) pathway and the actions of the CysLTs through their receptors made targeted drug development conceivable. The currently available compounds either inhibit leukotriene synthesis or prevent the effects of CysLTs by blocking the CysLT receptors in the lung.

CysLT₁ receptor antagonists improve airway obstruction in patients with asthma^{7,8} and inhibit airway obstruction elicited by different triggers of asthma such as allergens⁹ and exercise.^{10,11} CysLT₁ receptor antagonists can attenuate cellular inflammation and blunt activation of inflammatory cells in response to allergen.¹² However, very few data are available on the direct anti-inflammatory or antiremodeling effects of leukotriene antagonists *in vivo* in human beings.¹³

To evaluate the therapeutic benefits of this type of asthma medication, we studied the effect of the leukotriene receptor antagonist zafirlukast on the inflammatory cell influx in the airways of patients with mild asthma in response to inhaled provocation with LTE₄ sufficient to cause a 15% decline in FEV₁.

From ^athe Institute of Biomedicine, Department of Anatomy, University of Helsinki; and ^bthe Clinical Research Unit of Pulmonary Medicine, Department of Medicine, Helsinki University Central Hospital.

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Reprint requests: Lauri A. Laitinen, MD, PhD, FRCP, Hospital District of Helsinki and Uusimaa, POB 100, FIN-00029 HUS, Finland. E-mail: lauri.laitinen@hus.fi.

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METHODS

Volunteers

Nonsmoking subjects age 18 to 60 years with mild, intermittent asthma (diagnosed according to the American Thoracic Society guidelines) who had used only an inhaled short-acting β_2 -agonist as needed and demonstrated reversibility to inhaled β_2 -agonist and a positive response to bronchial challenge with histamine during the past 12 months were recruited from 2 centers in Finland and Estonia.

Study design

The study was a randomized, double-blind, placebo-controlled, parallel-group comparison of the effects of zafirlukast with a dose of 80 mg twice daily and of placebo on LTE₄-induced airway inflammation and pulmonary function. The experimental dose of zafirlukast used was selected to provide maximal antagonistic effects and was above that licensed for regular use.

After a 1-week screening period, the volunteers were randomized to receive either zafirlukast or placebo for a period of 6 weeks. The visits were at screening (week -1), at randomization (week 0), and after 3 and 6 weeks (week 6) of treatment. The study complied with good clinical practice, the protocol was reviewed by the local ethics committee of the study centers, and written informed consent was obtained from each participant.

Assessments

A challenge with inhaled LTE₄ was performed.³ At screening, the PD₁₅ was determined for all eligible volunteers. For each volunteer, the same individual doses were then used in the subsequent LT challenges performed at week 0 before randomization and after the 6-week treatment. The repeat challenge was performed 2 ± 1 hours after the last dose of the test medication.

Bronchial biopsy specimens were obtained during fiber optic bronchoscopy in accordance with the international standard¹⁴ and following the procedures used formerly^{6,15,16} before and from 4 to 6 hours after the challenge with inhaled LTE₄ at week 0 and after the 6-week treatment. On every occasion, 4 to 5 biopsy specimens were taken from the right lobar bronchus level of each patient by using cupped forceps. Care was taken to obtain biopsies from areas not yet touched by the bronchoscope or forceps.

FEV₁ was measured before the first bronchoscopy on each study day, before and after the challenge with inhaled LTE₄, and on recovery from the second bronchoscopy. The use of β_2 -agonists was withheld for 4 hours before Vitalograph (Bucks, United Kingdom) spirometry.¹⁷

The biopsy specimens were processed for immunohistochemistry as previously described.¹⁶ To detect eosinophils with the mouse monoclonal antibodies (Mab) EG1 and EG2 (1:50; Kabi Pharmacia Diagnostics AB, Uppsala, Sweden), the frozen sections were processed by the avidin-biotin-peroxidase method. Mast cells, T lymphocytes, neutrophils, and macrophages were identified with the Mab against mast cell tryptase (AA1, 1:500), CD3 (1:1000), neutrophil elastase (NP57, 1:2000, ELA), and BerMac3 (1:50) from Dako A/S (Glostrup, Denmark), respectively, and visualized with the alkaline phosphatase antialkaline phosphatase technique.¹⁶ The sections were examined under a Leitz (Wetzlar, Germany) Dialux 22 Eblight microscope. Negative controls were provided by omitting the primary Mab. The density of the inflammatory cells in the lamina propria of the entire sections was computed as described previously.¹⁶ For each cell type, the complete sections of the biopsies were photographed on color slide film. The slides were projected onto a properly calibrated 42-in $\times$ 60-in Kurta IS/THREE digitizing table (Kurta Corp, Phoenix, Ariz) to reach the final magnification of $\times 695$. The section area with all specifically stained cells was transferred into a computer with the use of a pointing device. The cell densities were

computed by the AutoCAD program, version 10.1 (Autodesk, Sausalito, Calif). The specimens were inspected blindly by the same investigator (A. Laitinen), and only the specimens with a well-preserved architecture of the bronchial mucosa were included in the analysis.

Statistical analysis

Using an estimate of the variation in the eosinophil count (SD approximately 200 cells/mm²) from a previous study⁶ and assuming a 2-sided significance level $\alpha = 0.05$, a study population of 12 to 13 subjects per treatment group was sufficient to detect a difference of 320 cells/mm² between the zafirlukast and placebo groups with 90% power. The difference of 320 cells/mm², though large, seemed appropriate because of the invasive nature of the procedures performed.

The analysis of covariance with the change as a result of LTE₄ at week 0 as covariate was used to test the change in the cell counts and pulmonary function because of the challenge with inhaled LTE₄ after the 6-week treatment. Appropriate post hoc testing assessed whether the 6-week treatment per se with zafirlukast compared with the placebo treatment had a significant effect on the cells or FEV₁ before the final LTE₄ challenge. The changes in the cell counts and FEV₁ from the week 0 baseline to the challenges with inhaled LTE₄ and the following 6 weeks of treatment were calculated for both treatment groups. If a significant covariate by treatment interaction was discovered in the cell counts, the results were calculated for the lower quartile, the median, and the upper quartile levels of the covariate to show that the treatment effect differed according to the level of the prechallenge covariate at week 0. The least mean square estimates of the treatment difference as well as the 95% CIs and *P* values were calculated. Where appropriate, the correlation analysis between the changes in the cell counts and lung function (FEV₁) was performed according to the Pearson method.

RESULTS

The groups were comparable with respect to age, sex, and pulmonary function at randomization (Table I). There were no statistically significant differences between the atopic and nonatopic groups at week 0 baseline in the response to LTE₄ inhalation, measured either as the number of eosinophils per square millimeter in the bronchial biopsies or as FEV₁. The atopic status did not significantly influence the treatment effect of zafirlukast versus placebo in the current study. Two volunteers from the placebo group were withdrawn, one for asthma exacerbation and the other for protocol violation. In the zafirlukast group, 2 volunteers were excluded from the analyses because their LTE₄ challenges were performed more than 12 hours after the last dose of the study drug. Of the 21 volunteers who completed the trial according to the protocol, all 4 biopsies were obtained from 19 volunteers.

Effects of inhaled LTE₄ at baseline

The challenge with inhaled LTE₄ at least doubled the numbers of airway mucosal eosinophils in 21 of 25 volunteers: +111 cells/mm² (95% CI, 80, 143). Only the data on EG2⁺ cells are included in the results because the analysis of the EG1⁺ cells gave results similar to those of EG2⁺ throughout the study (Fig 1). LTE₄ induced a marked neutrophilia and marginally increased the total

TABLE I. Volunteer characteristics at entry

Volunteer identification	Sex	Age (y)	FEV ₁ % predicted	PD ₁₅ LTE ₄ (nmol)	Atopy
Zafirlukast					
1	M	42	97	26.2	N
2	M	30	71	2.9	Y
3	F	52	97	26.2	N
4	F	46	80	43.7	N
5	F	31	79	2.9	Y
6*	M	25	107	43.7	Y
7	M	58	77	0.1	Y
8	F	21	111	1.0	Y
9*	M	52	73	43.7	N
10	M	51	131	8.7	Y
11	F	29	103	2.9	N
12*	F	42	85	2.9	N
Mean (±SD)		40 (±12)	93 (±18)	17.1 (±18.4)	
Placebo					
1	F	41	74	8.7	N
2*	F	18	86	61.2	Y
3	M	54	84	1.0	N
4	F	22	75	2.9	Y
5*	F	60	97	26.2	N
6	F	35	110	26.2	N
7	F	38	105	43.7	Y
8*	M	38	113	8.7	Y
9	M	37	84	1.0	N
10	M	40	87	0.02	N
11	M	52	120	43.7	Y
12	F	53	113	2.9	Y
13	F	19	109	43.7	N
Mean (±SD)		39 (±14)	97 (±16)	20.8 (±21.2)	

*Volunteers with not all 4 biopsies available.

lymphocyte numbers across the pooled data set: neutrophils, +62 cells/mm² (36, 88) and T lymphocytes, +56 cells/mm² (6, 107). There were no changes in the mast cells or macrophages (Fig 1; Table II).

The prechallenge FEV₁ averaged 3.27 L and 3.24 L in the placebo and zafirlukast groups, respectively. The challenge with inhaled LTE₄ decreased FEV₁ in the pooled data set by −0.57 L (−0.78, −0.37) or by 17%, and the change was equal in both study groups ($P > .05$; Fig 2). The post-LTE₄ inhalation FEV₁ response did not differ in either group between the screening week and the study baseline week. A significant inverse relationship between the increase in the number of EG2⁺ eosinophils and the fall in FEV₁ after inhaled LTE₄ was observed (Pearson correlation coefficient, −0.49; $P = .01$).

Cell counts after treatment and repeat LTE₄ challenge

After the 6-week treatment and before the repeat challenge with inhaled LTE₄, the count of eosinophils in the placebo group did not return to the baseline level but remained 61 cells/mm² (16, 106) above it (Fig 2). The eosinophil count in the placebo group was further significantly increased by the repeat challenge, with inhaled LTE₄ reaching +100 cells/mm² (38, 162) above the week 0 levels.

In contrast, the respective eosinophil count in the zafirlukast group returned even below the baseline level by week 6 (−5 cells/mm²; −50, 40), and only an insignificant increase was seen by further LTE₄ inhalation (+9 cells/mm²; −51, 70; Fig 2).

The prechallenge eosinophil counts were not comparable in the 2 treatment groups at week 6 (Fig 2). The superiority of the 6-week treatment with zafirlukast in comparison with that with placebo varied with the level of eosinophils at week 0. A further analysis between the zafirlukast and placebo treatments was made from the lowest to the highest quartile of the baseline eosinophil level. Differences in favor of zafirlukast were significant ($P = .015$) when estimated at the median eosinophil level and highly significant ($P = .004$) when estimated at the upper quartile eosinophil level (Table III).

The current data do not indicate any significant treatment effect of zafirlukast on neutrophils, lymphocytes, mast cells, or macrophages. In the placebo group, the neutrophil count remained 33 cells/mm² (3, 63) above the week 0 baseline until the completion of the trial and increased by 98 cells/mm² (42, 153) from the week 0 baseline with the LTE₄ inhalation at week 6. In the zafirlukast group, the neutrophil count returned to the pretreatment baseline at week 6 (+7 cells/mm²; −24, 39) but increased, although not significantly, in response to the

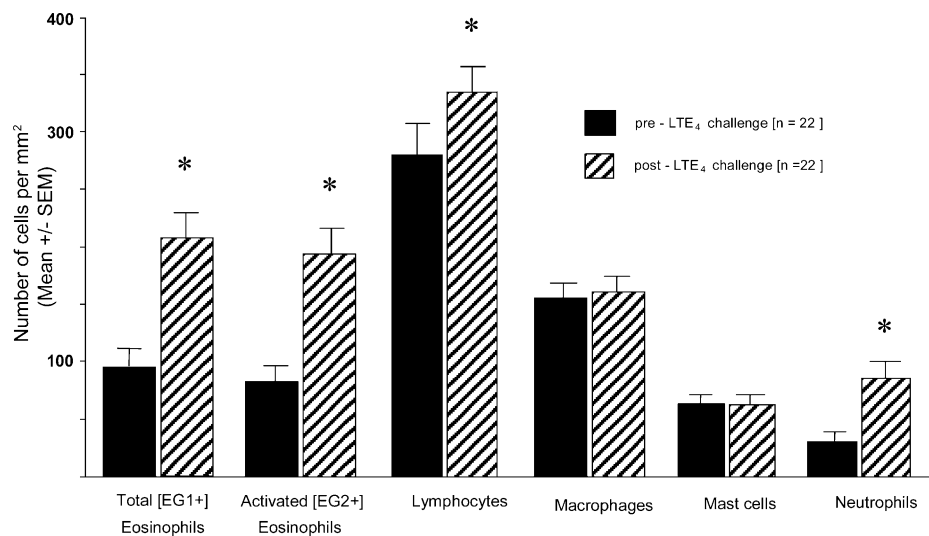


FIG 1. Counts of EG1⁺ and EG2⁺ eosinophils, lymphocytes, macrophages, mast cells, and neutrophils per square millimeter of the lamina propria before (closed columns) and after (hatched columns) provocation with inhaled LTE₄ at baseline (mean ± SEM; **P* < .05 vs prechallenge value).

TABLE II. Changes from week 0 baseline in bronchial biopsy cell counts in zafirlukast and placebo groups on challenges with inhaled LTE₄ and after 6 weeks of treatment; mean (95% CI)

Cell type	Zafirlukast (n = 9)				Placebo (n = 10)			
	Baseline number	Baseline challenge with inhaled LTE ₄	After 6 weeks of treatment	Repeat challenge with inhaled LTE ₄	Baseline number	Baseline challenge with inhaled LTE ₄	After 6 weeks of treatment	Repeat challenge with inhaled LTE ₄
EG2 ⁺ eosinophils	84	94 (29, 159)*	-5 (-50, 40)	9 (-51, 70)	90	127 (78, 176)*	61 (16, 106)*	100 (38, 162)*
Neutrophils	45	55 (17, 92)*	7 (-24, 39)	59 (-8, 125)	21	65 (24, 106)*	33 (3, 63)*	98 (42, 154)*
Lymphocytes	280	33 (-51, 118)	-30 (-151, 90)	-16 (-133, 102)	273	67 (-2, 136)	-4 (-95, 87)	55 (-45, 155)
Mast cells	71	4 (-19, 26)	-12 (-39, 14)	-9 (-39, 22)	63	-6 (-27, 14)	-14 (-35, 7)	-6 (-31, 20)
Macrophages	156	-16 (-74, 43)	-27 (-81, 26)	-32 (-104, 41)	138	35 (-9, 79)	19 (-28, 67)	32 (-15, 79)

*Significant change (*P* < .05) from baseline.

LTE₄ challenge at week 6 (+59 cells/mm²; -8, 125; Table II).

Lung function after treatment and repeat LTE₄ challenge

After treatment, FEV₁ in the placebo group failed to return to the week 0 prechallenge level but remained 0.41 L (-0.74, -0.07) below it before the repeat challenge. On the final week 6 challenge, the mean FEV₁ was further significantly decreased, 0.82 L (-1.44, -0.20) below the week 0 prechallenge baseline (Fig 2).

In contrast, FEV₁ in the zafirlukast group had returned to baseline before the final challenge, +0.06 L (-0.35, 0.48) above the week 0 prechallenge value, and remained unchanged (+0.05 L; -0.37, 0.48) on the final challenge (Fig 2).

The decline in FEV₁ induced in the placebo group by the pretreatment LTE₄ challenge persisted throughout the study and resulted at week 6 in an overall prechallenge FEV₁ difference of 0.33 L (-0.01, 0.66; *P* = .054)

between the treatments in favor of zafirlukast. After the final challenge at week 6, the mean FEV₁ was 0.88 L higher in the zafirlukast than in the placebo group (*P* = .001). No significant correlation existed between the change in the eosinophil count and the drop in FEV₁ produced by the LTE₄ inhalation challenge at week 6 either in the zafirlukast group (*R* = 0.04; *P* = .90) or in the placebo group (*R* = -0.04; *P* = .92).

DISCUSSION

The current study supports the evidence that inhalation challenge with LTE₄ provokes airway obstruction and rapid eosinophil recruitment to the airway mucosa of patients with asthma.^{6,18} The 6-week treatment with the CysLT₁ receptor antagonist zafirlukast at a high dose reduces the eosinophilia and inhibits airway obstruction on repeat inhalation of LTE₄.

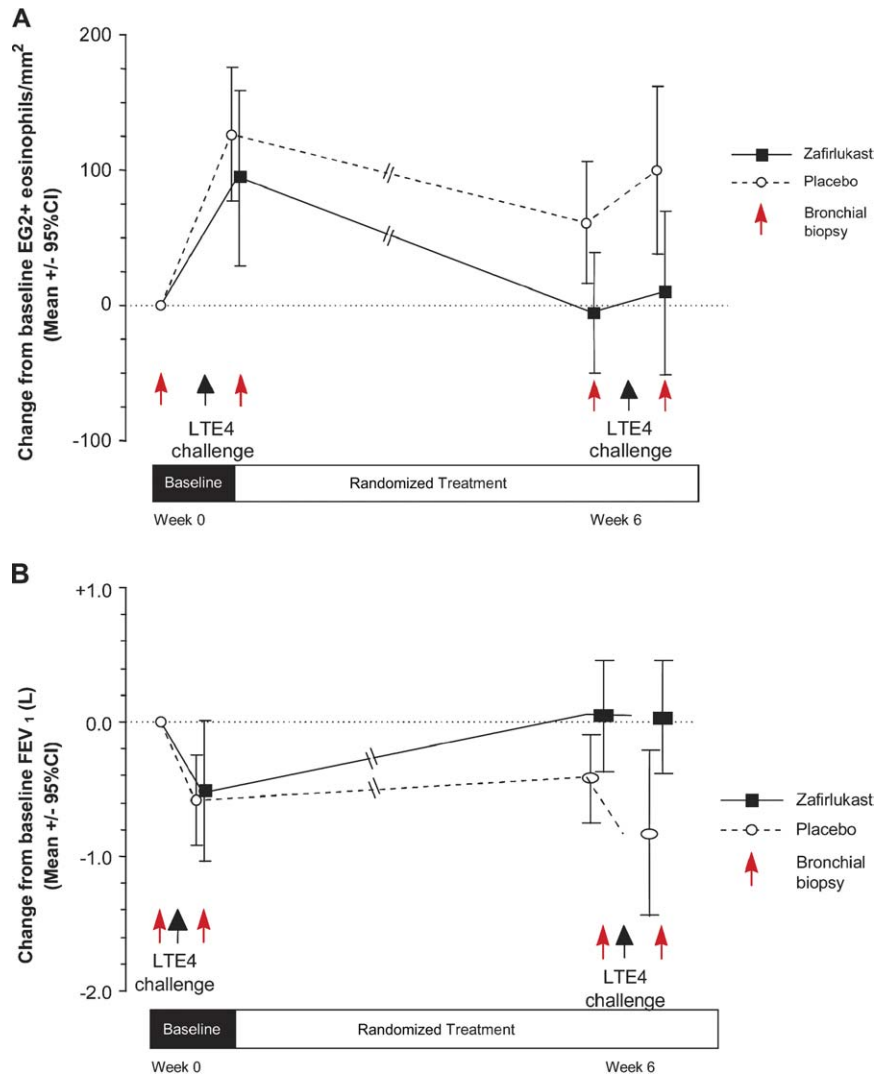


FIG 2. Changes from baseline for **(A)** eosinophils and **(B)** FEV₁ on challenges with inhaled LTE₄ and after 6 weeks of treatment: zafirlukast group (closed squares) or matching placebo (open circles) (mean and 95% CI).

TABLE III. Differences between 6-week zafirlukast and placebo treatments for eosinophils (cells/mm²) before and after the week 6 LTE₄ challenge*

	Lower quartile	Median	Upper quartile
Before week 6 challenge	+8 (−70, +86) [NS]	−64 (−119, −9) [<i>P</i> = .026]	−105 (−175, −34) [<i>P</i> = .006]
After week 6 challenge	−6 (−116, +105) [NS]	−99 (−175, −23) [<i>P</i> = .015]	−162 (−262, −62) [<i>P</i> = .004]

*The analysis is performed in 3 subsets according to week 0 baseline eosinophil counts: first to those ¼ of the volunteers who had the lowest eosinophil counts (lower quartile), then to those ¼ of the volunteers who had the highest eosinophil counts (upper quartile), and finally to those ½ of the volunteers who remained (median). Mean (95% CI) and *P* value.

The current study was designed with the expectation that the inflammatory cells would return to their baseline levels by 6 weeks after the LTE₄ challenge. The study used 2 LTE₄ challenges during the screening period. In the placebo group, the increased cell count, which became apparent after the second challenge at week 0, did not return to the baseline levels before the final challenge at week 6. The current results demonstrate that repeated

LTE₄ inhalation may cause a persistent airway eosinophilia that lasts at least 6 weeks. Moreover, in the placebo group, FEV₁ did not return to the baseline levels before the final challenge, whereas in the zafirlukast group, both the eosinophil count and the FEV₁ returned to the baseline. This suggests that the high cell count before the final challenge was not a random sampling error, but that the patients in the placebo group did not fully recover from the

effect of the 2 LTE_4 challenges at screening (weeks 0 and -1). The significant effects seen with zafirlukast when estimated at the median and upper quartile eosinophil levels (Table III) support the suggestion that, for some patients, the highest level of eosinophilia at week 0 may not have been a true baseline but represented an elevated level after the challenge performed at week -1. This in turn speaks in favor of the ability of LTE_4 -induced airway eosinophilia to form a basis of an amplification mechanism or set up an inflammatory cycle for further eosinophil recruitment.

The increase in eosinophils in response to LTE_4 inhalation is analogous to that demonstrated in earlier studies on sputum¹⁹ or on the airways of patients with asthma after LTD_4 or LTE_4 inhalation.^{6,18} Recently it was shown that 4 hours after challenge, LTE_4 caused significantly higher tissue eosinophilia in patients with asthma compared with LTD_4 , whereas only LTE_4 increased the number of eosinophils in sputum at 7 and 24 hours after challenge.¹⁸ In guinea pigs, a single inhalation exposure of LTD_4 has elicited an influx of eosinophils to the airways that was maintained as long as 4 weeks postchallenge.²⁰ By the time of the current study design, such scientific data were not yet available.

The current study is the first to show that a CysLT₁ receptor antagonist, zafirlukast, prevents eosinophilia induced by inhaled LTE_4 . However, in the current study, after 6 weeks of treatment with zafirlukast, the numbers of eosinophils did not normalize but decreased to the pretreatment baseline level, suggesting that the airway eosinophilia may be partly driven by a nonleukotriene-mediated pathway. The fact that neither the increase in eosinophils nor the airway obstruction was observed after the repeat LTE_4 inhalation after 6 weeks of zafirlukast treatment suggests an anti-inflammatory effect of zafirlukast mediated by the CysLT₁ receptor, which emphasizes the importance of this receptor in generating signals leading to eosinophil recruitment. Accordingly, *in vitro* results using blood eosinophils from atopic donors have shown that CysLTs directly upregulate eosinophil functions related to eosinophil recruitment.²¹

Normally, eosinophils are scarce in the human lung.²² Persistent high eosinophilia, as described in the current study, can result in a release of large quantities of proinflammatory mediators, markedly contributing to the development of an asthma exacerbation. In a recent study, it was shown that cytokine induction by human cord blood-dependent mast cells occurs through CysLT receptor pathways, and an autocrine amplification mechanism for IgE-dependent cytokine production exists via the CysLT receptors.²³ Stimulation of human cord blood-dependent mast cells by CysLT receptors may promote eosinophilia through IL-5, neutrophil recruitment through TNF- α , and lymphocyte chemotaxis through macrophage inflammatory protein 1.²³ This could explain, in part, why the current neutrophil and lymphocyte numbers increased in addition to eosinophilia in response to inhaled LTE_4 . The antieosinophil effect of zafirlukast seen in the current study can even imply an indirect antiremodeling action of

the drug, analogous to the action recently attributed to IL-5.²⁴ In particular, in the bronchial mucosa of patients with mild asthma, anti-IL-5 Mab reduces eosinophils expressing mRNA for TGF- β 1, which has been implicated in the remodeling events in asthma.²⁴ Also, the effects of zafirlukast and analogous CysLT₁ receptor antagonists may be directly dependent on the severity of the asthmatic inflammation, because the expression of the CysLT₁ receptor is upregulated by the T_H2 cytokines IL-4 and IL-13 in human monocytes and macrophages²⁵ as well as by the IL-5 in eosinophils,²⁶ in addition to the inhibition of the apoptosis of these cells.²⁷ In the future studies of CysLTs, the cytokines should also be measured.

Pharmacologically, at least 2 CysLT receptors have been defined in human beings.²⁸ Bronchoconstriction and eosinophilia are mediated by the CysLT₁ receptor, and zafirlukast, a CysLT₁ receptor antagonist, is therefore expected to alleviate these phenomena.²⁹ The expression of both the CysLT₁ receptor mRNA and protein has also been reported in blood eosinophils, pregranulocytic CD34⁺ cells, subsets of monocytes, and B lymphocytes.³⁰ High levels of matured plasma cells, which are the source of IgE antibody production, are seen in the airway mucosa of patients with asthma.⁶ In our recent study, evidence for linkage in chromosome 7p14-p15 was obtained for 3 phenotypes: asthma, a high level of IgE, and the combination of the phenotypes.³¹ High IgE levels in patients with asthma suggest that the patients are more predisposed to CysLT effects than normal subjects. However, a link between a recently described 133-kb asthma risk-conferring site containing 2 genes³² to CysLT effects and antileukotriene susceptibility remains to be elucidated.

Heterogeneity between patients with asthma in their response to treatment with both 5-LO inhibitors and CysLT receptor antagonists has been recognized.³³ By examining the genomic DNA, mutations in the promoter region of the 5-LO gene causing decreased transcriptional activity have been traced.³⁴ The occurrence of 4 non-responsive patients to inhaled LTE_4 in the current study supports the presence of unknown polymorphisms or mutations in association with the CysLT₁ receptor. At least 3 single nucleotide polymorphisms have been detected in the CysLT₁ receptor gene, but none has been related to asthma.³⁵ The finding of alleles, which may lead to differentially regulated gene activity, provides potential references to the patient response to treatment.

In conclusion, the study demonstrates that inhalation of LTE_4 provokes airway obstruction and bronchial eosinophilia that may persist for at least 6 weeks in patients with mild asthma. The treatment with the CysLT₁ receptor antagonist zafirlukast inhibits airways obstruction and eosinophil recruitment after repeat LTE_4 inhalation, suggesting that zafirlukast has anti-inflammatory actions mediated by the CysLT₁ receptor. The results of the current study show that high-dose zafirlukast inhibits airway obstruction and eosinophil recruitment after repeat LTE_4 inhalation. However, further studies are required to define the exact pattern of the dose-response of this effect.

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