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A M E R I C A N C O L L E G E O F
 **C H E S T**
P H Y S I C I A N S



Montelukast Treatment Attenuates the Increase in Myofibroblasts Following Low-Dose Allergen Challenge*

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Rationale: Airway remodeling is believed to be important in the pathophysiology of asthma, and myofibroblasts are increased in the airways of asthmatic individuals 24 h after allergen challenge. Leukotriene receptor antagonists exert antiinflammatory activity in asthma, but it is unknown whether they influence indices of airway remodeling. In the present study, we evaluated the effect of montelukast on airway myofibroblasts following low-dose allergen challenge (LDAC).

Methods: Stable subjects with mild asthma were included in a two-center, randomized, parallel-group study. A 2-week run-in period was followed by LDAC and endobronchial biopsy. Subjects were then randomized to receive either montelukast, 10 mg/d, or placebo (n = 10 in each group) for 8 weeks in a double-blind manner; at the end of the treatment period, subjects underwent a second LDAC and endobronchial biopsy. The effect of treatment on myofibroblasts, fibroblasts, and inflammatory cells was examined using electron microscopy techniques.

Results: Treatment with montelukast showed no significant difference by comparison with placebo but did show a significant within-group treatment-related decrease in airway wall myofibroblasts not seen in the placebo group. In addition, the montelukast-treated group also showed a significant within-group reduction in lymphomononuclear cells and increased neutrophils.

Conclusions: The results suggest that montelukast has an inhibitory effect on airway structural cells that play a key role in airway remodeling in allergic airway inflammation, and that montelukast may be a useful therapy to attenuate airway remodeling in asthma.

(CHEST 2006; 130:741-753)

Key words: airway remodeling; allergen challenge; asthma; leukotriene receptor antagonist; myofibroblast

Abbreviations: AHR = airway hyperresponsiveness; CFEG = cluster of free eosinophil granules; CysLT = cysteinyl leukotriene; CysLT1R = cysteinyl leukotriene 1 receptor; LDAC = low-dose allergen challenge; PC₂₀ = provocative concentration of methacholine causing a 20% fall in FEV₁; RER = rough endoplasmic reticulum; SMA = smooth-muscle actin

Asthma is characterized by structural changes of the airway wall, often termed *airway remodeling*. Features of remodeling include goblet-cell metapla-

sia, thickening of the reticular layer beneath the true basement membrane (lamina reticularis), smooth-muscle hyperplasia, and hyperplasia of fibroblasts

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and myofibroblasts.^{1–3} There is evidence that these structural changes are related to the airway hyperresponsiveness (AHR) present in asthma^{4,5} and may be related to the development of fixed airflow obstruction in individuals with long-standing chronic asthma.² Myofibroblasts, which have an enhanced ability to synthesize collagen, are increased after high-dose allergen challenge in the asthmatic airway⁶ and are believed to play a key role in the remodeling process.

It is not clear whether the changes of airway remodeling can be reversed by currently available therapies. Inhaled corticosteroids are the cornerstone of asthma treatment and are effective in decreasing asthma symptoms and airway inflammation.^{7,8} However, the effect of regular inhaled steroid treatment on AHR and airway remodeling is controversial^{9–13} and results in only modest attenuation of AHR and minimal effects on airway remodeling. Leukotrienes C₄, D₄, and E₄, known as the cysteinyl leukotrienes (CysLTs), play an important role in mediating bronchoconstriction and allergic airway inflammation in asthma,^{14,15} and have been shown to stimulate the production of transforming growth factor- β_1 from eosinophils in allergic inflammation.¹⁶ Montelukast, a CysLT₁ receptor (CysLT₁R) antagonist, attenuates early and late airway responses to allergen^{17,18} and allergen-induced sputum eosinophilia¹⁸ in asthmatics. In murine models of allergic airway remodeling, montelukast has also been shown to inhibit goblet-cell metaplasia, subepithelial fibrosis, and smooth-muscle hyperplasia.^{19,20} A study²¹ using fibroblasts obtained from BAL fluid in patients with interstitial lung disease has shown suppression of cell proliferation and α -smooth-muscle actin (SMA) production with montelukast. However, to date, no clinical studies have been conducted to examine the effect of CysLT receptor antagonists on myofibroblasts and other structural cells in the airways of asthmatic individuals.

Persistent AHR in asthma, which is often associated with ongoing low-grade airway inflammation, is likely due to repeated, relatively low-dose allergen exposure.²² Thus, single high-dose allergen challenge may be an incomplete model of naturally occurring asthma; instead, repeated low-dose allergen exposure may be a more valid model to evaluate antiinflammatory therapy.²³ Repeated low-dose allergen challenge (LDAC) has been shown to exacerbate AHR and inflammation, even when the patient has remained relatively asymptomatic^{24–26}; as such, it may be a more appropriate model of the natural fluctuations of asthma than high-dose allergen challenge. The specific objective of this study was to compare the effect of 8 weeks of treatment with montelukast, 10 mg/d, or the inhaled corticosteroid budesonide on structural and inflammatory airway

cells in subjects with asthma following LDAC. This relatively long period of treatment was selected because there have been no studies of the effect of montelukast on structural changes in the airway, and corticosteroids have been shown to modify airway remodeling only after weeks to months.^{9–13} Unfortunately, only four matched pairs of biopsy samples were available for comparison from the budesonide treatment arm, and no meaningful comparisons could be made. There were, however, sufficient biopsy samples available from the montelukast and placebo treatment arms, which make up the substance of this report. Transmission electron microscopy is the most reliable method to examine myofibroblasts,⁶ and all granulated cell types, including mast cells, eosinophils, and neutrophils, can be reliably distinguished from each other by their very different intracellular granule structure. In addition, cell degranulation can be readily identified.

MATERIALS AND METHODS

Subjects

Twenty nonsmoking subjects (15 women) with mild atopic asthma participated in the montelukast-vs-placebo phase of the study (Table 1). They all had symptoms of asthma for at least 1 year and were included in the study if their provocative concentration of methacholine causing a 20% fall in FEV₁ (PC₂₀) was < 16 mg/mL and if they demonstrated an early and late asthmatic response of $\geq$ 15% reduction in FEV₁ during screening allergen challenge.^{27,28} The subjects received no medication other than infrequent (less than twice weekly) inhaled β_2 -agonists and were not exposed to sensitizing allergens; none of the study participants had an asthma exacerbation or respiratory tract infection in the 4 weeks before entry into the study. No subject had been treated with leukotriene modifiers or with oral glucocorticosteroids in the previous 6 months, or with nasal or dermal glucocorticosteroids within 30 days prior to participation in the study. All subjects gave written informed consent, and the study was approved by the Hamilton Health Centres Hospital Ethics Committee and by Laval Hospital, St Foy, Quebec.

Study Design and Protocol

This was a two-center, randomized controlled trial in which participants received either montelukast, 10 mg/d, or placebo

Table 1—Baseline Subject Characteristics and Disease Severity Data During the Run-in Period*

Variables	Montelukast	Placebo
Subjects, No.	10	10
Female/male gender, No.	8/2	7/3
Mean age, yr	25	28
Time since diagnosis, yr	12 (1–26)	7 (0–32)
FEV ₁ , L	3.2 (2.02–5.10)	3.36 (2.18–5.18)
FEV ₁ , % predicted	88 (76–107)	88 (70–105)
PC ₂₀ , mg/mL†	2.12 (0.18–11.03)	1.66 (0.08–11.56)
Mean symptom score	0.5 (0.1–1.1)	0.4 (0–1.7)

*Data are presented as mean (range) unless otherwise indicated.

†Geometric mean.

over an 8-week treatment period (Fig 1). At the first visit after screening, a methacholine inhalation challenge was performed to determine baseline pretreatment, preallergen airway responsiveness. On the morning of the next 4 consecutive days, the subjects inhaled a low dose of allergen as previously described.²⁵ After the last day of LDAC, if the FEV₁ had not fallen by > 10% of the recorded value from the previous day, methacholine inhalation challenge was repeated. That afternoon, subjects underwent bronchoscopy and endobronchial biopsy. Subjects were then randomized to receive either montelukast, 10 mg po qd, in the evening (n = 10) or a placebo tablet (n = 10) for an 8-week period. After 8 weeks of treatment, subjects underwent a methacholine inhalation challenge to determine posttreatment preallergen airway responsiveness. Subjects then received a LDAC identical to that described for visit 1, with endobronchial biopsy being performed as before.

Randomization and Allocation Concealment

Treatment allocation was concealed from the investigators and participants for the duration of the study. All study medications were independently packaged and labeled by the hospital pharmacy. Placebo tablets were identical in appearance and labeling to montelukast tablets, both of which were supplied by Merck Research Laboratories (Merck Frosst Canada; Montreal, PQ, Canada); it should be noted that Merck Frosst did not sponsor the study. At the start of a treatment period, each subject was given a new coded container with sufficient study tablets. At the end of each treatment period, study medication was returned and compliance was monitored by counting the number of tablets remaining.

Outcome Measurements

The primary outcome was the effect of treatment on allergen-induced airway myofibroblasts and fibroblasts as identified by ultrastructural morphology, with secondary outcomes being the effect on allergen-induced airway wall inflammatory cells identified with electron microscopy. The effect of treatment on the thickness of the lamina reticularis was also examined.

Sample Size

We estimated that a sample size of 10 in each group would allow the study to be sufficiently powered (> 80%) to detect a clinically important difference of a three-fold increase in myofibroblasts after LDAC (on placebo) as opposed to baseline after diluent challenge. This was an approximation, as the only published study⁶ examining the numbers of myofibroblasts on ultrastructure was after high-dose allergen challenge.

Laboratory Procedures

Methacholine Inhalation Challenge: Methacholine inhalation challenge was performed using the method described by Cockcroft and colleagues.²⁹ Subjects inhaled through a mouthpiece attached to a Wright nebulizer (Roxon Medi-Tech; Montreal, PQ, Canada). Normal saline solution followed by doubling concentration increases in methacholine were nebulized for 2 min each. FEV₁ was measured at 30, 90, 180, and 300 s after each inhalation using a Collins water-sealed spirometer (Warren E. Collins; Braintree, MA) and kymograph. The test was terminated when FEV₁ had fallen to a level at least 20% below the

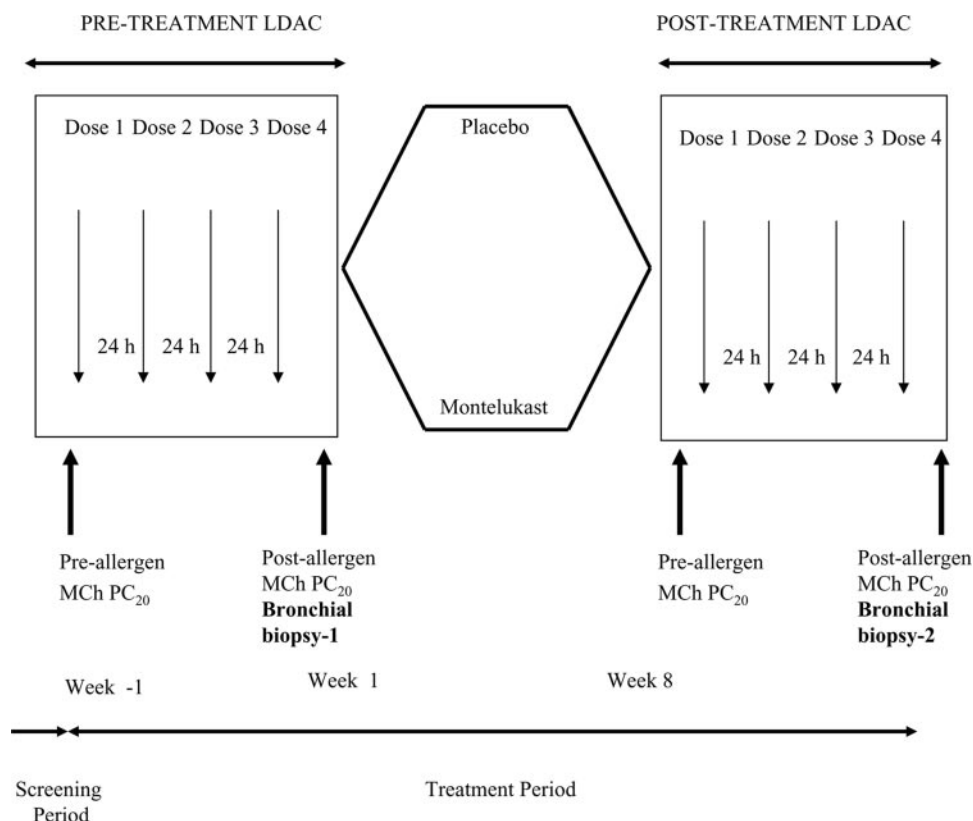


FIGURE 1. Study protocol. MCh = methacholine.

post-saline solution measurement. The PC₂₀ was calculated through linear interpolation of percentage fall in FEV₁ against the log-transformed methacholine concentration.²⁹

Allergen Inhalation Challenge: High-dose allergen challenge was performed during the screening process as previously described.²⁸ The concentration of allergen causing an early fall in FEV₁ of approximately 5% as measured in the high-dose screening challenge was used for the LDAC. The LDAC was commenced at least 30 days after the high-dose allergen challenge to allow the airway to return to normal. Subjects underwent LDAC over 4 consecutive days by inhaling a predetermined concentration of allergen administered as a single challenge for 2 min from a Wright nebulizer as described previously.²⁵ FEV₁ was measured at 10 min following inhalation and then at 10-min intervals for 40 min.

Fiberoptic Bronchoscopy and Endobronchial Biopsy: Bronchoscopy and endobronchial biopsy were performed according to the recommendations of the National Institutes of Health³⁰ as a day-case procedure. Two mucosal biopsy specimens were obtained from the subcarinae of the right lower lobe, fixed in 2% glutaraldehyde, buffered in 0.1 mol/L sodium cacodylate pH 7.4, and processed for transmission electron microscopy. Previous biopsy sites were avoided when identifiable.

Sample Processing for Transmission Electron Microscopy: After fixation for approximately 18 h, the biopsy specimens were rinsed in sodium cacodylate buffer, postfixed in 1% osmium tetroxide for 1 h, dehydrated in graded ethanol solutions, and then embedded in Spurr resin. One-micrometer-thick sections were stained with toluidine blue, and areas of intact submucosa were selected for further electron microscopic analysis. Ultrathin sections (90 nm) were cut and placed on a 200-mesh, thin-bar

copper grid and stained with uranyl acetate and lead citrate. The specimens were examined by a transmission electron microscope (CM10; Philips; Eindhoven; the Netherlands).

Ultrastructural Identification of Cells: The ultrastructural identification of cells was based on published criteria.^{6,9,31–37} Myofibroblasts were identified by the presence of fibronexi that are regarded as being crucial to the identification of myofibroblasts, as emphasized by Eyden³⁷ (Fig 2). Fibroblasts were identified by their bipolar morphology, rough endoplasmic reticulum (RER), and lack of other features such as cytoplasmic filaments or fibronexi (Fig 3, *top*, A). In addition to fibronexi, myofibroblasts also showed other typical features, including spindle-like cytoplasmic projections, dilated RER, crenated nuclear membranes, patchy basal lamina, intermediate or gap junctions, and pinocytotic vesicles with or without bundles of cytoplasmic microfilaments^{6,32,34,37,38} (Fig 3, *top*, B; *center left*, C; and *center right*, D). When the microfilaments were present, they tended to be scanty, to run parallel to the long axis of the cell, and were located peripherally. We have called these cells *type 1 myofibroblasts*. A minority of cells with fibronexi (and therefore classified as myofibroblasts) had an unusual morphology with numerous, prominent bundles of microfilaments distributed throughout the cytoplasm and absent or scanty RER (Fig 3, *bottom left*, E). In addition, a thick basal lamina around the perimeter of the cells was easily identified, although it was not completely continuous as is the case with smooth-muscle cells (Fig 3, *bottom right*, F). These cells were labeled as *type 2 myofibroblasts*. Eosinophils were characterized by a segmented nucleus and distinctive granules containing a crystalline core, with activated eosinophils undergoing cytolysis,³⁹ as well as clusters of free eosinophil granules (CFEGs) also being identi-

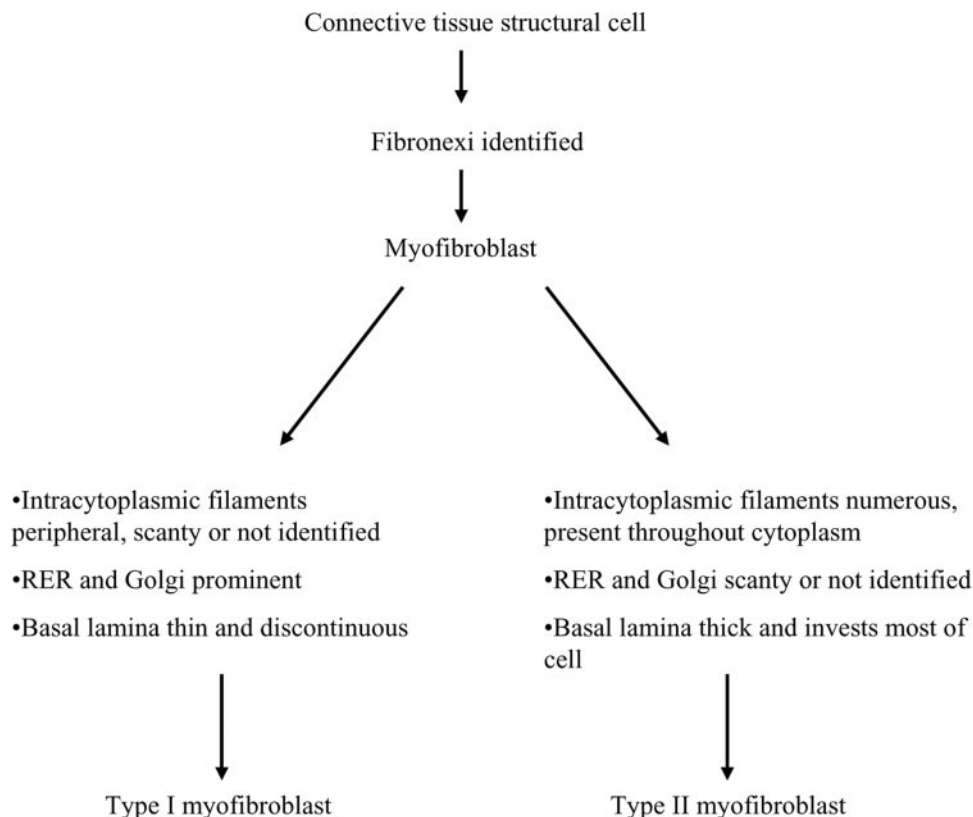


FIGURE 2. Classification of myofibroblasts into type 1 (the majority) and type 2 (the minority). Type 2 myofibroblasts were more differentiated toward smooth-muscle cells than type 1 myofibroblasts.

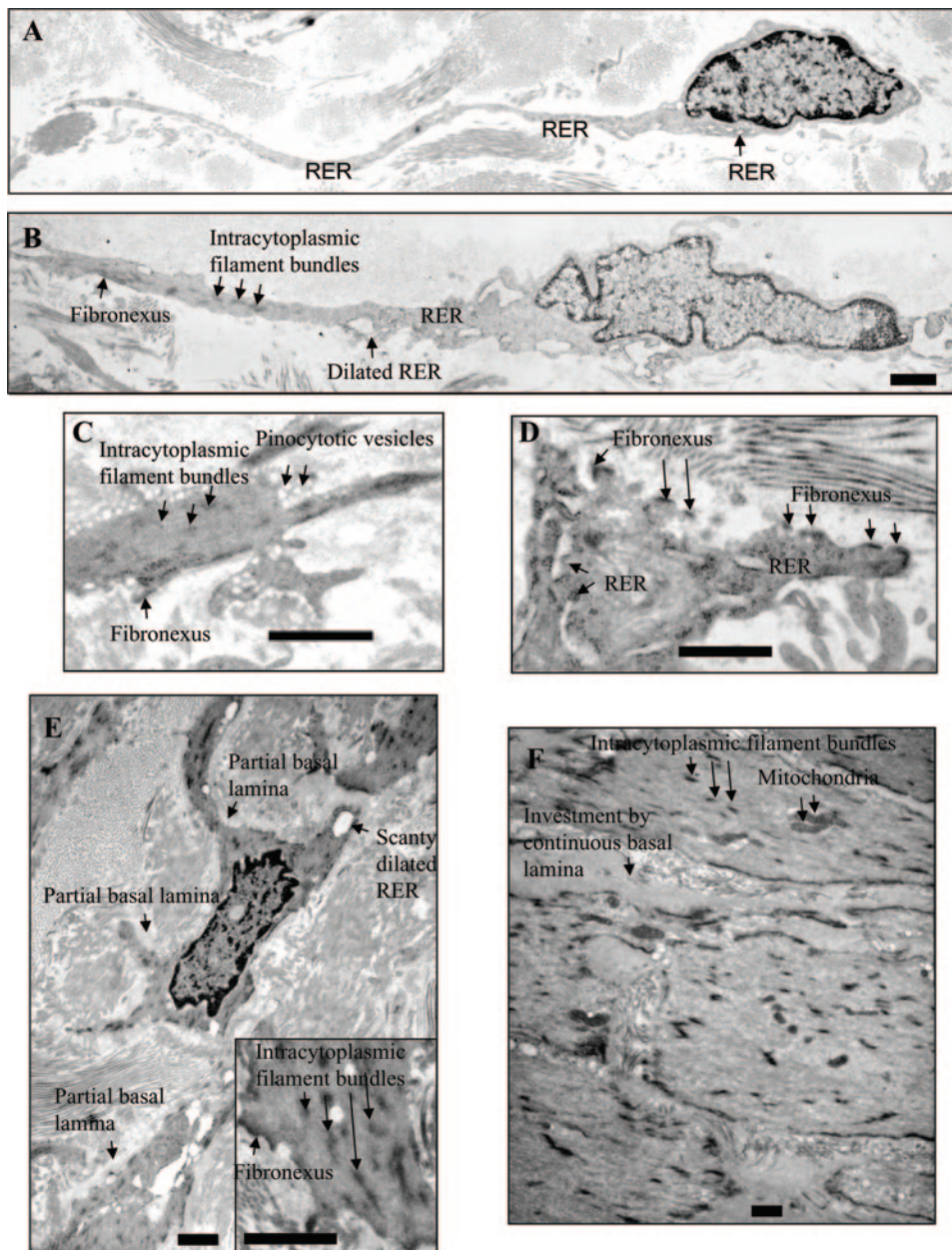


FIGURE 3. Electron microscopic appearance of mesenchymal cells of bronchial wall. *Top, A*: Relatively quiescent fibroblast with rough endoplasmic reticulum. *Top, B*: Myofibroblast (type 1) with RER and scanty cytoplasmic filament bundles. *Center left, C*: Myofibroblast with cytoplasmic filament bundles and prominent caveolae. *Center right, D*: Myofibroblast with prominent fibronexi and RER. *Bottom left, E*: Type 2 myofibroblast with numerous cytoplasmic filament bundles, prominent basal lamina, absent/scanty RER, and cellular processes that appear to be forming a continuous network with other myofibroblasts. *Bottom right, F*: Smooth muscle with thick, continuous basal lamina, numerous filaments, and mitochondria. Bar = 1 μ m.

fied³⁹ (Fig 4, *top left, A*; *top right, B*). Mast cells, basophils, and neutrophils were distinguished by their nuclei, characteristic granules, and presence/absence of cytoplasmic glycogen particles (Fig 4, *center left, C*; *center right, D*; Fig 5). Neutrophils were classified as cells with an intact cytoplasmic membrane and granules (Fig 5, *top left, A*) and those undergoing lysis with a degenerating nucleus and cytoplasmic membrane, and granules spilling out into extracellular matrix (Fig 5, *center, C*). Lymphocytes and monocytes were not always easy to distinguish, both

having scanty cytoplasm and a paucity of organelles with occasional mitochondria; therefore, they were classified as lymphomononuclear cells (Fig 4, *bottom, E*). Some cells could not be identified due to their lack of characteristic features or excessive degeneration and were labeled *difficult to classify*. Cells were only counted if the nucleus was identified.

Quantitation: All samples were coded and examined without knowledge of subject details. Cells were counted in a subepithelial zone 100- μ m thick by a single observer and expressed as the

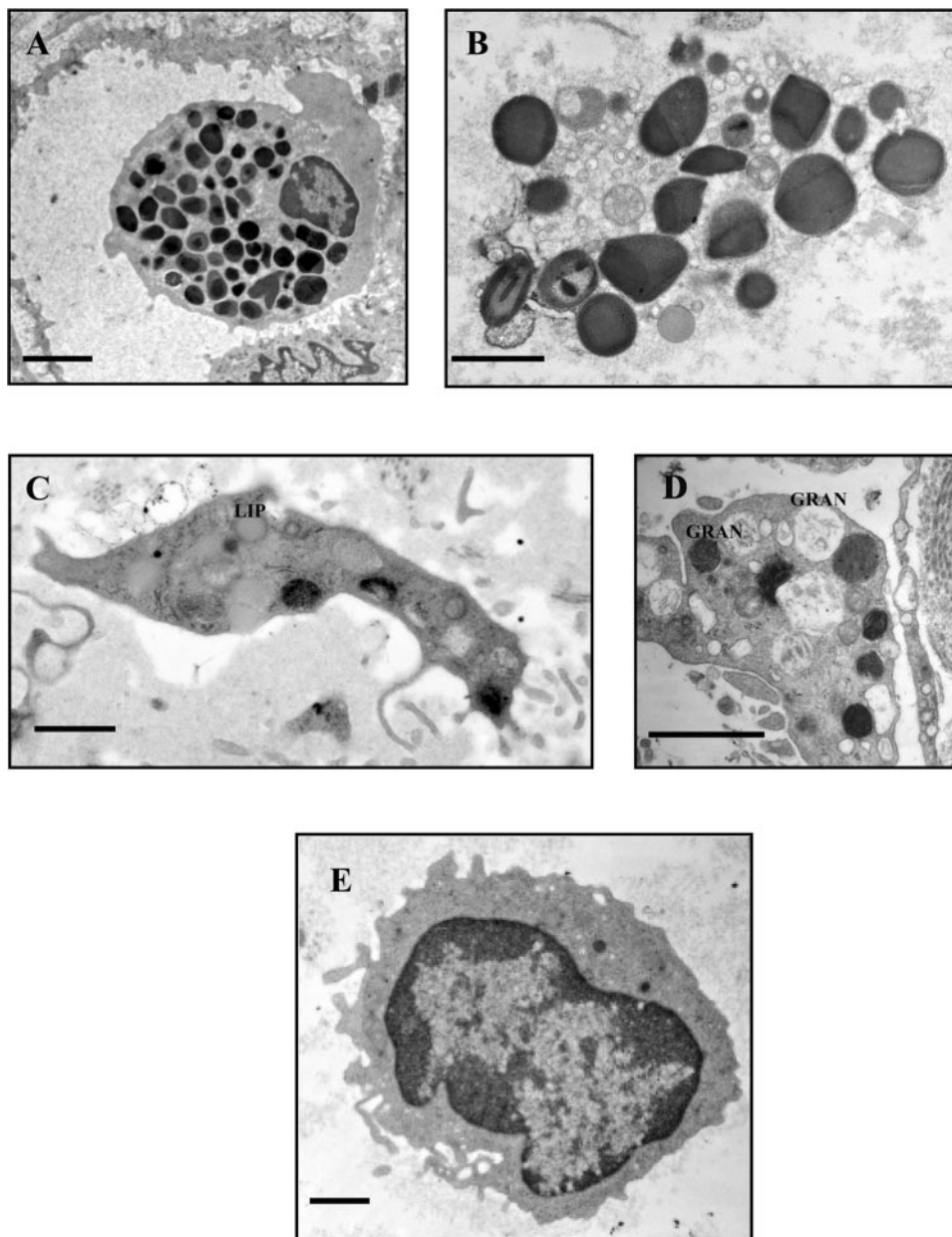


FIGURE 4. Electron microscopic appearance of inflammatory cells in bronchial wall. *Top left, A:* Intact eosinophil within blood vessel. *Top right, B:* CFEGs within the extracellular matrix. *Center left, C:* Mast cell with typical granules as well as lipid bodies. *Center right, D:* Mast cell partially granulated and degranulated. *Bottom, E:* Lymphomononuclear cell. LIP = lipid body; Gran = granule. Bar = 1 μ m.

number of positive cells per 0.1 mm² of bronchial submucosa excluding mucus glands, blood vessels, and smooth muscle. The sections were photographed and the area measured using an image analysis system (BQ System IV; R & M Biometrics; Nashville, TN). The resin-embedded sections prepared for electron microscopy and stained with toluidine blue were photographed at a magnification of $\times 200$ using a high-grade image analysis system (Leica Q500IW; Leica Microsystems; Wetzlar, Germany). A calibrated image analysis system (Openlab; Improvision; Lexington, MA) was used to measure the area and length of the lamina reticularis, taking care to only include well-orientated areas with intact bronchial epithelium. The thickness of the lamina reticularis was calculated by dividing the area by its length.⁴⁰

Statistical Analysis

The data were analyzed using a statistical program SPSS version 11.0.0 (SPSS; Chicago, IL). Data were expressed as mean and SEM unless otherwise indicated. The coefficient of variation for the error of repeat measurement for each cell type was $< 5\%$. Normality and variance assumptions were tested for all variables. The lamina reticularis values were logarithmically transformed, whereas ultrastructural data were best normalized by square-root transformation. Between-group comparisons prior to treatment were performed by one-way analysis of variance with a Bonferroni correction; the comparisons between groups after treatment were performed using a one-way analysis of covariance (data before treatment defined as covariables). Within-group analysis was performed using paired t

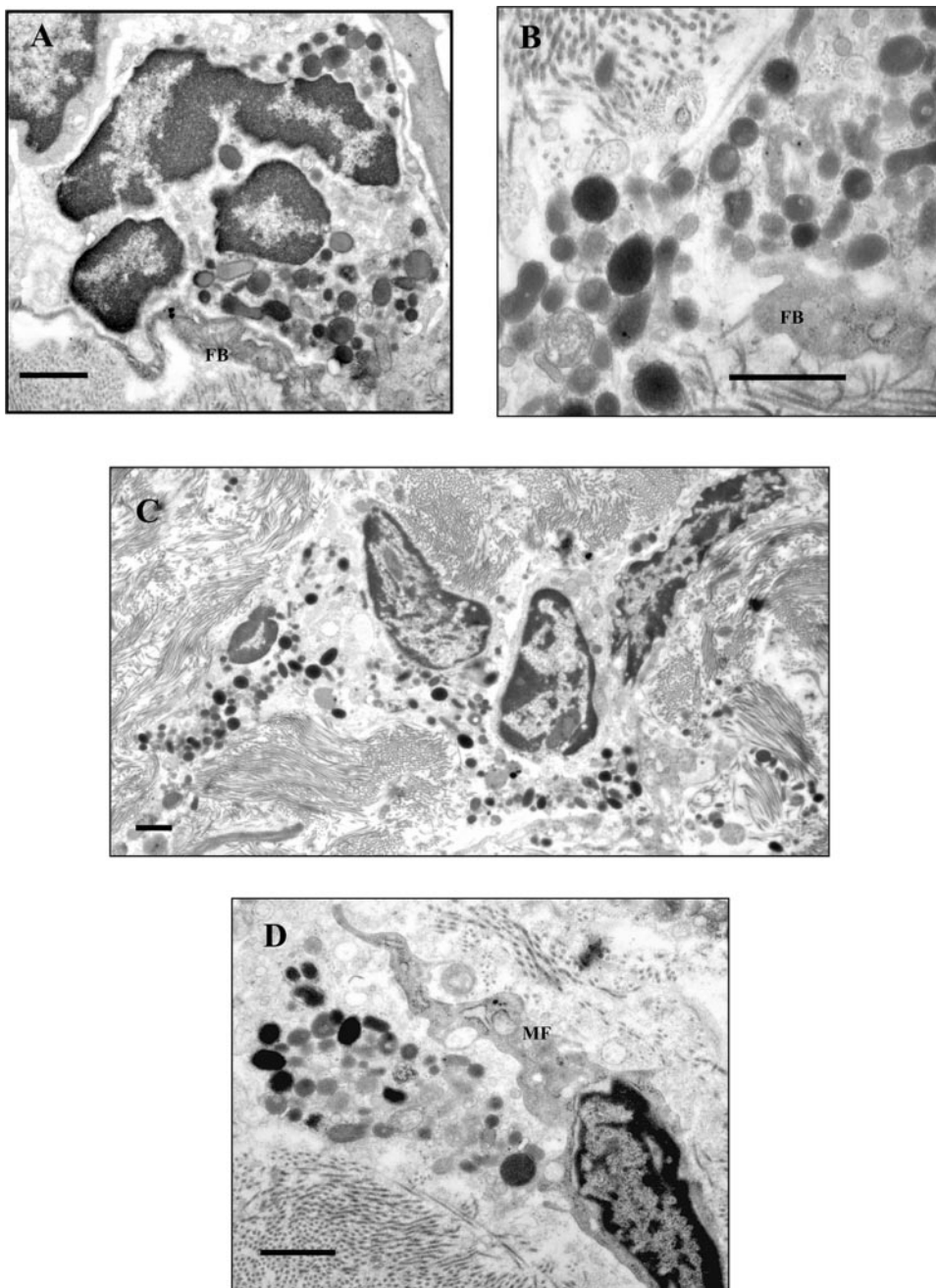


FIGURE 5. Electron microscopic appearance of inflammatory cells in the bronchial wall. *Top left, A:* Intact neutrophil with typical granules lying in close relation to fibroblasts. *Top right, B:* Neutrophil lysing with granules being released into the extracellular matrix in close apposition to a fibroblast. *Center, C:* Neutrophils in the process of lysing with released neutrophil granules lying within the extracellular matrix. *Bottom, D:* Collection of neutrophil granules in close apposition to a myofibroblast. FB = fibroblast; MF = myofibroblast. Bar = 1 μ m.

tests. Correlations between data were sought using a Pearson test. The results were considered significant if p values were < 0.05 (two tailed).

RESULTS

Lung Function

There were no significant differences between the pre-LDAC FEV₁ values in any group before or after

treatment. The FEV₁ after LDAC within the montelukast group was statistically significantly higher after treatment than before treatment ($p < 0.05$); the FEV₁ did not change significantly within the placebo group. The PC₂₀ showed a slight numeric increase after treatment but before LDAC in the montelukast group. However, no statistically significant differences could be detected within or between the groups in PC₂₀.

Adequate material was available for paired (pretreatment and posttreatment), ultrastructural studies from seven subjects in the placebo group and eight subjects in the montelukast group. Biopsy samples from the other three subjects in the placebo group and two patients in the montelukast group were technically inadequate to allow for paired ultrastructural examination. The average area of submucosa examined per patient was $42,740 \mu\text{m}^2$ (SEM, $3,835 \mu\text{m}^2$). There were no significant differences in variables when the montelukast-treated group was compared to the placebo-treated group before or after treatment (between-group analysis), and placebo had no significant effect on any variable (within-group analysis). Compared to the respective post-LDAC baseline values, montelukast treatment resulted in a significantly decreased number of total myofibroblasts ($p = 0.02$), lymphomononuclear cells ($p = 0.003$), and a trend to decreased macrophages ($p = 0.05$) and the subset of type 2 myofibroblasts ($p = 0.08$) [Fig 6]. The type 2 myofibroblasts, characterized by prominent bundles of cytoplasmic filaments and rare RER (Fig 3, *bottom left, E*), were present at any level in the submucosa, although they tended to occur further away from the epithelial layer than the type 1 myofibroblast. The type 2 myofibroblasts were often clustered in groups, with their cellular processes arranged in a network-like configuration, and had a prominent (although discontinuous) basal lamina (Fig 3, *bottom left, E*). Montelukast treatment also significantly increased the numbers of neutrophils ($p = 0.03$), with a significant increase in the number of lytic neutrophils ($p = 0.03$). Neutrophils and clusters of free neutrophil granules were often closely apposed to fibroblasts and myofibroblasts (Fig 5). There was no significant effect on eosinophils or mast cells.

Lamina Reticularis

The average length of lamina reticularis examined per subject was 2.4 mm (SEM, 0.17 mm). The thickness of the lamina reticularis in the placebo and montelukast groups was as follows: before treatment (mean, $9.2 \mu\text{m}$ [SEM, $0.47 \mu\text{m}$], and mean, $10.2 \mu\text{m}$ [SEM, $0.42 \mu\text{m}$]); after treatment (mean, $8.3 \mu\text{m}$ [SEM, $1.49 \mu\text{m}$], and mean, $9.6 \mu\text{m}$ [SEM $0.41 \mu\text{m}$]), respectively. There was no significant difference in the lamina reticularis thickness after treatment after within-group or between-group analysis. When compared to the cells identified by ultrastructure, the lamina reticularis thickness correlated with total fibroblasts/myofibroblasts ($r = 0.41$, $p = 0.02$) and lymphomononuclear cells ($r = 0.36$, $p = 0.04$) when analyzed before and after treatment; a positive

correlation with eosinophils ($r = 0.73$, $p < 0.01$) was only seen when analyzed before treatment.

DISCUSSION

This is the first study to examine the effects of montelukast on myofibroblasts and fibroblasts in allergen-induced airway inflammation. It demonstrates that 8 weeks of treatment with montelukast significantly reduced the numbers of myofibroblasts present in the airways of asthmatic subjects following LDAC compared to baseline values (Fig 6). These findings are consistent with those of Henderson et al,^{19,41} who demonstrated that montelukast inhibited allergen-induced changes of remodeling in a mouse model, reducing airway smooth-muscle cell hyperplasia by 80% and preventing subepithelial fibrosis. Montelukast treatment also significantly reduced the number of lymphomononuclear cells ($p = 0.003$), with a trend to decreased macrophages ($p = 0.05$) after LDAC, while the numbers of neutrophils increased ($p = 0.03$) [Fig 6]. We were unable to detect any significant difference in cell numbers between the montelukast-treated and placebo-treated groups after treatment, which may be related to our study being insufficiently powered to detect significant between-group differences.

There is experimental evidence indicating that leukotriene receptor antagonists are able to attenuate indexes of airway remodeling in allergic airway inflammation. CysLTs induce the release of fibroblast growth factor from alveolar macrophages,⁴² produce proliferative and synthetic responses in fibroblasts,⁴³ and can potentiate smooth-muscle cell proliferation^{44,45} and migration.⁴⁶ CysLTs are overexpressed in bleomycin-induced pulmonary fibrosis in mice, which is significantly attenuated in 5-lipoxygenase knockout mice.⁴⁷ The CysLT1R is present on bronchial smooth muscle, mast cells, eosinophils, neutrophils, macrophages, B-lymphocytes, and plasma cells^{48,49} but is upregulated in lung fibroblasts⁵⁰ and smooth muscle⁴⁵ by interleukin-13. Upregulation of the expression of the CysLT1R on human bronchial smooth-muscle cells results in a proliferative response to leukotriene D_4 , which can be prevented by pretreatment with montelukast.⁴⁵ In addition, the leukotriene D_4 -induced augmentation of collagen release in transforming growth factor- β -transformed cells and its potentiating effect on airway smooth-muscle proliferation are blocked by CysLT1R antagonists.^{43,44}

Although we did not have 10 pairs of samples in each group at the end of the study, previous studies^{51,52} have shown that a group of eight or more subjects has sufficient power to demonstrate within-

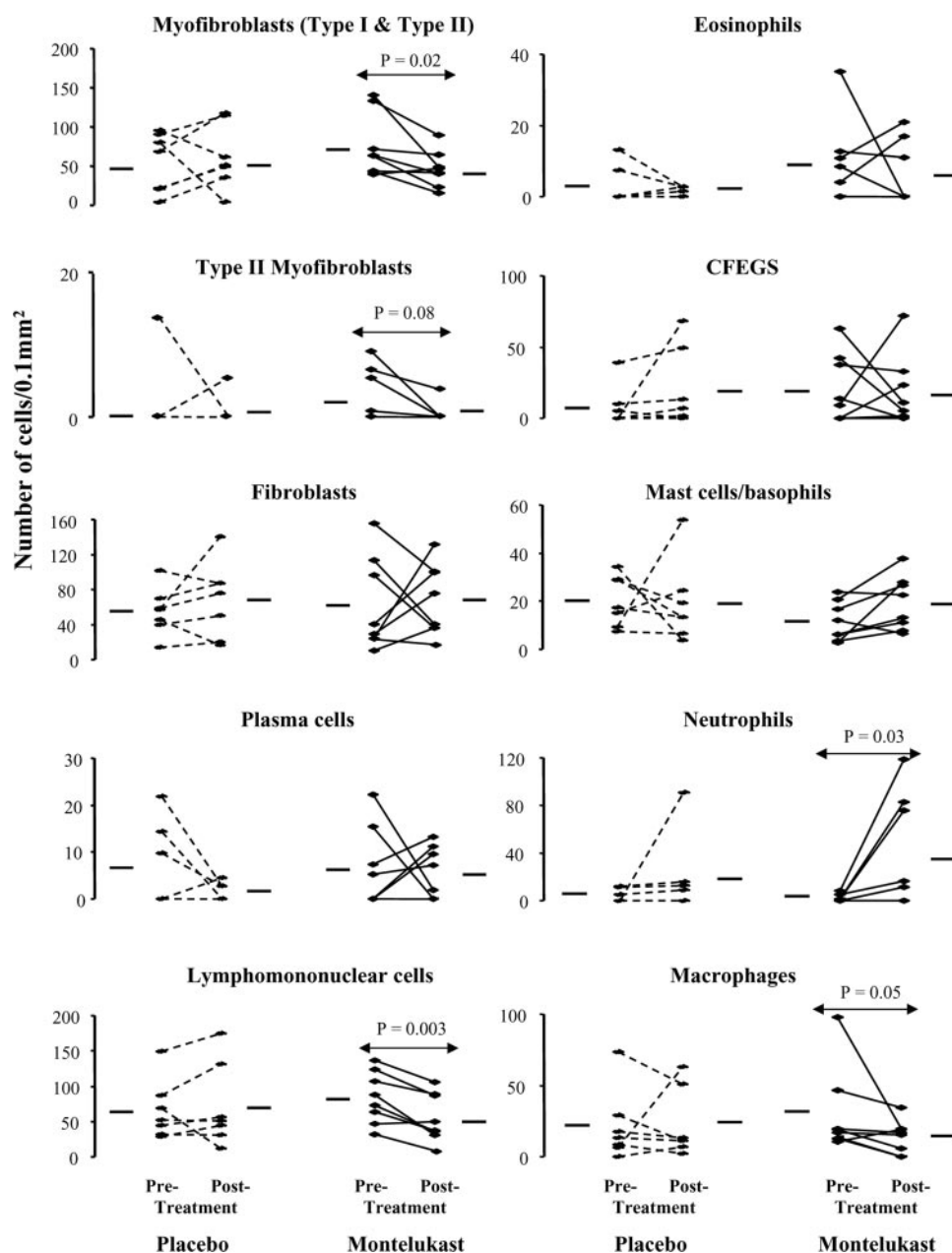


FIGURE 6. Cells identified on ultrastructure in bronchial submucosa after LDAC challenge before treatment and after 8 weeks of treatment with either placebo (dashed line) or montelukast (closed lines). All myofibroblasts (both types 1 and 2) were analyzed together; in addition, the subset of myofibroblasts (type 2 myofibroblasts) were also analyzed separately. There were seven pairs in the placebo group and 8 pairs in the montelukast group. Bars represent mean. Cell counts are expressed as number of positive cells/0.1 mm² of bronchial submucosa excluding mucus glands, blood vessels, and smooth muscle.

group differences in allergen-induced airway eosinophilia and in allergen-induced early and late asthmatic responses. In addition, Gیزیcki et al⁶ were able to demonstrate increased myofibroblasts after high-dose allergen challenge in seven subjects with asthma.

We utilized ultrastructural morphology to identify myofibroblasts, regarded as the “gold standard”

methodology.^{34,38} The first description of myofibroblasts was based on ultrastructural findings⁵³; according to other authorities,^{34,38} the features of myofibroblasts include fibronexi, gap and intermediate junctions, plasmalemmal attachment plaques, pinocytotic vesicles, well-developed RER, and Golgi areas.³² Within the cytoplasm are bundles of microfilaments present only focally and usually peripher-

ally, with interspersed dense bodies (Fig 3). The cell may be partially invested by a basal lamina. These details are not evident from light microscopy and require the high magnification afforded by transmission electron microscopy. Fibronexi have been emphasized as being a distinct, unique organelle for the identification of myofibroblasts,³⁷ and not all cells classified as such had identifiable cytoplasmic microfilaments. Since microfilaments within myofibroblasts are focal and peripheral, it was not surprising that they were not always apparent, since these are large, elongated cells and only part of the cell is evident in the sections. We should point out that our classification differs from that of Gizycki et al,⁶ who only included cells as myofibroblasts if they identified bundles of filaments in the cytoplasm.

Although immunohistochemistry for α -SMA may be positive in myofibroblasts, bronchial smooth muscle, vascular smooth muscle, and pericytes also express α -SMA. An important advantage of electron microscopy over immunologic methods is that the cells in the same section are simultaneously visualized and classified, avoiding the variability introduced when each cell type is identified in different tissue sections.⁵⁴ The average area of submucosa examined was 0.43 mm² and was therefore within the range of 0.3 to 0.5 mm² recommended by an international workshop on bronchial biopsy procedures.⁵⁴ All cells (other than blood vessels, smooth muscle, and glands) were photographed at high power and identified from glossy photographs, by an anatomic pathologist (M.M.K.).

In a previous study,⁶ it was demonstrated that subepithelial myofibroblasts were significantly increased 24 h after high-dose allergen challenge, and myofibroblast numbers have been shown to correlate with the thickness of the lamina reticularis.^{55,56} It is postulated that repeated allergen exposure with resulting increases in myofibroblasts produce thickened lamina reticularis and smooth-muscle hyperplasia, which persist even when minimal airway inflammation is present. Myofibroblasts have an enhanced ability to synthesize interstitial collagens, and those from asthmatic airways have been shown to release greater amounts of endothelin-1 and vascular endothelial growth factor, mitogens for smooth muscle, and vascular endothelial cells, respectively.⁵⁷ Myofibroblasts can generate and maintain contractile force,⁵⁸ and their expression of α -SMA increases their capacity to generate force.⁵⁹ The myofibroblast fibronexus is a specialized adhesion complex that uses transmembrane integrins to link intracellular actin with extracellular fibronectin fibrils,³² providing a mechanotransduction system that transmits the force generated by stress fibers to the surrounding extracellular matrix.⁶⁰ In turn, the

fibronexus may indicate a migratory phenotype, with the focal adhesions transmitting the force of the contractile apparatus to the matrix to pull the cell forward.⁶¹

It is tempting to speculate that CysLTs released during LDAC cause accumulation of myofibroblasts within the submucosa and that montelukast is able to attenuate this increase. However, it is unknown whether montelukast is affecting recruitment, proliferation, or survival of myofibroblasts. Indeed, the source of these cells is still controversial, with candidates including fibroblasts within the submucosa⁶² and circulating stem cells.⁶³ The type 2 myofibroblasts seen in this study are reminiscent of migrating smooth-muscle cells in culture, with a more contractile phenotype than is usually seen in myofibroblasts (Fig 3, *bottom left, E*), but clearly separate from the blocks of smooth muscle seen in some of the biopsies (Fig 3, *bottom right, F*). Bronchial smooth-muscle cells can migrate in culture,⁶⁴ and it has been proposed that in certain circumstances, cells with the features of myofibroblasts in the mucosa may represent de-differentiated smooth-muscle cells, arising from blocks of smooth muscle or even vascular smooth muscle, and should be referred to as *fibromyocytes*.⁶⁵ Interestingly, leukotriene D₄, in concert with epidermal growth factor, induces the proliferation of bronchial smooth-muscle cells that is partially inhibited by montelukast.⁶⁶

There was no effect of treatment with montelukast on the thickness of the lamina reticularis. This is not unexpected, since even with inhaled corticosteroid treatment a decrease in thickness of the lamina reticularis is controversial and it may require long-term treatment to see an effect.^{9–13} Interestingly, lipid bodies, the site of inducible eicosanoid production in eosinophils and basophils,⁶⁷ were observed in eosinophils, basophils, mast cells, and fibroblasts in this study (Fig 4, *center left, C*).

Only a few studies have examined the effect of CysLT1R inhibitors on inflammatory cells in the bronchial wall; in one study,⁶⁸ montelukast was administered in conjunction with corticosteroids, which makes it difficult to evaluate the montelukast effect alone. One study⁶⁹ that looked at bronchial biopsy samples in subjects treated with CysLT1R inhibitors and not corticosteroids showed that 4 weeks of treatment reduced activated eosinophils, T-cells, and mast cells. We cannot determine the phenotype of the lymphomononuclear cells in this study with the methods used. It is interesting that neutrophils were increased while lymphomononuclear cells and macrophages were decreased after LDAC after montelukast treatment, but the mechanism for this is unclear. The increased neutrophils were all in the lytic group, which essentially consists

of disrupted, degenerating neutrophils with extracellular collections of neutrophil granules. These are not the features of activated neutrophils, which remain intact with their granules becoming electron lucent following exocytosis of the contents. Occasionally, the extracellular granules were seen to be in close proximity to myofibroblasts (Fig 5, *bottom, D*), but the significance of this is not clear. Interestingly, it has recently been demonstrated that neutrophil serine proteases are able to induce detachment-induced apoptosis of human airway smooth-muscle cells limiting their hyperplasia, and it is conceivable that neutrophils may have a similar effect against myofibroblasts.⁷⁰ It has recently been shown that neutrophils express CysLT1R.⁴⁹

The lack of effect of montelukast on eosinophils may be due to the relatively low frequency of eosinophils at baseline in this group of patients with mild stable asthma, indicating that the signal is too small to show a treatment-related effect. A previous study⁶⁸ that examined bronchial biopsy samples before and after 8 weeks of treatment with montelukast, but without allergen challenge, also failed to detect any change in eosinophils.

A control diluent challenge was not performed at the start of the study; therefore, we cannot estimate the effect of LDAC alone relative to the unchallenged state. Similarly, as a bronchial biopsy was not performed at the end of the treatment period prior to allergen challenge, we cannot determine if montelukast prevented myofibroblast accumulation during the treatment period, after allergen challenge, or during both these periods. Although a previous study⁷¹ with LDAC demonstrated increased AHR and sputum eosinophilia, LDAC may not generate as strong a signal as high-dose allergen challenge with regard to the changes in myofibroblasts and inflammatory cells. In other studies,^{25,72} AHR and all inflammatory indexes returned to baseline 3 days after completion of LDAC; and one study⁷³ found only a nonsignificant trend for increased sputum eosinophils on day 2 of LDAC.

In summary, we have shown that 8 weeks of treatment with the CysLT1R antagonist montelukast is able to significantly attenuate myofibroblast accumulation following LDAC accompanied by significant effects on inflammatory cells. These observations are clinically important, as they suggest for the first time that pharmacologic therapy with leukotriene-modifying agents may be effective in preventing structural cell changes in the airways of asthmatic individuals.

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Montelukast Treatment Attenuates the Increase in Myofibroblasts Following Low-Dose Allergen Challenge

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