

Effects of leukotriene receptor antagonists on monocyte chemotaxis, p38 and cytoplasmic calcium

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Montelukast and zafirlukast, two cysteinyl leukotriene receptor antagonists (LTRAs), have been shown to have a beneficial effect on the clinical symptoms of asthma. LTRAs can inhibit eosinophil recruitment; however, little is known about their role in monocyte migration. We investigated whether montelukast and zafirlukast could suppress chemokine-induced chemotaxis of monocytes and signaling. Chemotaxis of monocytes from peripheral blood mononuclear cells (PBMCs), cord blood mononuclear cells (CBMCs), and THP-1 cells was evaluated using a 24-well transwell microchamber. $[Ca^{2+}]_i$ was measured with the fluorescence calcium indicator fura-2/AM photometry system. p38 MAPK expression was measured by Western blotting. Results showed that montelukast (1–100 μM) and zafirlukast (100 μM) significantly down-regulated monocyte chemoattractant protein-1 (MCP-1)-induced chemotaxis of THP-1 cells and human primary monocytes from PBMCs and CBMCs ($p < 0.05$, each comparison). Montelukast also abolished MCP-1-induced $[Ca^{2+}]_i$ and pp38 MAPK expression in THP-1 cells in a dose-dependent manner. These data demonstrate that montelukast is effective in down-regulating human monocyte chemotaxis induced by MCP-1. This effect may involve the down-regulation of MCP-1-induced $[Ca^{2+}]_i$ and p38 MAPK expression.

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Infiltrating eosinophils, macrophages, and monocytes comprise the largest population of cells within the airways of patients with allergic asthma and are associated with the induction of reversible airway hyperreactivity (1). Following endobronchial allergen challenge 4 h after allergen challenge, bronchoalveolar lavage fluid levels of RANTES, macrophage inflammatory protein-1 (MIP-1) α and monocyte chemoattractant protein-1 (MCP-1) were significantly increased in fluid obtained from the allergen-challenge site compared with the saline-challenge control site (2). MCP-1, a CC chemokine, mediates airway hyperreactivity in normal and allergic mice and plays an important role in monocyte recruitment

during asthma and allergic reactions. Anti-MCP-1 antibody treatments reduce by approximately 63% the influx of monocytes into the reaction sites (3). MCP-1 stimulates leukocyte chemotaxis to inflammatory sites such as rheumatoid arthritis, atherosclerosis, and asthma by use of the MCP-1 receptor, CCR2 (4). MCP-1 specific antibodies have been shown to neutralize MCP-1-mediated bronchial epithelial cell proliferation (5). The frequency of the -2518G polymorphism in the gene regulatory region of MCP-1 is significantly higher in asthmatic children than in controls also associated with an increased blood eosinophil level. This variant of MCP-1 might belong to the predictor gene set for asthma

(6). Integrin activation and chemotaxis are two important functions involved in monocyte transmigration. ERK is responsible for integrin activation. P38-MAPK is responsible for chemotaxis mediated by MCP-1 that is inhibited by the p38-MAPK inhibitor (7). Monocytes roll on the endothelial cells, interact with E-selectin, adhere to the endothelial cells by firm adhesion to ICAM-1 and VCAM-1, and then migrate into the subendothelium (8), MCP-1 increases adhesion of THP-1 cells to VCAM-1. The MCP-1-induced chemotaxis is also a potent stimulus for intracellular Ca^{2+} $[\text{Ca}^{2+}]_i$ mobilization in human monocytes; $[\text{Ca}^{2+}]_i$ increase represents a key event in chemokine-induced monocyte activation (9). The cysteinyl leukotrienes LTC₄, LTD₄, and LTE₄ induce many of the pathophysiological changes present in the lungs of patients with asthma, including airflow obstruction, mucus secretion, reduced mucociliary clearance, and inflammatory cell infiltration. Leukotriene receptor antagonists (LTRAs) prevent provoked asthmatic responses, ameliorate asthma symptoms, improve lung function, and reduce beta-agonist use in patients with persistent asthma. The neutralization of MCP-1 in allergic animals and instillation of MCP-1 in normal animals is related to leukotriene C₄ levels in the bronchoalveolar lavage and is directly induced in pulmonary mast cells by MCP-1 and (10) This indicates leukotrienes play an important role in MCP-1 expression. However, there are still no data about the influence of LTRAs on monocyte chemotaxis induced by MCP-1. We investigated whether montelukast could suppress chemokine-induced chemotaxis of monocyte and its detailed mechanisms to explore the other effects of LTRA on asthma and the potential effect on the cardiovascular diseases.

Materials and methods

The study protocol was approved by the Institution Review Board of Tri-Service General Hospital. Informed consent was obtained from three healthy adult volunteers and from each parent of cord blood donors.

Cell preparation

Peripheral blood samples were collected from three healthy individuals who had no personal or family history of allergy. Cord blood was harvested from the delivered placenta into a standard blood collection bag with citrate phosphate dextrose anticoagulant. Cord blood was collected from the delivered placenta to avoid any risk to

the mother or infant from the collection process. Blood samples were diluted with an equal volume of phosphate-buffered saline. Peripheral blood and cord blood mononuclear cells (PBMCs and CBMCs, respectively) were isolated by density-gradient centrifugation (Lymphoprep, Oslo, Norway). Human primary and cord blood monocytes were isolated from PBMCs and CBMCs by magnetic bead sorting with anti-CD14 monoclonal antibody (mAb) (MACS, Milteny Biotec, Germany).

The human monocytic cell line THP-1 (American Type Culture Collection, Rockville, MD, USA) was cultured in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin and 100 $\mu\text{g}/\text{ml}$ of streptomycin at 37°C, and 5% CO₂ in a humidified incubator (9).

Chemotactic assay

Monocyte chemotaxis was measured by using a 24-well Micro Chemotaxis Transwell (Corning

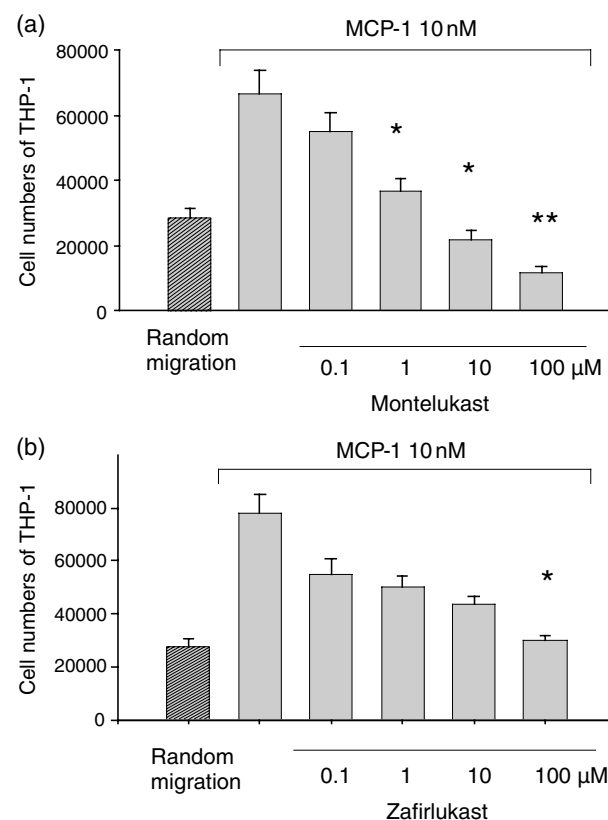


Fig. 1. Effect of montelukast (a) and zafirlukast (b) on MCP-1-induced THP-1 monocyte chemotaxis. Monocyte chemotaxis was measured by using transwell chamber with a diaphragm of polycarbonate membrane (5 μm pore size). *p < 0.05; **p < 0.01; indicates significant suppression of MCP-1-induced chemotaxis.

Costar, Cambridge, MA, USA). The cells were resuspended at $3 \times 10^6/\text{ml}$ in the presence or absence of montelukast, zafirlukast, or MK-886 (Cayman Chemicals, Ann Arbor, MI, USA) at various concentrations for 2 h and then loaded into the upper chamber of the Micro Chemotaxis chamber. The chemoattractant MCP-1 (10 nM, R&D Systems, Minneapolis, MN, USA) or leukotriene D4 (2.5 $\mu\text{g}/\text{ml}$, Cayman Chemicals, Ann Arbor, MI, USA) (in RPMI medium with 1 mg/ml of bovine serum albumin) was added to the lower chamber. The lower and upper chambers were separated by a polycarbonate membrane (5- μm pore size). The THP-1 cells were left to transmigrate for 2 h at 37°C in a humidified atmosphere with 5% CO_2 . After the incubation, the number of monocytes that migrated to the lower compartment was determined by counting the cells under light microscopy. The inhibition percentage was expressed as mean $\pm$ SEM for four separate experiments performed in duplicate.

Measurement of $[\text{Ca}^{2+}]_i$

Calcium flux assays were performed according to a method described previously (11, 12). For

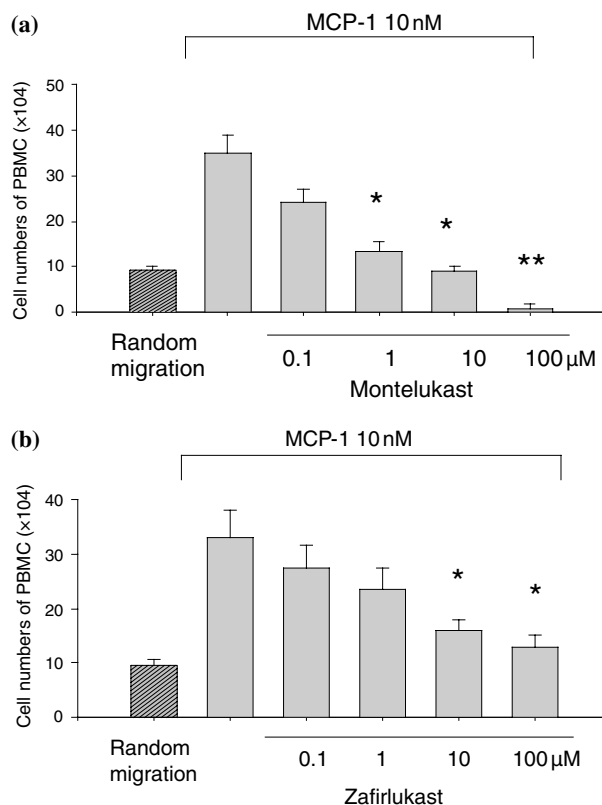


Fig. 2. Effects of montelukast (a) and zafirlukast, (b) on MCP-1-induced human primary monocytic cell (from PBMC) chemotaxis.

calcium measurements, recombinant human MCP-1 was obtained from R & D Systems, Minneapolis, MN, USA. Monocytic THP-1 cells were suspended at a density of $2 \times 10^6/\text{ml}$ in Hanks' balanced salt solution containing 1.3 mM CaCl_2 , 10 mM HEPES (pH 7.3), and 1% fetal calf serum. The cells were loaded with 10 mM Fura-2 AM (Molecular Probes Inc., Eugene, OR, USA) for 1 h at 30°C with occasional shaking. Loaded cells were washed twice with centrifugation and resuspended at a concentration of $10^6/\text{ml}$. The temperature of the cell suspension was kept at 37°C, and immediately before each assay the cells were collected by centrifugation, resuspended in 2 ml of Hanks' balanced salt solution/HEPES/FCS and added to a cuvette in a temperature-controlled holder (37°C), with continuous stirring. The cells were illuminated alternately by 340 and 380 nm excitation light. The emitted fluorescence over the excitation period at each wavelength was measured by a photomultiplier. The F340-to-F380 ratio (fura-2 signal intensities at 340 and 380 nm excitation light, respectively) was used as an indicator of $[\text{Ca}^{2+}]_i$.

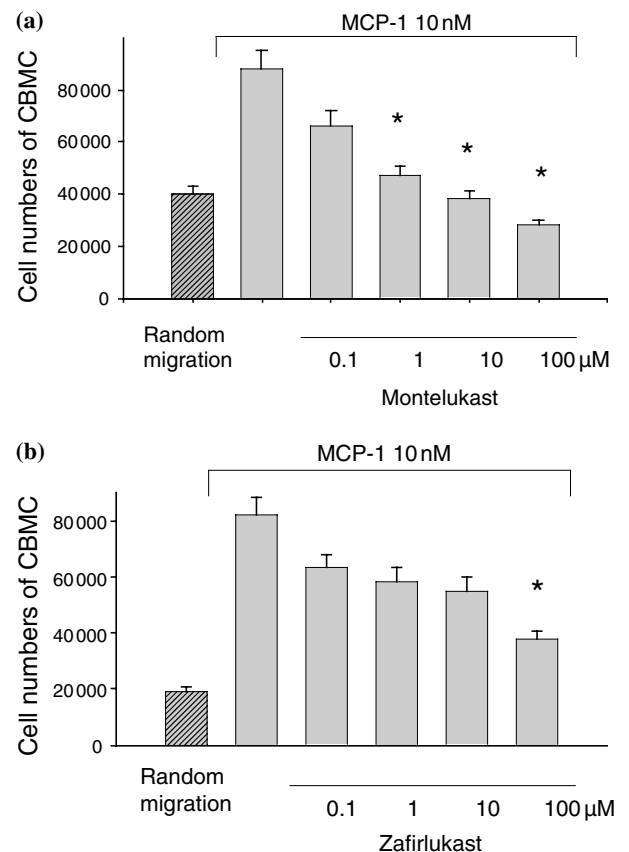


Fig. 3. Effects of montelukast, (a) and zafirlukast (b) on MCP-1-induced cord blood monocytic cell (from CBMC) chemotaxis.

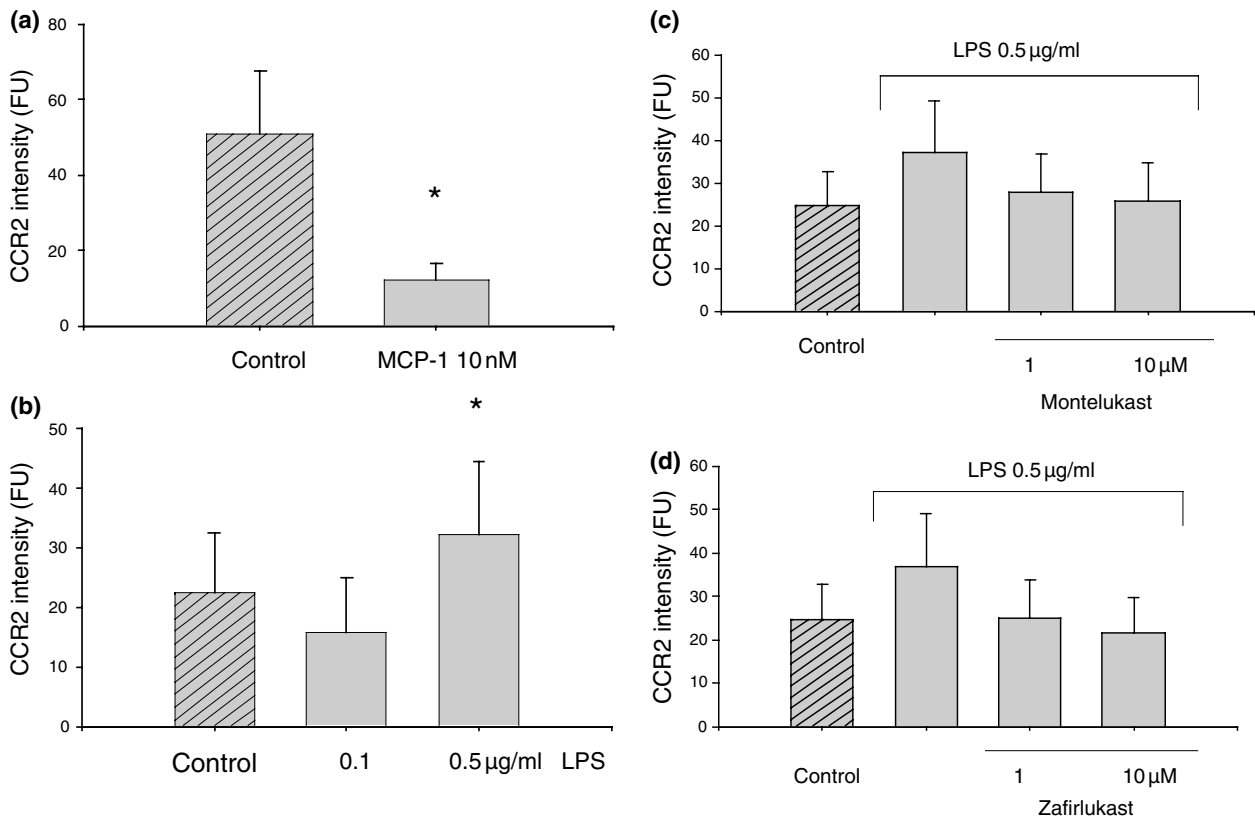


Fig. 4. CCR2 expression of THP-1 cell under different stimulation. (a) MCP-1 (10 nM) suppressed CCR2 expression on THP-1 cells ($p < 0.05$). (b) LPS 0.5 $\mu\text{g/ml}$ but not 0.1 $\mu\text{g/ml}$ induced CCR2 expression on THP-1 cells significantly ($p < 0.05$). Neither montelukast (c) nor zafirlukast (d) suppressed LPS-induced CCR2 expression on THP-1 cell significantly ($p > 0.05$).

The $[\text{Ca}^{2+}]_i$ was measured by testing the relative ratio of the fluorescence emission intensities ($[\lambda]_{\text{ex}} 505 \text{ nm}$) excited at both $[\lambda]_{\text{em}} 340$ and 380 nm , using the AR-CM-MIC Cation Measurement System (Spex Industries Inc., Metuchen, NJ, USA).

Flow cytometry

THP-1 Cells (1×10^6 cells/ml) were washed in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) (PBS-A), and then blocked with human purified IgG at 0.2 mg/ml (Sigma, St Louis, MO, USA) for 15 min at room temperature. Cells (0.5×10^6) were incubated with the indicated CCR2 antibody (R&D Systems) at 10 $\mu\text{g/ml}$ for 45 min at 4°C , and subsequently washed twice in cold PBS-A. In control experiments, an irrelevant isotype-matched control mAb was used. Flow cytometry was performed on a FACScan (Becton Dickinson, San Jose, CA, USA).

CCR2 is the receptor of MCP-1 on monocytes and is an important factor in MCP-1-induced chemotaxis of monocytes (7). We next examined

whether montelukast and zafirlukast suppressed CCR2 expression. The THP-1 cells were incubated with LPS or MCP-1 to determine whether montelukast or zafirlukast could suppress LPS or MCP-1-induced CCR2 expression of THP-1 cells.

MAPK assay

After treatment with or without montelukast (0.1 and 10 μM), the cells were stimulated with MCP-1 (10 nM) and lysed with equal volumes of ice-cold 150 μl lysis buffer. After centrifugation at 13,000 g for 15 min, equal amounts of cell lysates were analyzed by Western blot with anti-p38-MAPK and anti-phospho-p38-MAPK antibodies. Immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibody and the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Chalfont, UK).

ELISA assay

To evaluate whether MCP-1 induces the secondary release of cysteinyl leukotrienes (CysLTs),

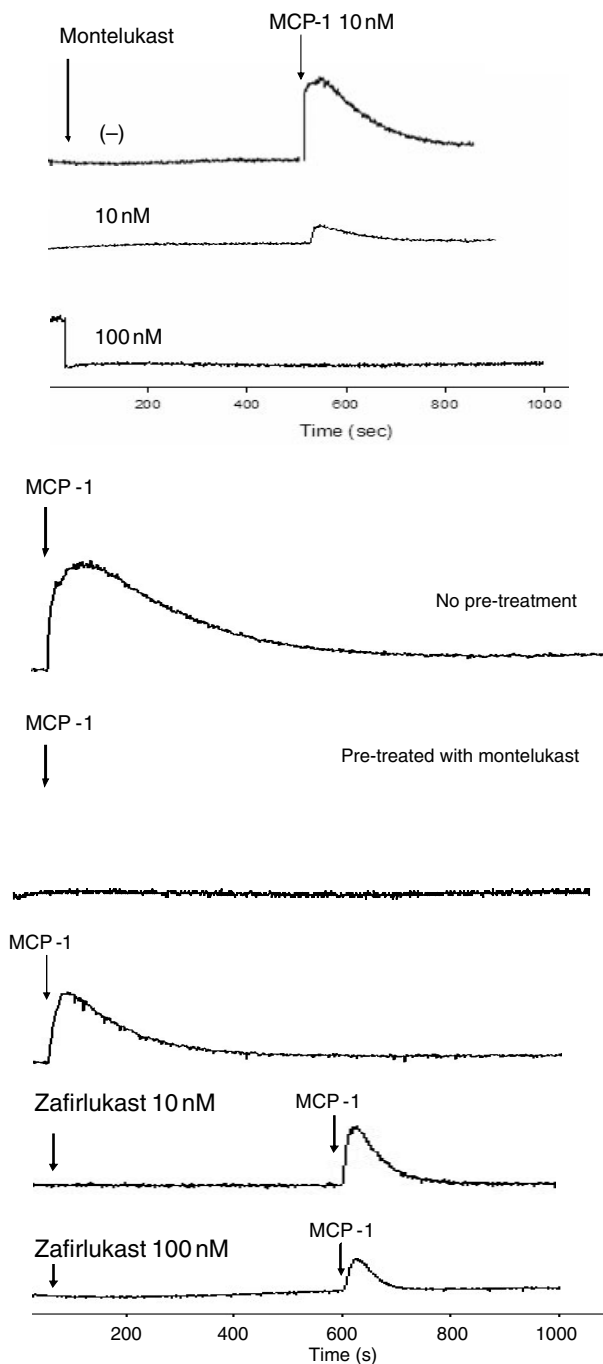


Fig. 5. (a) Effect of montelukast on MCP-1-induced intracellular calcium concentration ($[Ca^{2+}]_i$) change in human THP-1 monocytes. (b) Two hours pre-treatment with montelukast 100 nM suppressed the MCP-1-induced peak $[Ca^{2+}]_i$ in THP-1 cells completely. (c) Effect of zafirlukast on monocyte chemoattractant protein-1 (MCP-1)-induced intracellular calcium concentration ($[Ca^{2+}]_i$) change in human THP-1 monocytes.

which may then act at CysLT receptors to produce $[Ca^{2+}]_i$ and chemotactic responses, the THP-1 cells were treated with MCP-1 (0–100 nM). Cell supernatant was collected at 2

and 24 h. Concentrations of leukotriene B₄ (LTB₄) and CysLTs were determined by a commercially available ELISA assay system (R&D Systems, Minneapolis, MN, USA). Assays were performed using the protocols recommended by the manufacturer.

Statistical analysis

All data are presented as mean $\pm$ SD, except results of the chemotaxis assay. One-way ANOVA was used for all statistical comparisons, and the Student–Newman–Keuls test was conducted for multiple comparisons. A $p < 0.05$ was considered indicative of significant between-group differences. SigmaStat software (Jandel Scientific, Erkrath, Germany) was used for all statistical analysis.

Results

Montelukast and zafirlukast suppress chemotaxis of THP-1

Cell migration was $2.8 \pm 0.5 \times 10^4$ cells in the absence of MCP-1 (random migration), increasing to $6.6 \pm 0.6 \times 10^4$ cells after MCP-1 stimulation. THP-1 cells were incubated with montelukast or zafirlukast to determine whether MCP-1-induced chemotaxis of monocytes was inhibited. Montelukast (1, 10 and 100 μ M) and zafirlukast (100 μ M) suppressed MCP-1-induced THP-1 monocytic cell chemotaxis significantly at different doses (Fig. 1a and b). However, montelukast suppressed MCP-1-induced monocyte chemotaxis more effectively than zafirlukast at doses of 10 μ M (30% vs. 53%, respectively, in the lower chamber; $p < 0.05$) and 100 μ M (17.5% vs. 37.5%, respectively; $p < 0.05$).

Montelukast and zafirlukast suppress chemotaxis of human primary monocytes

Cell migration of human primary monocytes (from PBMCs) was $10.3 \pm 1.5 \times 10^4$ cells in the absence of MCP-1, increasing to $35.8 \pm 3.8 \times 10^4$ cells after MCP-1 stimulation. Migration of human primary monocytes was more effective than that of THP-1 cells (10.3×10^4 vs. 2.8×10^4 cells, respectively; $p < 0.05$) in the absence of MCP-1 and after MCP-1 stimulation (35.8×10^4 vs. 6.6×10^4 cells, respectively; $p < 0.05$). Montelukast suppressed MCP-1-induced monocyte chemotaxis more effectively than zafirlukast (22% vs. 49%, respectively, in the lower chamber; $p < 0.05$) and 100 μ M (28% vs. 39%, respectively; $p < 0.05$) (Fig. 2a and b). The effect of montelukast

ast and zafirlukast on MCP-1-induced monocyte chemotaxis was dose-dependent.

Montelukast and zafirlukast suppress chemotaxis of human cord blood monocytes

Chemotaxis of human cord blood monocytes (from CBMCs) was less effective than that of adult primary monocytes (4×10^4 vs. 10.3×10^4 cells, respectively) in the absence of MCP-1 and after MCP-1 stimulation (9×10^4 vs. 35.8×10^4 cells, respectively) (Fig. 3a and b). Montelukast suppressed MCP-1-induced monocyte chemotaxis more effectively than zafirlukast at doses of $10 \mu\text{M}$ (39% vs. 49%, respectively, in the lower chamber; $p < 0.05$) and $100 \mu\text{M}$ (30% vs. 39%, respectively; $p < 0.05$) (Fig. 3a and b). The effect of montelukast and zafirlukast on MCP-1-induced monocyte chemotaxis was dose-dependent.

Montelukast and zafirlukast did not suppress CCR2 of THP-1 cells

After 4 h incubation, MCP-1 (10 nM) suppressed CCR2 expression on THP-1 cells ($p < 0.05$) (Fig. 4a). LPS $0.5 \mu\text{g/ml}$, but not $0.1 \mu\text{g/ml}$, significantly induced CCR2 expression on THP-1 cells ($p < 0.05$) (Fig. 4b). In contrast, there was no significant suppression effect of montelukast or zafirlukast on LPS-induced CCR2 expression on THP-1 cells ($p > 0.05$) (Fig. 4c and d).

Montelukast and zafirlukast suppress $[\text{Ca}^{2+}]_i$ of human monocytes

The increase in $[\text{Ca}^{2+}]_i$ represents a key event in chemokine-induced monocyte activation. A rapid, transient and concentration-dependent in-

crease in $[\text{Ca}^{2+}]_i$ was observed with MCP-1 treatment (10 nM). Treatment with montelukast (1–100 μM) reduced MCP-1-induced peak $[\text{Ca}^{2+}]_i$ in THP-1 cells (Fig. 5a). Once THP-1 was pre-treated with montelukast 100 μM for 2 h, MCP-1 did not induce any $[\text{Ca}^{2+}]_i$ influx wave (Fig. 5b). Treatment with zafirlukast (10–100 μM) also reduced MCP-1-induced peak $[\text{Ca}^{2+}]_i$ in THP-1 cells (Fig. 5c). However, zafirlukast (10–100 μM) did not suppress MCP-1-induced peak $[\text{Ca}^{2+}]_i$ in THP-1 cells as effectively as did montelukast.

Montelukast suppresses p38 expression of THP-1 cells

MCP-1-mediated p38-MAPK signaling is also an important factor in the MCP-1-induced chemotaxis of monocytes (7). Pre-treatment with montelukast (10 nM) for 2 h suppressed MCP-1-induced p38 MAPK expression on THP-1 cells (Fig. 6).

MCP-1 did not induce LTB₄ and cysteinyl leukotriene expression of THP-1 cells. MCP-1 did not induce LTB₄ and cysteinyl leukotriene expression of THP-1 cells at various concentrations for 2 h (Fig. 7a) and 24 h. LTD₄ did induce chemotaxis of THP-1, which was inhibited by montelukast (1 μM) (Fig. 7b). MK-886 (0.1–100 μM) did not inhibit MCP-1-induced chemotaxis of THP-1 cells.

Discussion

Chemokines play an important role in the pathophysiology of asthma and allergy. Airway hyperreactivity may be mediated by MCP-1 through CCR2 because allergen-induced, as well as direct MCP-1 instilled-induced changes in airway hyperreactivity are significantly attenu-

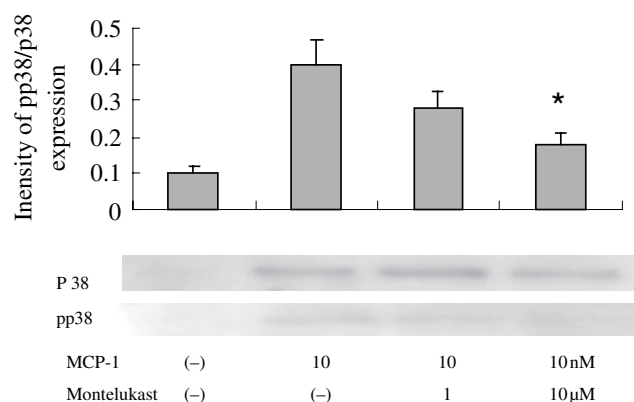


Fig. 6. Suppressive effect of montelukast on MCP-1-induced p38 and pp38 in THP-1 monocytic cells.

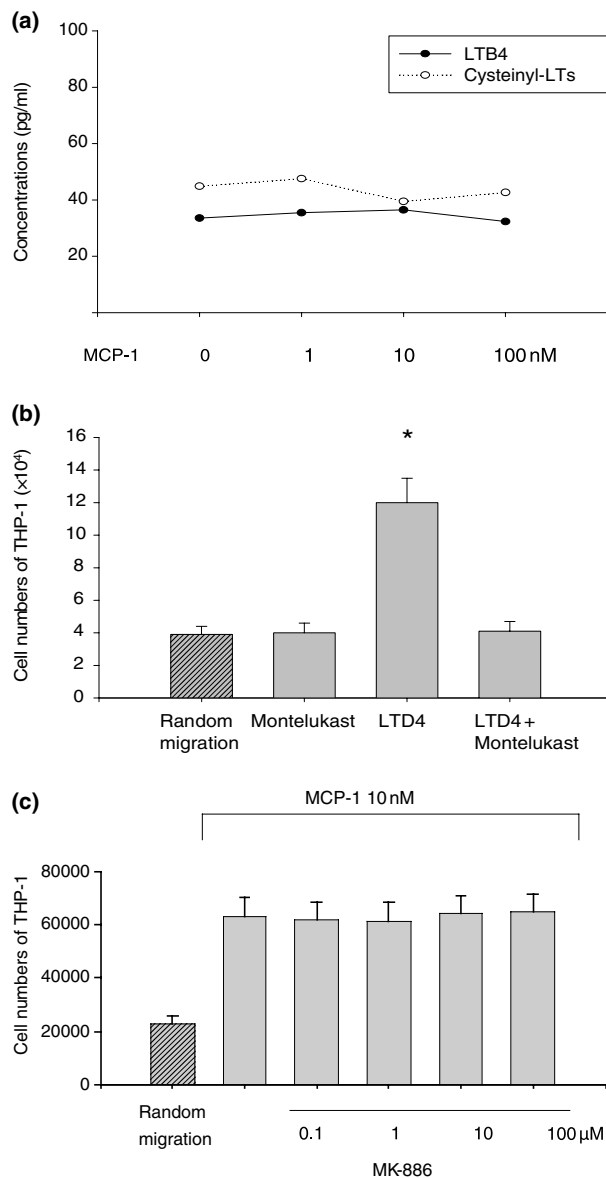


Fig. 7. (a) MCP-1 (1–100 nM) cannot induce LTB4 and cysteinyl leukotriene expression of THP-1 cell for 2 h. (b) LTD4 could induce chemotaxis of THP-1 cell. However, the effect could be inhibited by montelukast (1 μ M). (c) MK-886 could not inhibit MCP-1-induced chemotaxis of THP-1 cell.

ated in CCR2^{-/-} mice (3). Monocyte infiltration involved with MCP-1 and its hematopoietic cell receptor, CCR2, is important in inflammatory disorders of the lung (1, 2). MCP-1 stimulates leukocyte chemotaxis to inflammatory sites, such as those that occur in rheumatoid arthritis, atherosclerosis and asthma, by using CCR2 (4). Activation of leukocytes and migration from the circulation to areas of inflammation appear to be important factors in the immunological responses associated with asthma. MCP-1 plays a key role in monocyte recruitment by integrin

activation and by promoting migration to the vessel wall (7). MCP-1 and CCR2 are potentially important therapeutic targets for the treatment of hyperreactive airway disease and for repair of injured bronchial epithelial cells in asthma (3, 5).

Montelukast provides some anti-inflammatory effects, as reflected by its ability to reduce exhaled nitric oxide levels and peripheral blood and airway eosinophils, the pivotal cells in the airway inflammation of asthma (13). Montelukast has been found to reduce airway eosinophilic inflammation by down-regulation of Mac-1 expression and by eosinophil chemotaxis in patients with chronic asthma (13). Cysteinyl leukotrienes are important inflammatory factors in asthma and allergic diseases, and montelukast is a competitive antagonist of these substances at the cysteinyl leukotriene type-1 receptor 1. In this study, the two LTRAs inhibited the chemotaxis of monocytes by suppressing intracellular $[Ca^{2+}]_i$. Thus, LTRAs may improve asthmatic symptoms due, in part, to the suppression of MCP-1-induced monocyte infiltration in the lung. Transmigration of monocytes to the subendothelial space is the initial step of atherosclerotic plaque formation and inflammation (7). Monocyte and MCP-1 are important in the immunological and inflammatory processes associated with the pathogenesis and progression of cardiovascular diseases, such as atherosclerosis (14–16), acute coronary syndrome (17) and congestive heart failure (CHF) (18). Therefore, LTRAs may be potential therapeutic medications for cardiovascular diseases.

Integrin activation and chemotaxis are important functions involved with monocytes for transmigration. MCP-1 stimulated β 1 integrin-dependent, but not β 2 integrin-dependent cell adhesion in a time-dependent manner. ERK is responsible for integrin activation, and p38-MAPK and Rho are responsible for chemotaxis mediated by MCP-1. Rho and the Rho kinase are upstream of p38-MAPK in MCP-1-mediated signaling (7). In this study, the suppressing effect of montelukast on MCP-1-mediated p38-MAPK signaling also contributed to the suppression of chemotaxis in THP-1 cells. CCR2 is the receptor of MCP-1 on monocytes, responsible for chemotaxis of monocytes. So we initially hypothesize that montelukast might suppress MCP-1-induced chemotaxis of monocytes by down-regulation of CCR2 expression. However, we found that MCP-1 could not upregulate CCR2 expression but enhance monocyte chemotaxis that can be inhibited by LTRA. In contrast, we found that

both LTRAs couldn't suppress LPS-induced CCR2 expression of THP-1 cells significantly. Taken together, LTRAs may suppress MCP-1 induced monocyte chemotaxis in the post-receptor step.

In our study, the key issues were whether secondary release of leukotrienes mediated the ability of MCP-1 to increase $[Ca^{2+}]_i$, phosphorylate p38 and induced chemotaxis in monocytes. There is evidence that exogenous LTD4 can trigger intracellular Ca^{+2} (19, 20). LTB4 is a potent chemoattractant, and LTD4 also can induce chemotaxis of THP-1 cells (Fig. 7b). However, we found that MCP-1 could not induce cysteinyl leukotrienes (LTC4/D4/E4) or leukotriene B4 production by THP-1 cells (Fig. 7a). Thus, anti-leukotriene drugs that inhibit MCP-1-induced chemotaxis unlikely do so by inhibiting the secondary release of leukotrienes.

Another key issue in the present study was whether LTRAs inhibited MCP-1-induced monocyte chemotaxis via the $cysLT_1$ (or LT_2) receptors. We showed that both LTRAs had IC_{50} values of about $10 \mu M$ in the present study. These IC_{50} values were much higher than the IC_{50} value of zafirlukast ($0.6 \mu M$) on LTD4-induced tracheal mucus secretion (21) and the IC_{50} value of montelukast (about $4 \mu M$) in inhibiting specific binding of leukotriene D4 to guinea pig lung (22). These LTRAs may, therefore, not act as specific $cysLT_1$ antagonists. Further studies are necessary to clarify the effect of LTRAs on the chemotaxis of other inflammatory cells.

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