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Cysteinyl leukotrienes induce monocyte chemoattractant protein 1 in human monocytes/macrophages

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Summary

Background Monocytes/macrophages have a cysteinyl leukotriene 1 (CysLT1) receptor, but its function is poorly understood.

Objective To elucidate the biological function of the CysLT1 receptor of human monocytes/macrophages.

Methods We examined the production of TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, monocyte chemoattractant protein 1 (MCP-1), macrophage colony-stimulating factor (M-CSF), and eotaxin induced by CysLTs (leukotriene (LT)C4, -D4, and -E4) in THP-1 cells, a human monocytic leukaemia cell line, and peripheral blood $CD14^+$ monocytes/macrophages. Moreover, we examined the effect of CysLTs on the expression of β-chemokine receptor 2B (CCR2B) as the receptor of MCP-1 by Western blot analysis.

Results ELISA revealed that CysLTs induced MCP-1 in THP-1 cells and peripheral blood $CD14^+$ monocytes/macrophages, but not other cytokines. PCR demonstrated that CysLTs increased MCP-1 mRNA expression in THP-1 cells, and Western blotting showed that CysLTs increased the expression of CCR2B in THP-1 cells. Moreover, we demonstrated that pranlukast, a CysLT1 receptor antagonist, blocked MCP-1 production by CysLTs in THP-1 cells almost completely, and partially inhibited MCP-1 release by CysLTs in peripheral blood $CD14^+$ monocytes/macrophages and CCR2B expression by CysLTs in THP-1 cells.

Conclusion CysLTs induce MCP-1 and increase CCR2B expression in human monocytes/macrophages.

Keywords CCR2B, cysteinyl leukotrienes, monocyte chemoattractant protein 1, monocytes/macrophages, pranlukast

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Introduction

Various roles of cysteinyl leukotrienes (CysLTs), such as leukotriene C4 (LTC4), -D4, and -E4, have been reported in bronchial asthma [1–4]. CysLTs induce contraction of the tracheal muscle [1]. CysLTs have potent effects on leucocyte trafficking, airway mucus secretion, and collagen synthesis [2–4]. Normal peripheral blood leucocytes, such as basophils, eosinophils, B lymphocytes, and monocytes/macrophages, have a CysLT1 receptor [5]. However, the function of these cells, especially monocytes/macrophages, is poorly understood.

Alveolar macrophages (AM) are the most abundant cells not only in the alveoli and distal air spaces but also in the conducting airways [6]. In asthma, AM are activated [7]. Activated AM may participate in the inflammatory events associated with allergic disease of the lower airways, including release of cytokines, chemokines, arachidonic acid metabolites, products of activated O_2, and by direct interaction with T lymphocytes [8–11].

We examined cytokine production, including TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, monocyte chemoattractant protein 1 (MCP-1), macrophage colony-stimulating factor (M-CSF), and eotaxin, of human monocytes/macrophages induced by CysLTs to elucidate the biological function of the CysLT1 receptor of human monocytes/macrophages.

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Methods

Cell culture and stimulation conditions

THP-1 cells [12], a human monocytic leukaemia cell line that has a CysLT1 receptor [13], obtained from the American Type Culture Collection, were maintained at 37 °C under humidified 5% CO_2 as stationary cultures. The cells were grown in RPMI 1640 medium containing 10% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood from 10 healthy medication-free volunteers, with informed consent, by Histopaque 1077 (Sigma

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Chemical Co., St Louis, MO, USA) gradient centrifugation and washing. Purification of individual cell subpopulations was achieved with a high magnetic gradient Mini MACS purification system (Miltenyi, Sunnyvale, CA, USA). $CD14^+$ monocytes/macrophages were isolated by depletion of non-monocytes (negative selection) with a Monocyte Isolation Kit II (Miltenyi). The purity of the isolated cells was determined using the respective fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (Becton-Dickinson Biosciences, San Diego, CA, USA) and flow cytometric analysis (FACScan; Becton-Dickinson Biosciences). Cells were exposed to LTC4, -D4, or -E4 (Sigma Chemical Co). Some samples were pretreated with pranlukast, a CysLT1 receptor antagonist, provided by ONO Pharmaceutical Co. (Osaka, Japan) for 30 min before the addition of CysLTs. Supernatant fluid, both before and after the addition of CysLTs, was harvested for determination of cytokine levels and then stored at $-20\,^{\circ}C$.

Determination of the concentrations of cytokines

The concentrations of TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-8, and IL-10 in the supernatant fluid were measured with a cytometric bead array (CBA) kit (BD PharMingen, San Diego, CA, USA) according to the manufacturer's instructions, as described previously [14–16], with modification of the data analysis, using GraphPad Prism software (GraphPad Prism Software, San Diego, CA, USA). Briefly, a CBA involves a series of beads exhibiting discrete fluorescence intensities at 670 nm. Each series of beads is coated with a monoclonal antibody against a single cytokine, and a mixture of the beads can detect six cytokines in one sample. A secondary phycoerythrin (PE)-conjugated monoclonal antibody stains the beads proportionately to the amount of bound cytokine. After fluorescence intensity calibration and electronic colour compensation procedures, standard and test samples were analysed with a FACScan flow cytometer equipped with CellQuest software (BD PharMingen). Data were transferred to GraphPad Prism. Starting with standard dilutions, the software performed log transformation of the data and then fitted a curve to 10 discrete points using a four-parameter logistic model. The calibration curve created for each cytokine was used to estimate the cytokine concentrations in the samples. The detection limits for TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-8, and IL-10 were 2.8, 7.2, 2.6, 2.6, 2.5, 3.6, and 2.8 pg/mL, respectively.

The concentrations of MCP-1, M-CSF, and eotaxin in the supernatant fluid were determined with sandwich-type ELISA kits (R&D Systems, Minneapolis, MN, USA). The detection limits for MCP-1, M-CSF, and eotaxin were 5.0, 9.0, and 5.0 pg/mL, respectively.

Reverse transcriptase-polymerase chain reaction

Total RNA was prepared from each cell type using a TRIzol reagent (Invitrogen, Leek, the Netherlands). RT-PCR was performed with Gene Amp and an oligo dT primer (Applied Biosystems, Foster City, CA, USA) for RT, and Taq polymerase (Roche Diagnostics GmbH, Mannheim, Germany) for PCR. The primers used were as follows: (1) MCP-1: forward, 5′-ACTGAAGCTCGCACTCTC-3′, and reverse, 5′-CTTGGGTTGTGGAGTGAG-3′; and (2) GAPDH: forward, 5′-ACCACAGTCCATGCCATCAC-3′, and reverse, 5′-TCCACCACCCTGTTGCTGTA-3′. Quantitation of bands was conducted with a Kodak Digital Science 1D (Eastman Kodak Company, New Haven, CT, USA).

Western blot analysis

Total cell lysates were obtained by the incubation of cell samples in ice-cold lysis buffer (1 mM ethylene diamine tetraacetic acid, 0.2 mM phenylmethylsulphonyl fluoride) with protease inhibitors (1 μM leupeptin and 1 μM pepstatin) and centrifugation to remove debris (12 000 ***g*** for 10 min at 4 °C). The protein concentrations of the samples were determined with a Bio-Rad (Hercules, CA, USA) protein concentration reagent. Samples containing 20 μg of protein were separated in denaturing 10% polyacrylamide gels and then transferred to polyvinylidene difluoride membranes. After three washes in TBST (40 mM Tris-HCl, pH 7.6, 300 mM NaCl and 0.5% Tween-20), the membranes were incubated with a 1 : 1000 dilution of goat polyclonal anti-β-chemokine receptor 2B (CCR2B) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TBST containing 5% non-fat dry milk at room temperature for 1 h. After three washes in TBST, the membranes were incubated with a 1 : 2500 dilution of horseradish peroxidase-conjugated rabbit anti-goat IgG (Bio-Rad) for 1 h at room temperature. Immunoreactive proteins were detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA) and analysed by radiography. Quantification of bands was conducted with a Kodak Digital Science 1D.

Statistical analysis

The values of cytokine concentrations are expressed as means ± SD. Statistical analysis was performed with the Wilcoxon's matched paired test, with a *P*-value of less than 0.05 being taken as significant.

Results

Figure 1 demonstrates MCP-1 release by the stimulation of LTC4, -D4, and -E4 for 4 h in THP-1 cells. CysLTs (10^{-6}–10^{-8} M) induced MCP-1 in THP-1 cells, and LTC4 and -D4 significantly induced MCP-1 more than -E4 ($P<0.01$). Interestingly, 10^{-7} M of LTC4 and -D4 significantly induced MCP-1 more than 10^{-6} and 10^{-8} M of those, respectively ($P<0.01$). CysLTs (10^{-6} to 10^{-8} M) did not induce TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, M-CSF, or eotaxin in THP-1 cells (Table 1). The time course of MCP-1 production was examined by incubating THP-1 cells with 10^{-6} to 10^{-8} M LTD4 during a 24 h period (Fig. 2). MCP-1 production could be observed 1 h after the addition of LTD4, and gradually increased over 24 h.

The concentrations of MCP-1 in the culture fluid of THP-1 cells exposed to CysLTs, in the presence or absence of pranlukast, for 4 h are shown in Fig. 3. The production of MCP-1 induced by CysLTs was significantly inhibited by 10^{-6}–10^{-8} M pranlukast. The effect of pranlukast was dose related. The time courses of the inhibitory effects of

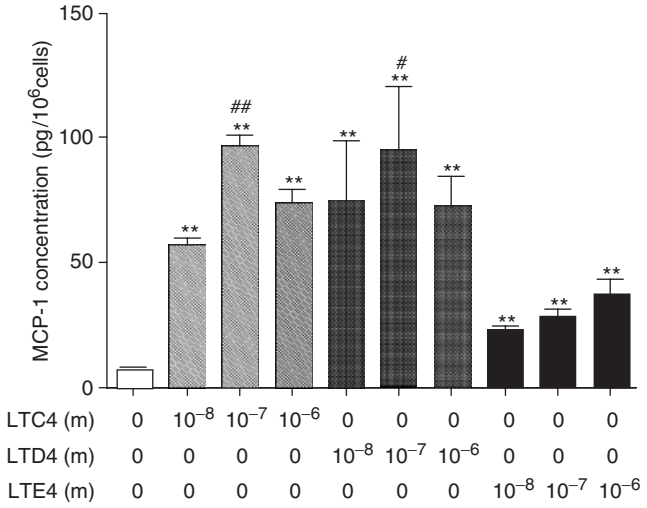

Fig. 1. Monocyte chemoattractant protein-1 (MCP-1) production, as measured by ELISA, in THP-1 cells stimulated with LTC4, -D4, or -E4 for 4 h. Data ($n = 8$) are presented as means ± SD. $^{**}P < 0.01$, compared with the culture fluid of cells not treated with LTC4, -D4, or -E4. $\#\#P < 0.01$, and $\#P < 0.05$, compared with the culture fluid of cells treated with 10^{-6} or 10^{-8} M LTC4, or -D4.

Table 1. Other cytokine productions stimulated by LTD4 in THP-1 cells and peripheral blood $CD14^+$ monocytes/macrophages (pg/mL)

	THP-1 cells		Peripheral blood $CD14^+$ monocytes/macrophages	
	Medium only	$+10^6$ M LTD4	Medium only	$+10^6$ M LTD4
TNF-α	<2.8	<2.8	<2.8	<2.8
IL-1β	<7.2	<7.2	<7.2	<7.2
IL-2	<2.6	<2.6	<2.6	<2.6
IL-4	<2.6	<2.6	3.0 ± 0.6	2.9 ± 0.5
IL-6	2.9 ± 0.4	3.1 ± 0.6	3.2 ± 0.8	3.3 ± 0.9
IL-8	<3.6	<3.6	4.2 ± 0.5	4.4 ± 0.7
IL-10	<2.8	<2.8	<2.8	<2.8
M-CSF	<9.0	<9.0	<9.0	<9.0
Eotaxin	<5.0	<5.0	<5.0	<5.0

M-CSF, macrophage-colony stimulating factor.

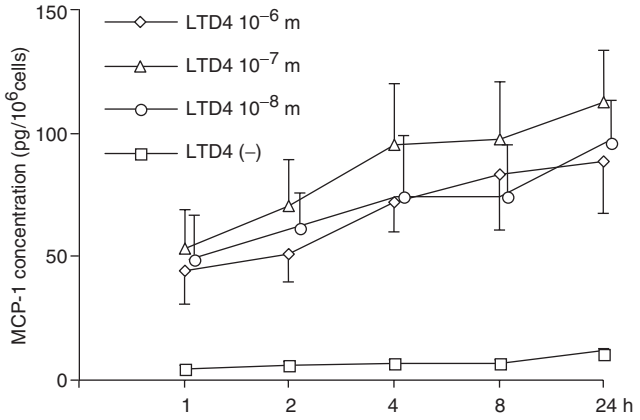

Fig. 2. Kinetics of monocyte chemoattractant protein 1 (MCP-1) production, as measured by ELISA, in THP-1 cells stimulated with LTD4. Data ($n = 8$) are presented as means ± SD.

pretreatments with 10^{-7} and 10^{-8} M pranlukast on MCP-1 production induced by 10^{-6}–10^{-8} M LTD4 were examined in THP-1 cells over 24 h. Pranlukast similarly blocked MCP-1 production induced by 10^{-6}–10^{-8} M LTD4 during the experiment (data not shown).

The purity of the $CD14^+$ cells obtained after negative selection with the MACS system was 87.1 ± 5.4%. The MCP-1 production induced by CysLTs and the inhibitory effect of pranlukast on MCP-1 production were examined by incubating peripheral blood $CD14^+$ monocytes/macrophages with CysLTs for 4 h, with some samples being pretreated with 10^{-6}–10^{-8} M pranlukast (Fig. 4). CysLTs (LTC4, -D4, and -E4) induced MCP-1 in peripheral blood $CD14^+$ monocytes/macrophages (192.2 ± 25.2 pg/10^5 cells, 211.4 ± 20.6 pg/10^5 cells, and 179.5 ± 28.0 pg/10^5 cells, respectively). Pretreatment with pranlukast significantly inhibited MCP-1 production induced by CysLTs in peripheral blood $CD14^+$ monocytes/macrophages in a dose-related manner. CysLTs (10^{-6}–10^{-8} M) did not induce TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, M-CSF, or eotaxin in peripheral blood $CD14^+$ monocytes/macrophages (Table 1).

RT-PCR demonstrated that MCP-1 mRNA expression was significantly increased 4 h after the addition of 10^{-6} M CysLTs in THP-1 cells ($P < 0.05$) (Fig. 5). Moreover, pretreatment with 10^{-6} M pranlukast significantly decreased MCP-1 mRNA expression caused by the stimulation of CysLTs in THP-1 cells ($P < 0.05$).

The effect of MCP-1 on the expression of CCR2B, an MCP-1 receptor [17], was examined using Western blot analysis (Fig. 6). The expression of CCR2B increased significantly 24 h after the introduction of 10^{-6} M CysLTs into THP-1 cells ($P < 0.05$). Moreover, pretreatment by 10^{-6} M pranlukast significantly decreased CCR2B expression stimulated by CysLTs in THP-1 cells ($P < 0.05$).

Discussion

A previous study demonstrated that LTD4 activates mitogen-activated protein kinase in THP-1 cells [13]. Macrophage inflammatory protein-1α (MIP-1α), TNF-α, and nitric oxide were produced in NR8383 cells, a rat AM cell line, exposed to lipopolysaccharide pretreated with LTD4 [6]. Our present study showed that LTC4, -D4, and -E4 induced MCP-1 in THP-1 cells and $CD14^+$ monocytes/macrophages, and increased CCR2B expression in THP-1 cells. LTC4, -D4, and -E4 enhance autocrine MCP-1 production by increasing CCR2B expression in monocytes/macrophages. Our present data suggest that MCP-1 is produced via the bonding of LTC4, -D4, and -E4 to the CysLT receptor because pranlukast inhibits MCP-1 production induced by LTC4, -D4, and -E4. MCP-1 production induced by 10^{-7} M LTC4 and -D4 was significantly higher than that induced by 10^{-6} M LTC4 and -D4. Ménard and Bissonnette [6] reported that MIP-1α production induced by 10^{-11} M LTD4 in AM was more than that induced by 10^{-6}, 10^{-8}, 10^{-10}, 10^{-12}, or 10^{-14} M LTD4. Therefore, it is unlikely that cytokine production induced by CysLTs is dose related. Our present data revealed that LTC4 and LTD4 were equally potent in stimulating MCP-1 release in THP-1 cells, and LTC4, LTD4,

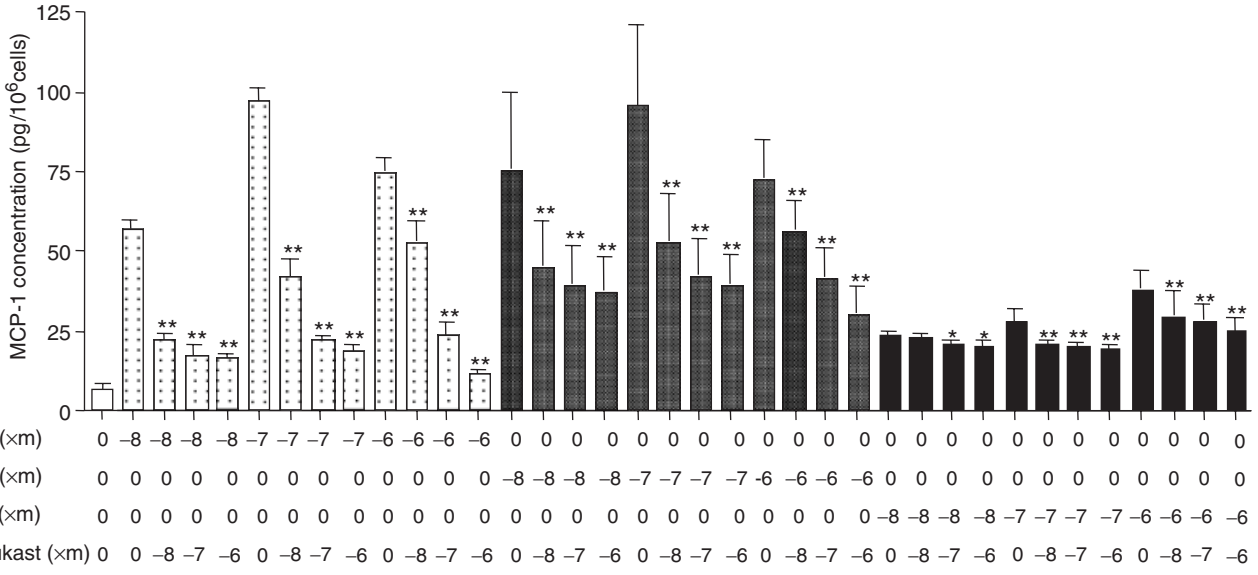

Fig. 3. Inhibitory effect of pranlukast on monocyte chemoattractant protein 1 (MCP-1) release, as measured by ELISA, in THP-1 cells stimulated with LTC4, -D4, or -E4 for 4 h (cells pretreated with pranlukast for 30 min before LTC4, -D4, or -E4 treatments). Data ($n = 8$) are presented as means ± SD. $^{**}P < 0.01$, and $^{*}P < 0.05$, compared with the culture fluid of cells treated with LTC4, -D4, or -E4 only (no pretreatment).

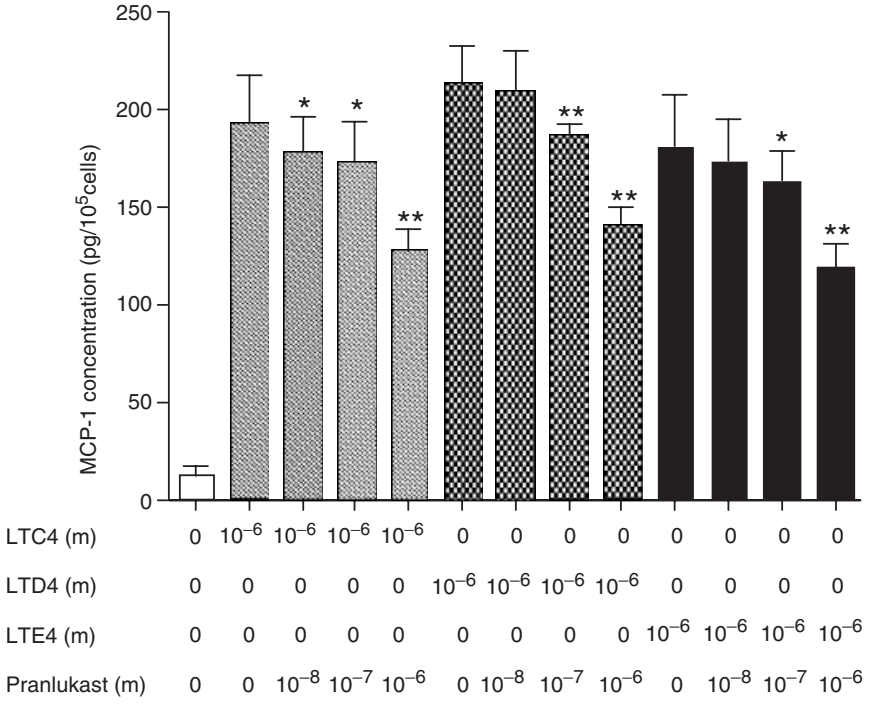

Fig. 4. Monocyte chemoattractant protein 1 (MCP-1) production and the inhibitory effect of pranlukast on MCP-1 release, as measured by ELISA, in peripheral blood $CD14^+$ monocytes/macrophages stimulated with LTC4, -D4, or -E4 for 4 h (cells pretreated with pranlukast for 30 min before LTC4, -D4, or -E4 treatments). Data ($n = 10$) are presented as means ± SD. $^{**}P < 0.01$ and $^{*}P < 0.05$, compared with culture fluid of cells treated with LTC4, -D4, or -E4 only.

and LTE4, in peripheral blood $CD14^+$ monocytes/macrophages. LTD4 would be expected to be about tenfold more potent for calcium flux at the CysLT1 receptors than LTC4 (and 100-fold more than LTE4) [18, 19]. The mechanism of MCP-1 production via CysLT1 receptors of monocytes/macrophages stimulated by CysLTs is different from that of the contraction via CysLT1 receptors of airway smooth muscle stimulated by CysLTs. It is likely that the potencies of LTC4, LTD4, and LTE4 for MCP-1 production from monocytes/macrophages are different from those for the contraction of airway smooth muscle via calcium flux.

MCP-1 is part of the C–C chemokine β subfamily and exhibits its most potent chemotactic activity towards monocytes and T lymphocytes [20]. MCP-1 has multiple functions, such as causing an increase in cytosolic calcium levels, stimulating superoxide anion production and lysosomal

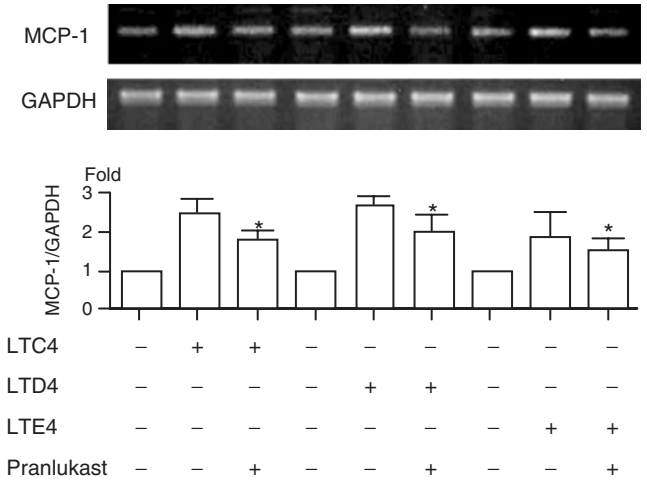

Fig. 5. Monocyte chemoattractant protein 1 (MCP-1) mRNA expression and the inhibitory effect of pranlukast on MCP-1 mRNA expression, as measured by RT-PCR, in THP-1 cells stimulated with 10^{-6} M LTC4, -D4, or -E4 for 4 h (cells pretreated with 10^{-6} M pranlukast for 30 min before LTC4, -D4, or -E4 treatments). Representative data are shown. After densitometric analysis, data ($n = 4$) were expressed as fold-increase above untreated cells and presented as means ± SD. *$P < 0.05$ vs. cells treated with LTC4, -D4, or -E4 only.

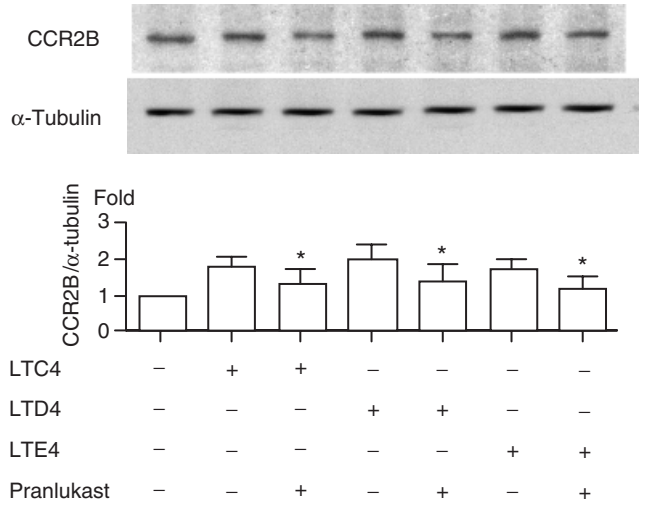

Fig. 6. CCR2B expression and the inhibitory effect of pranlukast on CCR2B expression, as measured by Western blot analysis, in THP-1 cells stimulated with 10^{-6} M LTC4, -D4, or -E4 for 24 h (cells pretreated with 10^{-6} M pranlukast for 30 min before LTC4, -D4, or -E4 treatments). Representative data are shown. After densitometric analysis, data ($n = 4$) were expressed as fold-increase above untreated cells and presented as means ± SD. *$P < 0.05$ vs. cells treated with LTC4, -D4, or -E4 only.

enzyme release in monocytes [21]. MCP-1 provokes mast cell aggregation [22] and histamine release in mast cells [23]. Moreover, MCP-1 induces chemotaxis and calcium mobilization in eosinophils [24]. MCP-1 induces LTC4 in the bronchoalveolar lavage of mice, pulmonary mast cells of mice, and human basophil leucocytes [25, 26]. A positive feedback loop might exist in the expression of MCP-1 and the CysLTs. MCP-1 causes not only the migration of monocytes but also of eosinophils and basophils, and induces basophil histamine release [20]. It is likely that CysLT-induced MCP-1 in monocytes/macrophages, such as AM, is related to the pathogenesis of bronchial asthma. In asthma, AM are activated and participate to create the cytokine milieu [7]. MCP-1 induced by AM and/or AM activated by MCP-1 may modify pulmonary inflammation in asthma. Our present study demonstrated that other cytokines, including TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, M-CSF, and eotaxin, stimulated with CysLTs were not produced in THP-1 cells or peripheral blood $CD14^+$ monocytes/macrophages. A previous paper reported that IL-4 was produced in PBMC from mite antigen-positive asthmatic patients stimulated with the mite antigen [27]. It is likely that cytokines other than MCP-1 were not produced in THP-1 cells or peripheral blood $CD14^+$ monocytes/macrophages because the stimulator was not a specific allergen.

A CysLT1 receptor antagonist inhibits contraction of the tracheal muscle [1]. Pranlukast has some actions in asthma other than as an antagonist of the CysLT1 receptor, including immunomodulation [27]. We have previously demonstrated that pranlukast inhibits TNF-α-induced nuclear factor-κB activation in monocytes/macrophages [28]. Pranlukast could exert its anti-inflammatory action via the inhibition of MCP-1 production induced by CysLT in monocytes/macrophages. Our present data demonstrated that 10^{-6} M pranlukast could block CysLT-induced MCP-1 release completely in THP-1 cells, but could only block about one-quarter of the MCP-1 release in peripheral blood $CD14^+$ monocytes/macrophages. The partial effect of pranlukast in peripheral blood $CD14^+$ monocytes/macrophages suggests that a part of the MCP-1 response may be mediated by CysLT2 receptors, not CysLT1 receptors. The purity of the $CD14^+$ cells obtained after negative selection with the MACS system was 87.1 ± 5.4%. MCP-1 might be produced by the interaction of mononuclear cells other than monocytes. There might be toxic effects of 10^{-6} M pranlukast on cells that have a reduced MCP-1 expression. In adults administered a single oral dose of 225 mg, C_{max} was 642.3 ± 151.0 ng/mL (1.31 ± 0.31 μM) [29], but this value included pranlukast bound to plasma protein. The concentration of free pranlukast was probably at least tenfold lower than this [30]. Therefore, we think that 10^{-7} and 10^{-8} M pranlukast are clinical doses, and 10^{-6} M is a pharmacological dose. However, the contribution of the anti-inflammatory activity of pranlukast at oral therapeutic doses in asthmatic patients remains unclear. We speculate that an additional study, involving the modulation of the regulatory system for monocytes/macrophages, on bronchial asthma is needed.

In conclusion, CysLTs induce MCP-1 and increase CCR2B expression in human monocytes/macrophages *in vitro*, and pranlukast inhibits CysLT-induced MCP-1 production and CCR2B expression in the cells.

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