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Neutralization of leukotriene C_4 and D_4 activity by monoclonal and single-chain antibodies

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ABSTRACT

Background: Cysteinyl leukotrienes (LTs) are key mediators in inflammation. To explore the structure of the antigen-recognition site of a monoclonal antibody against LTC_4 (mAbLTC), we previously isolated full-length cDNAs for heavy and light chains of the antibody and prepared a single-chain antibody comprising variable regions of these two chains (scFvLTC).
Methods: We examined whether mAbLTC and scFvLTC neutralized the biological activities of LTC_4 and LTD_4 by competing their binding to their receptors.
Results: mAbLTC and scFvLTC inhibited their binding of LTC_4 or LTD_4 to $CysLT_1$ receptor ($CysLT_1R$) and $CysLT_2$ receptor ($CysLT_2R$) overexpressed in Chinese hamster ovary cells. The induction by LTD_4 of monocyte chemoattractant protein-1 and interleukin-8 mRNAs in human monocytic leukemia THP-1 cells expressing $CysLT_1R$ was dose-dependently suppressed not only by mAbLTC but also by scFvLTC. LTC_4- and LTD_4-induced aggregation of mouse platelets expressing $CysLT_2R$ was dose-dependently suppressed by either mAbLTC or scFvLTC. Administration of mAbLTC reduced pulmonary eosinophil infiltration and goblet cell hyperplasia observed in a murine model of asthma. Furthermore, mAbLTC bound to $CysLT_2R$ antagonists but not to $CysLT_1R$ antagonists.
Conclusions: These results indicate that mAbLTC and scFvLTC neutralize the biological activities of LTs by competing their binding to $CysLT_1R$ and $CysLT_2R$. Furthermore, the binding of cysteinyl LT receptor antagonists to mAbLTC suggests the structural resemblance of the LT-recognition site of the antibody to that of these receptors.
General significance: mAbLTC can be used in the treatment of inflammatory diseases such as asthma.

1. Introduction

Cysteinyl leukotrienes (LTs) including LTC_4, LTD_4 and LTE_4 are key mediators in inflammation [1,2]. Biosynthesis of cysteinyl LTs is initiated by conversion of arachidonic acid to LTA_4 catalyzed by 5-lipoxygenase. Then, the unstable epoxide LTA_4 is conjugated with reduced glutathione to produce LTC_4 which is further converted to LTD_4 and LTE_4 by the sequential cleavage for the tripeptide adduct of glutamate and glycine [2]. The biological activities of the cysteinyl LTs are mediated by at least two known G protein-coupled receptors, $CysLT_1$ receptor ($CysLT_1R$) and $CysLT_2$ receptor ($CysLT_2R$) [3]. The human and mouse $CysLT_1Rs$ bind LTD_4 with higher affinity than LTC_4 [4,5]. The human $CysLT_2R$ binds LTC_4 and LTD_4 equally [6], whereas the mouse $CysLT_2R$ shows higher affinity to LTC_4 than to LTD_4 [5]. $CysLT_1R$ couples to $G_{q/11}$ and/or $G_{i/o}$ depending on cell types, whereas $CysLT_2R$ couples to $G_{q/11}$ and activation of these receptors elicits intracellular calcium mobilization [3]. $CysLT_1R$ is the molecular target of the antiasthmatic drugs pranlukast, zafirlukast and montelukast, which show efficacy in blocking inflammatory actions in the airways and improving airway function [7–9]. The functional characterization of $CysLT_2R$ had been limited, although its roles in bleomycin-induced pulmonary fibrosis [10] as well as atopic dermatitis [11] were recently reported. BAY-u9773 has been known as a

Abbreviations: BAL, bronchoalveolar lavage; CHO, Chinese hamster ovary; $CysLT_1R$, $CysLT_1$ receptor; $CysLT_2R$, $CysLT_2$ receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HE, hematoxylin and eosin; IL-8, interleukin-8; LT, leukotriene; mAbLTC, monoclonal antibody against leukotriene C_4; MCP-1, monocyte chemoattractant protein-1; OVA, ovalbumin; PAS, periodic acid-Schiff; scFvLTC, single-chain variable fragment comprising variable regions of heavy and light chains of monoclonal antibody against leukotriene C_4

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nonselective antagonist for $CysLT_2R$, but two selective $CysLT_2R$ antagonists, HAMI3379 and $BayCysLT_2$ were recently developed [12,13].

Matsumoto et al. previously prepared a monoclonal antibody against LTC_4 (mAbLTC) for immunoaffinity purification and radioimmunoassay of this bioactive eicosanoid in human synovial fluid [14]. To explore the structure of the active site of the antibody recognizing the antigen, we isolated full-length cDNAs for heavy and light chains of the monoclonal antibody and deduced their primary structures [15]. Furthermore, we constructed an expression plasmid encoding a single-chain variable fragment antibody comprising variable regions of heavy and light chains of the original monoclonal antibody and expressed it in COS-7 cells [15]. The expressed fragment termed as scFvLTC showed high affinity and specificity to LTC_4 but lower affinity to LTD_4 and LTE_4, although a slight reduction of affinity to these LTs was observed as compared with mAbLTC [15]. The aim of this study was to examine whether mAbLTC and scFvLTC neutralized the biological activities of LTC_4 and LTD_4 by competing their binding to their receptors. Furthermore, we examined whether mAbLTC bound to antagonists for these receptors and investigated the structural similarity of the LT-recognition site of antibody to that of receptors.

2. Materials and methods

2.1. Animals

Male C57BL/6 J mice were obtained from Charles River Laboratories, Japan (Yokohama, Japan), and were used under protocols that were approved by the Experimental Animal Care and Use Committee of Okayama Prefectural University. All mice were maintained in a temperature-controlled (25 °C) facility with a 12-hour light/12-hour dark cycle and were fed a normal rodent chow diet (CE-2, CLEA Japan, Tokyo, Japan). Food and water were available ad libitum.

2.2. Materials

LTC_4, LTD_4, pranlukast, MK-571, BAY-u9773, HAMI3379, $BayCysLT_2$, goat anti-mouse IgG-coated plates and Ellman's reagent containing the substrate to acetylcholinesterase were purchased from Cayman Chemical (Ann Arbor, MI). LTC_4-acetylcholinesterase conjugate was a gift from Dr. K. Maxey of Cayman Chemical. Oligodeoxyribonucleotides were synthesized by Hokkaido System Science (Sapporo, Japan). An expression vector pCXN2 having a powerful CAG promoter [16] was kindly provided by Dr. J. Miyazaki of Osaka University. Hybridoma cells producing mAbLTC [14] were cultured in serum-free GIT medium (Wako, Osaka, Japan). The mAbLTC in the culture medium was purified using protein A-Sepharose CL4B (GE Healthcare, Piscataway, NJ) column chromatography and the buffer was changed to phosphate-buffered saline at pH 7.4 by gel-filtration before use [15].

2.3. Preparation of scFvLTC

The scFvLTC was prepared as described previously [15]. Briefly, an expression plasmid encoding an scFvLTC (pCXN2-scFvLTC) [15] was introduced into COS-7 cells by the DEAE-dextran method. The culture medium was collected and subjected to Ni-NTA agarose (Qiagen, Valencia, CA) column chromatography. After washing with 50 mM sodium phosphate buffer at pH 8.0 containing 300 mM NaCl, 0.05% Tween 20 and 20 mM imidazole, the hexahistidine-tagged scFvLTC was eluted with 250 mM imidazole in 50 mM sodium phosphate buffer at pH 8.0 containing 300 mM NaCl and 5 mM NaN_3. After desalting and concentration, the eluted fractions were reloaded onto a Ni-NTA agarose. The purified scFvLTC was obtained as described above except that 50 mM imidazole was contained in the washing buffer, and the buffer was changed to phosphate-buffered saline at pH7.4 before use.

2.4. Cell culture

Chinese hamster ovary (CHO) cells were cultured at 37 °C with 5% CO_2 in Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin. Human monocytic leukemia THP-1 cells obtained from RIKEN Cell Bank (Tsukuba, Japan) were maintained at 37 °C with 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal bovine serum. Mouse macrophage-like J774A.1 cells obtained from Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan) were maintained at 37 °C with 5% CO_2 in DMEM medium supplemented with 10% fetal bovine serum. The cells were subcultured every 3–4 days.

2.5. Transfection of human $CysLT_1R$ and $CysLT_2R$ expression vectors

cDNAs of human $CysLT_1R$ and $CysLT_2R$ were isolated from human leukocyte cDNA library [5], and ligated to EcoRI site of pCXN2 expression vector. CHO cells were transfected with the plasmid using lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 3 days, the transfected cells were split at a ratio of 1: 100 in culture medium containing 1.5 mg/ml geneticin. After 2 weeks we picked clones, and identified receptor-expressing cells by dot-blot analysis of RNA with alkaline phosphatase-labeled cDNA probes corresponding to the full-length coding sequences of human $CysLT_1R$ and $CysLT_2R$ using AlkPhos Direct Labelling Module (GE Healthcare). The cells permanently expressing human $CysLT_1R$ and $CysLT_2R$ were designated as CHO-$CysLT_1$ and CHO-$CysLT_2$, respectively. Mock-transfected cells were established by transfection of the parental pCXN2.

2.6. Measurement of intracellular calcium concentrations

CHO-$CysLT_1$, CHO-$CysLT_2$ and THP-1 cells were suspended in Hepes-Tyrode's-BSA buffer (10 mM Hepes-NaOH at pH 7.4, 140 mM NaCl, 2.7 mM KCl, 0.49 mM $MgCl_2$, 2 mM $CaCl_2$, 12 mM $NaHCO_3$, 5.6 mM D-glucose, 0.37 mM NaH_2PO_4 and 0.1% (w/v) of fatty acid-free BSA) and loaded with 5 μM fura 2-AM at 37 C for 1 h, washed twice and resuspended in Hepes-Tyrode's-BSA buffer to a concentration of 2×10^6 CHO cells/ml and 5×10^6 THP-1 cells/ml. Receptor antagonists or antibodies were added. After stirring at 37 C for 5 min, LTC_4 or LTD_4 was added, and fluorescence was measured using a fluorescence spectrophotometer (F-2000, Hitachi, Tokyo, Japan), with emission wavelength set at 510 nm and excitation wavelength at 340 nm and 380 nm. The intracellular calcium concentration was calculated with the Grynkiewicz formula [intracellular calcium concentration $= K_d \times (F_0/F_s) \times [(R - R_{min}) / (R_{max} - R)]$]. In this formula R is the experimentally derived 340/380 nm fluorescence ratio, R_{min} and R_{max} are the values of ratio pairs in the presence of nominally zero and saturating calcium, respectively. K_d is the fura-2 dissociation constant (224 nM at 37 C), and F_0 and F_s are proportionality coefficients for free- and calcium-bound fura-2, respectively (measured at 380 nm excitation). R_{max} was measured in the buffer containing 10 μM ionomycin and 20 mM $CaCl_2$ and R_{min} in the buffer containing 10 μM ionomycin and 10 mM EGTA.

2.7. Quantitative RT-PCR analysis

THP-1 cells were seeded into 35 mm-dishes at a density of 1×10^5 cells/dish in RPMI 1640 and preincubated at 37 C for 24 h. Receptor antagonists or antibodies were added 5 min before stimulation of the cells. After addition of LTC_4 or LTD_4, the cells were incubated for 1 h. Total RNA was isolated from the cells using an RNeasy mini kit (Qiagen) according to the manufacturer's protocol. First-strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen) and oligo$(dT)_{20}$ as a primer. The quantitative RT-PCR analyses were performed using a Bio-Rad iQ5 real time PCR detection system (Hercules, CA). Monocyte chemoattractant protein-1 (MCP-1)-specific primers were: upstream, 5′-GCTCAGCCAGATGCAATCAA-3′ and downstream,

5′-CCTTGGCCACAATGGTCTTG-3′, resulting in a 151-bp product. Interleukin-8 (IL-8)-specific primers were: upstream, 5′-CTTGGCAGCC TTCCTGATTT-3′ and downstream, 5′-CTCAGCCCTCTTCAAAAACT-3′, resulting in a 265-bp product. Human $CysLT_1R$-specific primers were: upstream, 5′-AAGGTACTCCAGTGCCAGAAAGAG-3′ and downstream, 5′-TAGTGTCATGGCATGTGGCAGAAG-3′, resulting in a 103-bp product. Human $CysLT_2R$-specific primers were: upstream, 5′-TGCAACCATCCA TCTCCGTAT-3′ and downstream, 5′-TGTGCAGTTCCTGCTGTTGTTAT-3′, resulting in a 74-bp product. Primer sequences for housekeeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were: upstream, 5′-ATGAGAAGTATGACAACAGCCTCAAG-3′ and downstream, 5′-TCAT GAGTCCTTCCACGATACCAAAG-3′, generating a 117-bp product. A quantitative RT-PCR mixture (20 μl) contained 1 μl of template cDNA, 0.25 μM each primer and 10 μl of SsoAdvanced SYBR Green Supermix (Bio-Rad). The cycling protocol consisted of 95 C for 30 s, 40 cycles of denaturation at 95 C for 5 s and annealing/extension at 62 C for 10 s, and plate read. To confirm the amplification specificity, we analyzed the amplified products by 10% polyacrylamide gel electrophoresis. We also performed a melting curve analysis at the end of cycling. The nucleotide sequences of the PCR products were confirmed using an Applied Biosystems 3130 Genetic Analyzer (Foster City, CA) with a BigDye terminator cycle sequencing kit (Applied Biosystems). All samples were analyzed for GAPDH expression in parallel in the same run. The analysis of the quantitative RT-PCR was done using the ΔCt cycle threshold method ($\Delta Ct = Ct$ (MCP-1, IL-8, $CysLT_1R$ or $CysLT_2R$)$-Ct$ (GAPDH)). Relative gene expression was obtained by $\Delta\Delta Ct$ methods ($\Delta\Delta Ct = \Delta Ct$ (*sample*)$-\Delta Ct$ (*control*)). Difference in the expression levels was determined according to the following formula: $2^{-\Delta\Delta Ct}$ [17].

2.8. Platelet aggregation

Blood was carefully collected in a plastic syringe containing 15 mM sodium citrate from the inferior vena cava of anesthetized C57BL/6 J mice. Blood from 3 to 5 mice was pooled and centrifuged at 180 ×*g* for 5 min at room temperature for preparation of platelet-rich plasma. The platelets were washed twice and resuspended in calcium-free Hepes-Tyrode's-BSA buffer (10 mM Hepes-NaOH at pH 7.4, 140 mM NaCl, 2.7 mM KCl, 0.49 mM $MgCl_2$, 12 mM $NaHCO_3$, 5.6 mM D-glucose, 0.37 mM NaH_2PO_4 and 0.1% (w/v) of fatty acid-free BSA) at a concentration of 1.0×10^8 platelets/ml. A 100-μl aliquot of washed mouse platelets was preincubated at 37 °C for 1 min with constant stirring at 1,400 rpm in the absence or presence of receptor antagonists or antibodies and then incubated with LTC_4 or LTD_4. Platelet aggregation was measured as the change in light transmission for 10 min using a MCM HEMA TRACER 712 (MC Medical, Tokyo, Japan). The extent of aggregation was estimated quantitatively by measuring the maximum curve height above the baseline level.

2.9. RT-PCR analysis

Expression of $CysLT_2R$ in mouse platelets was evaluated by RT-PCR analysis. Total RNA was extracted from mouse platelets using Sepasol (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized using Superscript III reverse transcriptase and random primers. Mouse $CysLT_2R$-specific primers were: upstream, 5′-TGCTTTTGGAAGAGAGAAGAGTCCA-3′ and downstream, 5′-AAAGCCATTTCCCAAGGCTC-3′, resulting in a 606-bp product. Mouse $CysLT_1R$-specific primers were: upstream, 5′- TTAAATTCACCATC TTCCTGCTTTGG-3′ and downstream, 5′- AGCCTTCTCCTAAAGTTTCCAC-3′, resulting in a 1,013-bp product. DNA amplification was carried out using Ex Taq DNA polymerase (Takara Bio, Shiga, Japan) under the following conditions: denaturation at 94 C for 30 s, annealing at 55 C for 30 s and extension at 72 C for 1 min. The equal amount of aliquots from 25, 30, 35 and 40 thermocycles was electrophoresed in 1.5% agarose gel. The nucleotide sequences of the PCR products were confirmed using an Applied Biosystems 3130 Genetic Analyzer (Foster City, CA) with a BigDye terminator cycle sequencing kit (Applied Biosystems).

2.10. Asthmatic mouse model

Thirty-six male C57BL/6 J mice of 6 weeks of age were divided into six groups. Mice were immunized with ovalbumin (OVA) as previously reported [18,19]. Briefly, mice were actively sensitized by intraperitoneal injections of 50 μg of OVA with 1 mg of alum as an adjuvant on days 0 and 12. They were exposed to OVA aerosol (1% w/v diluted in sterile physiologic saline) using an ultrasonic nebulizer NE-U07 (OMRON, Kyoto, Japan) for 30 min on days 22, 26 and 30. Mice received an intraperitoneal injection of vehicle (Veh), 250 μg of MK-571 (MK), 100, 250 and 500 μg of mAbLTC 30 min before each OVA exposure. Control mice were intraperitoneally injected with saline and exposed to saline in the same manner (Cont). Bronchoalveolar lavage (BAL) was performed 24 h after the last OVA exposure. Mice were sacrificed by exsanguination

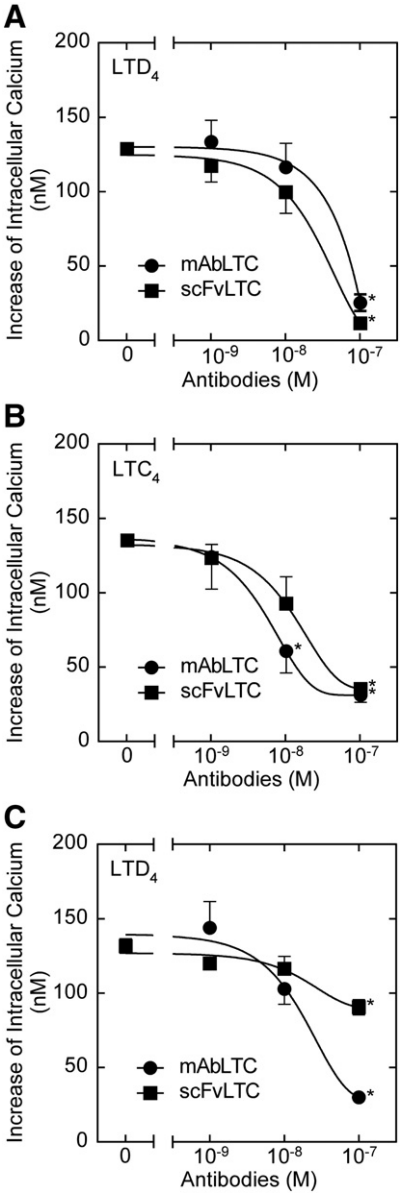

Fig. 1. Effect of mAbLTC and scFvLTC on intracellular calcium concentration in CHO-$CysLT_1$ and CHO-$CysLT_2$ cells. The cells were loaded with 5 μM fura 2-AM for 1 h at 37 °C. CHO-$CysLT_1$ cells were stimulated with 0.1 nM LTD_4 (A) in the absence or presence of the indicated concentrations of mAbLTC (circles) or scFvLTC (squares). CHO-$CysLT_2$ cells were stimulated with 0.1 nM LTC_4 (B) or 0.1 nM LTD_4 (C) in the absence or presence of the indicated concentrations of mAbLTC (circles) or scFvLTC (squares). The increase in intracellular calcium concentration calculated from the fluorescence ratio (340/380 nm) is shown. The data represent means ± SEM of triplicate experiments. $^*P < 0.05$ from the cells without mAbLTC or scFvLTC using one-way ANOVA followed by Dunnet's post hoc test.

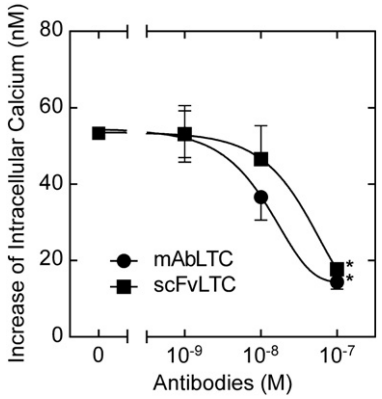

Fig. 2. Effect of mAbLTC and scFvLTC on intracellular calcium concentration in THP-1 cells. The cells were loaded with 5 μM fura 2-AM for 1 h at 37 °C and then stimulated with 5 nM LTD_4 in the absence or presence of the indicated concentrations of mAbLTC (circles) or scFvLTC (squares). The increase in intracellular calcium concentration calculated from the fluorescence ratio (340/380 nm) is shown. The data represent means ± SEM of triplicate experiments. *$P < 0.05$ from the cells without mAbLTC or scFvLTC using one-way ANOVA followed by Dunnet's post hoc test.

under pentobarbital anesthesia. The trachea was cannulated and the lungs were lavaged three times with 1 ml of phosphate-buffered saline at pH 7.4 containing 0.05 mM EDTA and 0.1% BSA. Total cells in BAL fluid were counted using a hemocytometer. Aliquots of cells were centrifuged to attach to glass slides using a cytocentrifuge (SC-2, Tomy, Tokyo, Japan) and stained using Diff-Quik (Sysmex, Kobe, Japan), and the numbers of eosinophils, macrophages and lymphocytes were determined under light microscopic observation of at least 400 total cells. Alternatively, the lungs were fixed in 10% formalin in 100 mM sodium phosphate buffer at pH 7.4 followed by embedding in paraffin, and 3-μm sections were stained with hematoxylin and eosin (HE) for inflammatory cells and with periodic acid-Schiff (PAS) for mucin-producing goblet cells.

2.11. Enzyme immunoassay

The binding of $CysLT_1R$ and $CysLT_2R$ antagonists to mAbLTC was analyzed by an enzyme immunoassay. The 96-well plate coated with an anti-mouse IgG was incubated with 50 μl of 4 ng/ml mAbLTC in the presence of 50 μl of indicated concentrations of antagonists and a fixed amount of LTC_4 acetylcholinesterase conjugate at 4 °C for 18 h. After washing, wells were developed by the addition of Ellman's reagent containing a substrate of acetylcholinesterase, and the plate was read at 415 nm absorbance.

2.12. Statistical analysis

Data are expressed as means ± SEM of multiple experiments. The statistical analysis was performed using one-way ANOVA followed by Dunnet's post hoc test. Significant difference was based on a $P < 0.05$. Sigmoidal dose–response curve fitting was performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA).

3. Results

3.1. Effect of monoclonal and single-chain antibodies on binding of LTC_4 and LTD_4 to their receptors

The binding of LTC_4 and LTD_4 to $CysLT_1R$ and $CysLT_2R$ is known to increase the intracellular calcium concentration [6,20]. The basal level of intracellular calcium concentration in CHO-$CysLT_1$ cells was 131 ±

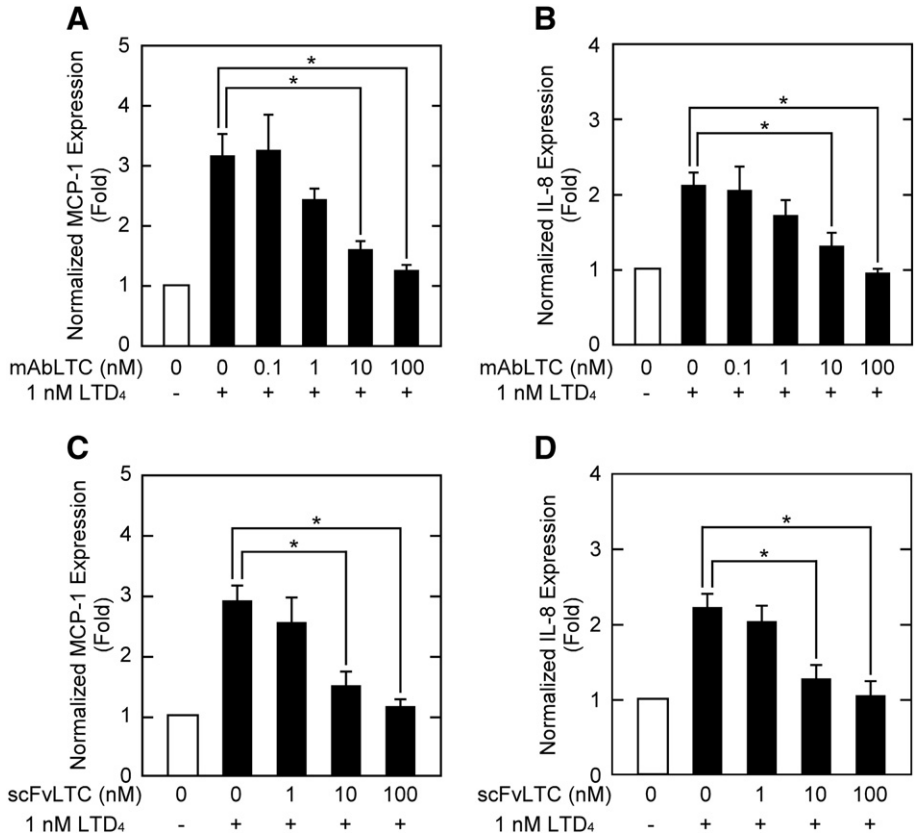

Fig. 3. Effect of mAbLTC and scFvLTC on MCP-1 and IL-8 gene expression induced by LTD_4 in THP-1 cells. THP-1 cells were stimulated with 1 nM LTD_4 for 1 h at 37 °C in the absence or presence of the indicated concentrations of mAbLTC (A and B) or scFvLTC (C and D). The mRNA levels of MCP-1 (A and C) and IL-8 (B and D) in the cells were determined using a quantitative RT-PCR. Relative mRNA levels of MCP-1 and IL-8 compared with that in unstimulated cells is shown. Values are normalized by GAPDH expression and shown as means ± SEM of triplicate experiments. *$P < 0.05$ from the cells without mAbLTC or scFvLTC using one-way ANOVA followed by Dunnet's post hoc test.

11 nM. LTD_4 at 0.1 nM evoked an increase in intracellular calcium concentration by 129 ± 4 nM above basal levels in CHO-$CysLT_1$ cells. LTC_4 at 10 nM induced a slight increase in intracellular calcium. CHO-$CysLT_1$ cells pretreated with selective $CysLT_1R$ antagonist (100 nM pranlukast or 100 nM MK-571) did not respond to LTD_4. As shown in Fig. 1A, pretreatment of CHO-$CysLT_1$ cells with 100 nM mAbLTC or 100 nM scFvLTC significantly suppressed the calcium mobilization induced by 0.1 nM LTD_4. In CHO-$CysLT_2$ cells, the basal level of intracellular calcium concentration was 138 ± 18 nM. LTC_4 and LTD_4 at 0.1 nM induced an increase in intracellular calcium concentration in CHO-$CysLT_2$ cells by 135 ± 3 nM and 132 ± 5 nM. The response was inhibited by 100 nM HAMI3379, a selective $CysLT_2R$ antagonist, but was not inhibited by 100 nM pranlukast and 100 nM MK-571. As shown in Fig. 1B and C, preincubation of CHO-$CysLT_2$ cells with mAbLTC or scFvLTC significantly suppressed the calcium mobilization induced by 0.1 nM LTC_4 or 0.1 nM LTD_4. The basal level of intracellular calcium concentration in mock-transfected cells was 147 ± 6 nM. The cells did not respond to either LTC_4 or LTD_4 up to 100 nM. ATP at 10 μM elicited an increase in intracellular calcium concentration to the same level among CHO-$CysLT_1$, CHO-$CysLT_2$ and mock-transfected cells (data not shown). These results indicate that mAbLTC and scFvLTC inhibit the binding of LTs to their receptors.

3.2. Effect of monoclonal and single-chain antibodies on induction of proinflammatory cytokines in THP-1 cells expressing LT receptor

We examined whether mAbLTC and scFvLTC neutralized the activity of LTD_4 by inhibition of its binding to $CysLT_1R$ expressed in human monocytic leukemia THP-1 cells. The basal level of intracellular calcium concentration in THP-1 cells was 151 ± 3 nM. LTD_4 at 5 nM induced a rapid increase in intracellular calcium concentration by 53 ± 2 nM above basal levels. The cells did not respond to LTC_4 up to 100 nM. MK-571 at 100 nM reduced the LTD_4-elicited calcium mobilization, whereas 100 nM HAMI3379 did not inhibit the calcium response, confirming that THP-1 cells express functionally active $CysLT_1R$ but not $CysLT_2R$ [21]. RT-PCR analysis showed the expression of $CysLT_1R$ mRNA but not $CysLT_2R$ mRNA (data not shown). As shown in Fig. 2, treatment of THP-1 cells with mAbLTC or scFvLTC dose-dependently suppressed the LTD_4-elicited calcium mobilization and 100 nM of either antibody completely blocked the calcium response of the cells. When THP-1 cells were stimulated with 1 nM LTD_4 for 1 h, expression levels of MCP-1 and IL-8 mRNA examined by quantitative RT-PCR were increased by 3.0-fold and 2.2-fold, respectively, as compared with unstimulated cells. The upregulation of MCP-1 and IL-8 mRNA by LTD_4 was completely inhibited by 100 nM MK-571 but not by 100 nM HAMI3379, indicating that LTD_4 induced these cytokines by $CysLT_1R$ stimulation. The upregulation of these cytokines was suppressed not only by addition of 5 mM EGTA but also by treatment of the cells with BAPTA-AM, an intracellular calcium chelator (data not shown), confirming the results in a previous report indicating that the induction of these cytokines was mediated by intracellular calcium increase [22]. As shown in Fig. 3, induction of MCP-1 and IL-8 mRNA was dose-dependently suppressed by mAbLTC or scFvLTC and significantly suppressed at more than 10 nM. The mRNA level of $CysLT_1R$ did not significantly change (data not shown). These results indicate that mAbLTC and scFvLTC neutralize the activity of LTD_4 on THP-1 cells by inhibiting its binding to $CysLT_1R$.

3.3. Effect of monoclonal and single-chain antibodies on aggregation of mouse platelets induced by LT receptor stimulation

We examined whether mAbLTC and scFvLTC neutralized the activity of LTC_4 and LTD_4 on the aggregation of mouse platelets by inhibition of binding to $CysLT_2R$. Mouse platelets readily aggregated in response to 2 nM LTC_4 and 10 nM LTD_4. LTC_4- and LTD_4-induced platelet aggregation

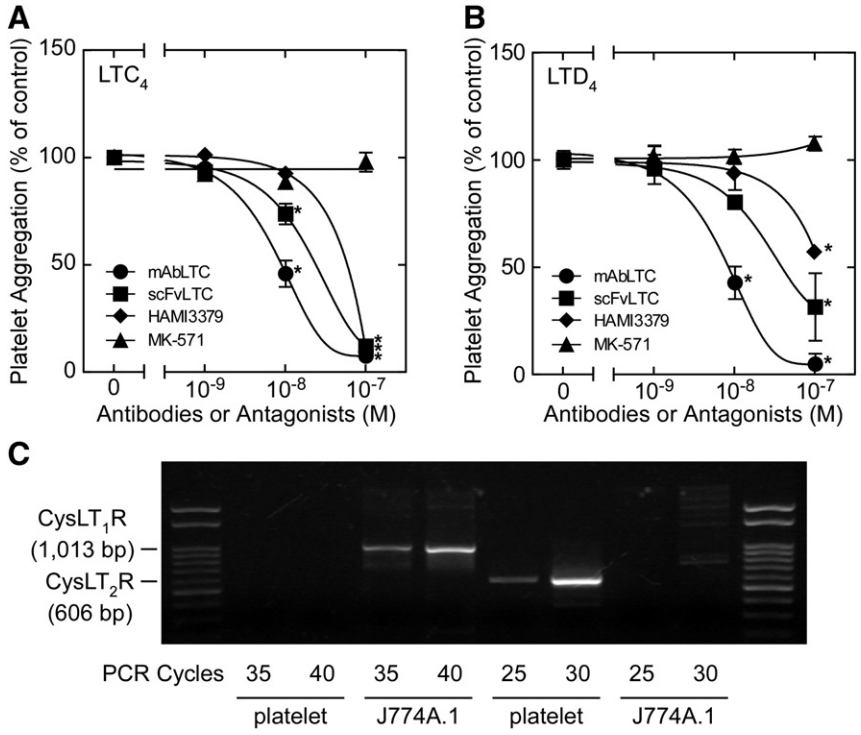

Fig. 4. Effect of mAbLTC and scFvLTC on platelet aggregation. The washed mouse platelets were stimulated with 2 nM LTC_4 (A) or 10 nM LTD_4 (B) in the absence or presence of the indicated concentrations of mAbLTC (circles), scFvLTC (squares), MK-571 (triangles) or HAMI3379 (lozenges). Receptor antagonists or antibodies were added 1 min before stimulation of the cells. The platelet aggregation is presented as the percent of control, which was measured in the presence of LTC_4 or LTD_4 and in the absence of the antibodies and antagonists for $CysLT_1R$ and $CysLT_2R$. The data represent means ± SEM of triplicate experiments. *$P < 0.05$ from the platelets without antibodies or antagonists using one-way ANOVA followed by Dunnet's post hoc test. Expression of $CysLT_2R$ in mouse platelets was evaluated by RT-PCR analysis (C). Total RNA (1 μg) extracted from mouse platelets and J774A.1 cells was subjected to RT-PCR with gene-specific primers. Aliquots from 35 and 40 cycles for $CysLT_1R$ amplification and aliquots from 25 and 30 cycles for $CysLT_2R$ amplification were subjected to agarose gel electrophoresis followed by ethidium bromide staining.

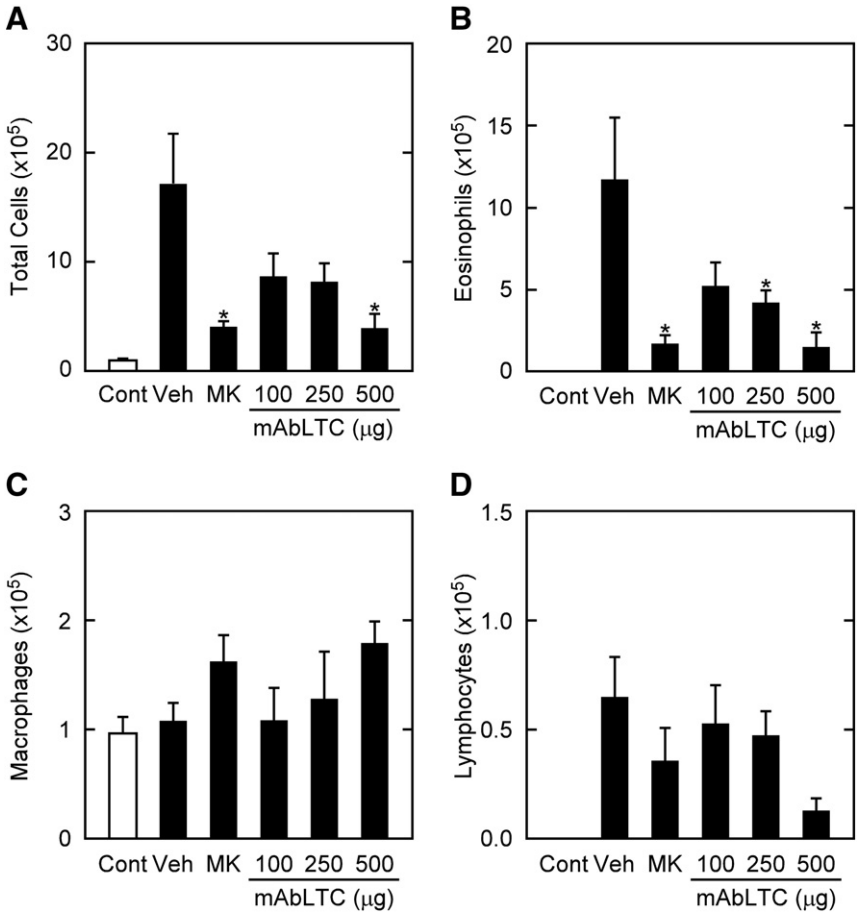

Fig. 5. Effect of mAbLTC on OVA-induced infiltration of inflammatory cells in mouse airway. OVA exposed mice were treated with either vehicle (Veh), MK-571 (250 μg per mouse, MK) or mAbLTC (100, 250 and 500 μg per mouse) intraperitoneally prior to every OVA aerosol challenge. BAL fluid was collected 24 h after the last exposure of saline or OVA aerosol. Control mice were intraperitoneally injected with saline and exposed to saline in the same manner (Cont). The numbers of total cells (A), eosinophils (B), macrophages (C) and lymphocytes (D) in BAL fluid of mice were shown. The data represent means ± SEM of 6 mice. *$P < 0.05$ from the mice with vehicle using one-way ANOVA followed by Dunnett's post hoc test. Histological examination of lung sections of mice stained with HE (E–H) and with PAS (I–L) 24 h after the last exposure of saline (Cont, E and I) or OVA (F–H and J–L) aerosol. OVA exposed mice were treated with vehicle (Veh, F and J), 250 μg of MK-571 (MK, G and K) and 500 μg of mAbLTC (H and L). Bar denotes 100 μm.

was suppressed by 100 nM HAMI3379 but not by 100 nM MK-571 (Fig. 4A and B), indicating that LTC_4 and LTD_4 aggregated mouse platelets by $CysLT_2R$ stimulation (T. Shimizu, personal communication). As shown in Fig. 4A and B, platelet aggregation with LTC_4 and LTD_4 was dose-dependently inhibited by either mAbLTC or scFvLTC and completely blocked by 100 nM mAbLTC. The scFvLTC at 100 nM completely inhibited LTC_4-induced platelete aggregation and significantly suppressed LTD_4-induced platelet aggregation. RT-PCR analysis demonstrated the expression of $CysLT_2R$ mRNA but not $CysLT_1R$ mRNA (Fig. 4C). As a control experiment, only $CysLT_1R$ mRNA was detected in mouse macrophage-like J774A.1 cells. These results indicate that mAbLTC and scFvLTC neutralize the activity of LTC_4 and LTD_4 on mouse platelets by inhibiting their binding to $CysLT_2R$.

3.4. Effect of monoclonal antibody on ovalbumin-induced murine model of asthma

To evaluate the effect of mAbLTC on the activity of cysteinyl LTs in vivo, we applied the antibody to an OVA-induced murine model of asthma. As shown in Fig. 5A–D, OVA exposed mice had an increase in the number of total cells, eosinophils and lymphocytes in BAL fluid as compared with the control mice. OVA exposed mice treated with 250 μg MK-571 showed an 80–90% decrease in the number of eosinophils recovered in BAL fluid. Treatment of the mice with mAbLTC dose-dependently suppressed the infiltration of eosinophils and 250 to 500 μg of mAbLTC significantly decreased the number of eosinophils in BAL fluid. Observations of lung sections by light microscopy showed that exposure of OVA aerosol induced marked infiltration of inflammatory cells, particularly eosinophils (Fig. 5F), into perivascular and peribronchiolar area as compared with those exposed to saline (Fig. 5E). Treatment of mice with 500 μg mAbLTC (Fig. 5H) as well as 250 μg MK-571 (Fig. 5G) inhibited the infiltration of inflammatory cells. Goblet cell hyperplasia shown by positive PAS staining was also abated by treatment with mAbLTC (Fig. 5I–L). Lungs of control mice were free of cellular infiltration and goblet cell hyperplasia.

3.5. Binding of LT receptor antagonists to monoclonal antibody

To investigate whether mAbLTC bound to antagonists for $CysLT_1R$ and $CysLT_2R$, we applied an enzyme immunoassay using mAbLTC bound to the 96-well plate. Among tested compounds, mAbLTC bound to HAMI3379 and $BayCysLT_2$, selective $CysLT_2R$ antagonists, as well as BAY-u9773, a nonselective antagonist, whereas the mAbLTC did not bind to pranlukast and MK-571, selective $CysLT_1R$ antagonists (Fig. 6).

4. Discussion

In the present study, we demonstrated that mAbLTC and scFvLTC neutralized the activities of LTC_4 and LTD_4 on CHO cells overexpressing human $CysLT_1R$ or $CysLT_2R$ as well as THP-1 cells with $CysLT_1R$ and mouse platelets with $CysLT_2R$. Furthermore, mAbLTC significantly inhibited inflammatory cell infiltration in a murine model of asthma.

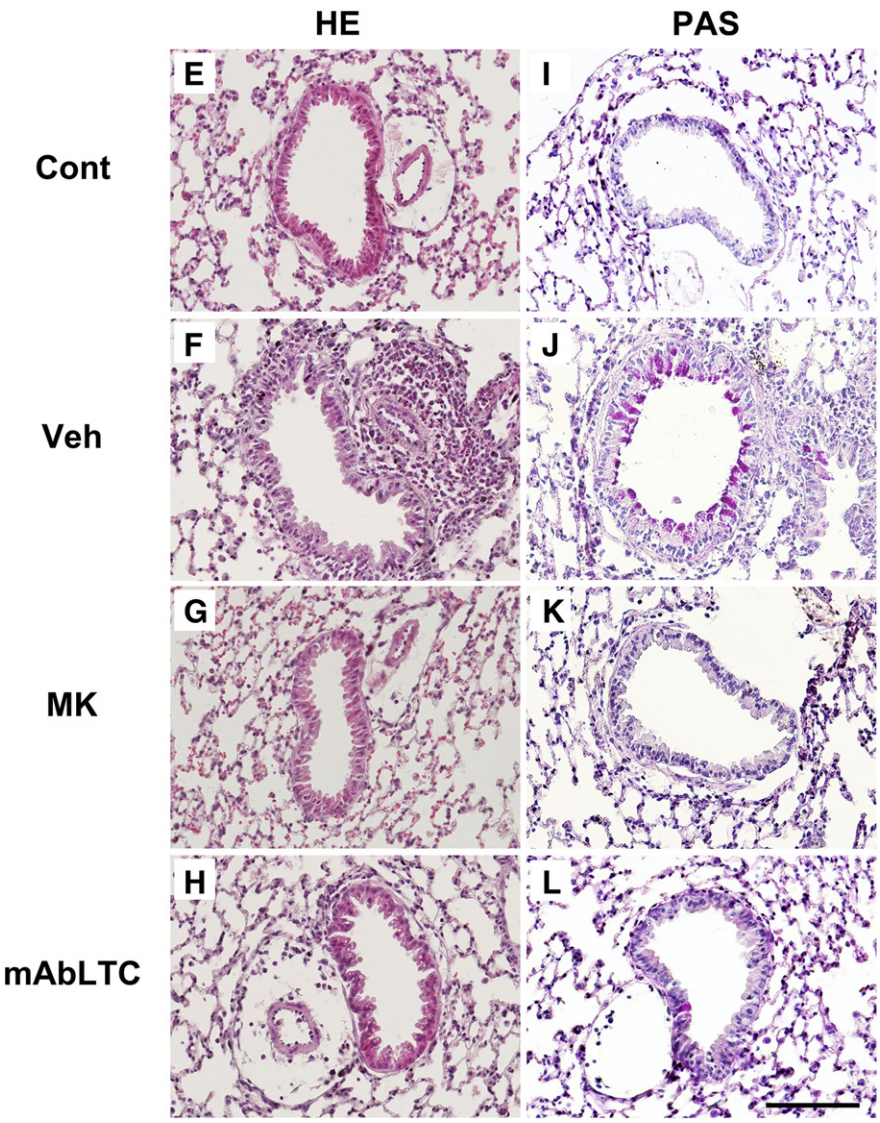

Fig. 5 (*continued*).

Previously Mnich et al. described the generation of a monoclonal antibody that neutralized the biological activities of prostaglandin E_2 in vitro and in vivo [23]. Our present work is the first report of a monoclonal antibody and a single-chain variable fragment antibody that block LT-dependent responses. The capacity of scFvLTC to neutralize the activity of LTC_4 and LTD_4 was reduced as compared with mAbLTC, supporting a slight reduction of affinity to LTs of scFvLTC [15]. Moreover, mAbLTC and scFvLTC showed lower affinity to LTD_4 than that to LTC_4 [15], supporting the results that required amount of these antibodies to neutralize the LTD_4 activity was higher than that to neutralize the LTC_4 activity.

THP-1 cells responded to LTD_4 and increased intracellular calcium concentration [20,24]. Furthermore, LTD_4 induced gene expression of MCP-1 and IL-8 in THP-1 cells as previously reported [25–27]. We confirmed that calcium chelators, EGTA and BAPTA-AM, suppressed the upregulation of these cytokines by LTD_4, indicating that a calcium-dependent pathway was involved in the induction of these cytokines. A previous study showed that LTD_4 activated mitogen-activated protein kinase through a calcium-dependent protein kinase Cα and Raf-1 pathway in THP-1 cells expressing $CysLT_1R$ [24]. MCP-1 induction by $CysLT_1R$ stimulation was mediated by extracellular signal-regulated kinase 1/2 and c-jun N-terminal kinase in a mitogen-activated protein kinase and nuclear factor-κB pathway in THP-1 cells [27]. Thompson et al. demonstrated that nuclear factor-κB played a key role in the signaling driven by the $CysLT_1R$ that led to the induction of IL-8 expression [26].

Stimulation of $CysLT_2R$ in mouse platelets induced platelet aggregation (T. Shimizu, personal communication). Platelet activation is achieved through various surface receptors that include G protein-coupled receptors, integrins and glycoproteins receptors [28,29]. Intracellular calcium mobilization is a key signaling event in platelet activation [30]. In mouse platelets, LTC_4 and LTD_4 induced an increase in intracellular calcium concentration and the response was inhibited by HAMI3379, a selective $CysLT_2R$ antagonist, but was not inhibited by MK-571, a selective $CysLT_1R$ antagonist (data not shown). The results suggest that $CysLT_2R$ stimulation by LTC_4 and LTD_4 leads to intracellular calcium mobilization and platelet aggregation in mouse platelets.

The selective $CysLT_1R$ antagonists are clinically used in the treatment of asthma [8,9,31]. In the present study, we reported that eosinophil infiltration and goblet cell hyperplasia observed in a murine model of asthma were markedly attenuated by mAbLTC administration. Consistent with these findings, previous studies indicated that $CysLT_1R$ blockade significantly reduced the allergen-induced increase in the number of eosinophils and goblet cells in the airways [32–34]. Eosinophils as well as mast cells release cysteinyl LTs that develop airway

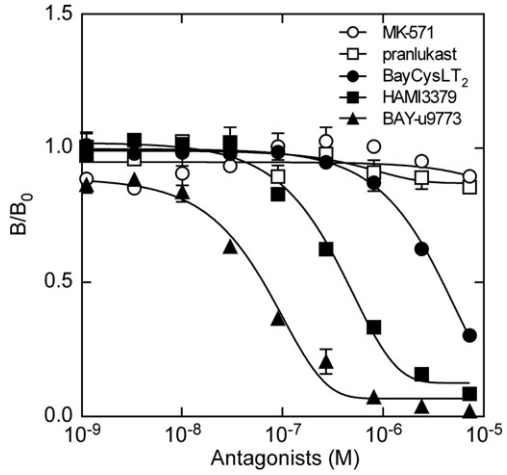

Fig. 6. Binding of antagonists for $CysLT_1R$ and $CysLT_2R$ to mAbLTC. The 96-well plate was precoated with 200 pg of mAbLTC, and LTC_4 tracer was added to the well in the absence or presence of indicated concentrations of MK-571 (open circles), pranlukast (open squares), $BayCysLT_2$ (closed circles), HAMI3379 (closed squares) or BAY-u9773 (closed triangles). Results are expressed as a ratio of the absorbance of a sample well (B) to that of the well without antagonists (B_0). The data represent means ± SEM of triplicate experiments.

inflammation [35]. These findings suggest that antiasthmatic effects of mAbLTC are attributed to inhibiting the binding of cysteinyl LTs to $CysLT_1R$. At present our recombinant scFvLTC is expressed only at low levels in COS-7 cells. A more efficient expression system needs to be established to reveal the effects of scFvLTC in vivo.

The results that our antibodies inhibited the binding of LTs to $CysLT_1$-R and $CysLT_2R$ in vitro and in vivo suggested the structural similarity of the LT-binding site in cysteinyl LT receptors to that in mAbLTC. Interestingly, mAbLTC bound to $CysLT_2R$ antagonists but not to $CysLT_1R$ antagonists. Identification of amino acids involved in binding of antigen in mAbLTC by X-ray crystallographic analysis and site-directed mutagenesis of scFvLTC may contribute to the understanding the structure of the LT-binding site of cysteinyl LT receptors.

It is reported that either the lack of cysteinyl LT production as in LTC_4 synthase-null mice or the $CysLT_2R$ deficiency significantly attenuated bleomycin-induced chronic pulmonary inflammation and fibrosis which were conversely increased in $CysLT_1R$-null mice [10,36]. On the other hand, $CysLT_2R$-deficient mice showed a significant increase in *Dermatophagoides farinae*-induced pulmonary inflammation [37]. Therefore, elimination of LT activity using antibodies rather than blocking of the specific LT receptor might be ideal for prevention of such receptor interaction [37,38], although some therapeutic antibodies are immunogenic even after humanization [39]. The development of scFv with altered affinity and specificity to LTs may be possible by site-directed mutagenesis, which is now in progress in our laboratory.

In conclusion, mAbLTC and scFvLTC neutralized LT activity. Anti-tumor necrosis factor α (Infliximab), anti-IL-6 receptor (Tocilizumab) and anti-IgE (Omalizumab) are developed as medical agents and clinically used in the treatment of various inflammatory diseases including Crohn's disease, rheumatoid arthritis and asthma [40–42]. Our antibodies which could suppress the activity of LT through $CysLT_2R$ might contribute the treatment of idiopathic pulmonary fibrosis, for which no efficient interventions are available.

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