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Functional Characterization of Human Cysteinyl Leukotriene 1 Receptor Gene Structure

Grzegorz Woszczek,* Rafał Pawlicka,† Hai-Yan Qi,* Sahrudaya Nagineni,* Sura Alsaaty,* Carolea Logun,* and James H. Shelhamer,‡*

The 5-lipoxygenase pathway has been strongly implicated in the pathogenesis of chronic inflammatory disorders, such as bronchial asthma and atherosclerosis. Cysteinyl leukotrienes (cysLTs), 5-lipoxygenase pathway products, are recognized now not only as important factors in asthmatic inflammation, but also as mediators of cell trafficking and innate immune responses. To study a role of cysLTs in inflammatory reactions we have characterized the gene structure of human cysteinyl leukotriene receptor type 1 (cysLT1R). The cysLT1R gene consists of 5 exons that are variably spliced and a single promoter region with multiple transcription start sites. Four different cysLT1R transcripts were identified. RT-PCR showed dominant and wide expression of the transcript I, containing exons 1, 4, and 5, with the strongest presence in blood leukocytes, spleen, thymus, lung, and heart. The expression of cysLT1R is functionally regulated at the transcriptional level by IL-4 through a STAT6 response element localized to the proximal cysLT1R promoter region. IL-4 stimulation increased cysLT1R mRNA (real-time PCR) and surface protein expression (flow cytometry) in a time-dependent fashion. CysLTs (LTD4 and LTE4) induced an increased production of a potent monocyte chemoattractant CCL2 (MCP-1) in IL-4-primed THP-1 cells in a dose-dependent manner. This effect was effectively inhibited by the cysLT1R-selective antagonist MK571 in a dose-dependent manner and only partially by a nonselective cysLT1R/cysLT2R inhibitor BAY-u9773, implying a cysLT1R-mediated mechanism. Thus, cysLTs signaling through cysLT1R might contribute to inflammatory reactions by cooperating with IL-4 in enhanced CCL2 production in human monocyctic cells. The Journal of Immunology, 2005, 175: 5152–5159.

Cysteinyl leukotrienes (cysLTs)3 (LTC4, LTD4, LTE4) are recognized now as important inflammatory mediators that initiate and potentiate many biological responses. CysLTs are generated from arachidonic acid through the 5-lipoxygenase (5-LO) pathway by limited cell types, such as eosinophils, mast cells, macrophages, basophils, and dendritic cells (1, 2). CysLTs, described primarily as lipid mediators involved in pathogenesis of airways inflammation and related symptoms, such as bronchoconstriction, mucous secretion, and airway hyperresponsiveness, are recognized now as well as important mediators of cell trafficking and innate immune responses (3, 4). The biological action of cysLTs is mediated via two known G-protein-coupled receptors: cysLT type I receptor (cysLT1R) and cysLT type II receptor (cysLT2R). The human cysLT1R (5, 6) and cysLT2R (7–9) have been cloned and characterized. Genes for both receptors were reported to be intronless in the protein encoding sequence, but the full cysLTR gene structures have not been elucidated. Characterization of the promoter regions of cysLT receptor genes would help to define the pathways involved in the receptor expression and signaling. The cysLTs are key mediators of trafficking eosinophils and other leukocytes to sites of inflammation. Acting directly through the cysLT1R receptors, cysLTs attract eosinophils to airways in allergic inflammation (10). It has been shown that cysLT1R antagonists may block eosinophil chemotaxis in rat model of asthma (11) as well as in human studies in asthmatics (12). But more and more data support the hypothesis that cysLTs might also play an important regulatory role in trafficking of other cells, such as dendritic cells (DC), T cells and monocytes, probably indirectly through the regulation of CC chemokine synthesis. Recently, it has been shown that LTC4 promotes chemotaxis to CCL19 and mobilization of DC from the epidermis (13). Pretreatment of mild asthmatics with a cysLT1R antagonist decreased the number of circulating myeloid dendritic cells, demonstrating a significant role of cysLTs in DC trafficking in vivo (14). Other studies suggest that cysLTs produced by macrophages may participate in the recruitment of inflammatory cells, i.e., monocytes and T cells in human diseased tissue through mechanisms that involve CC- and CXC-type chemokines. In mouse and human arteries, LTD4 strongly stimulated expression of CCL3 (MIP-1α) in macrophages and CXCL2 (MIP-2) in endothelial cells, pointing to cysLTs as important mediators in the pathogenesis of atherosclerosis and arterial aneurysm formation (15). CCL2 (MCP-1) is one of the major chemokines responsible for attracting monocytes to sites of atherosclerotic inflammation (16). It has been shown that LTB4, but not cysLTs, can increase CCL2 expression, underlining a link between the LTB4 pathway and atherosclerosis (17). However, the influence of cysLTs on synthesis of CCL2 in inflammatory conditions, when many mediators cooperate to generate the final response has not been studied yet.

Both allergic and atherosclerotic inflammatory reactions are known to be strongly influenced by the prototypical Th2 type cytokine, IL-4. The actions of IL-4 are not limited to the initiation of Th2 responses, but may also stimulate other cellular responses that
contribute to manifestations of allergic or cardiovascular diseases (18–20). IL-4 stimulation might also have a potentiating effect on cysLTs mediated trafficking of immune cells. It has been shown that IL-4 up-regulates direct chemotactic response to cysLTs by increasing expression of cysLT1Rs on human monocytes/macrophages (21), probably by a transcriptionally regulated mechanism, but lack of information about the cysLT1R promoter structure makes this mechanism difficult to prove.

In the present study, we defined the full structure of the gene encoding human cysLT1R and analyzed the mechanism of IL-4 stimulation on cysLT1Rs mediated synthesis of CC chemokine, CCL2 in a human monocytic cell line.

Materials and Methods

Cell culture

THP-1 (acute monocytic leukaemia) and U937 (human histiocytic lymphoma) cells were obtained from American Type Culture Collection and were grown at 37°C with 5% CO₂ in RPMI 1640 medium supplemented with 10% FBS and 2 mM l-glutamine (BioSource International). PBMC were isolated using BD Vacutainer CPT method (BD Biosciences).

RACE

5’- and 3’-RACE were performed using the Gene Racer kit (Invitrogen Life Technologies) according to the manufacturer’s protocol. Total RNA was extracted from PBMC, U937, and THP-1 cells using QIAshredder columns and RNeasy kits and was treated with DNase (all obtained from Qiagen). The GeneRacer RNA oligo (containing 5’ adaptor) was ligated to 5’ decapped, full length mRNA and reverse transcribed using SuperScript III reverse transcriptase and the Gene Racer oligo dT (containing 3’ adaptor). The obtained cDNA was amplified by PCR (touchdown PCR followed by nested PCR) with gene-specific primers (GSP): 3’-RACE GSP-5’-CATAAACCTTGTCTCTGGCTGAC; 3’-RACE nested GSP-5’TCCACCGTGACTTATGTACCCAG; 5’-RACE GSP-5’-AGACCAACA CGGAGAGAGGACAGTGTGC; 5’-RACE nested GSP-5’-GCGGAAGCT CATCAATAGTGCTGAC; and adaptor GeneRacer primers supplied with the kit. The nested PCR products were gel purified, cloned into pCR4- Topo vector (Invitrogen Life Technologies) and sequenced using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

To check for rare transcripts and confirm 5’-untranslated region (UTR) gene structure a set of RT-PCRs were performed with upstream primers located in the region of potential transcription start sites (primers UP1–UP7, Fig. 1C) and downstream primer localized to the coding region of cysLT1R: R: DO1–5’.TGAACATATAAGCCACAGC. All reactions were conducted using Platinum TaqDNA Polymerase High Fidelity (Invitrogen Life Technologies) under the following conditions: 94°C for 2 min, followed by 35 cycles 94°C for 30 s, 56°C for 30 s, and 68°C for 10 min. PCR products were identified on 2% agarose gel, cloned, and sequenced.

RT-PCR analysis of alternative transcripts expression

Total RNAs (5 μg) from various human tissues (BD Biosciences) were reverse transcribed using the SuperScript III first strand synthesis system (Invitrogen Life Technologies) and PCR performed to characterize the expression pattern of transcripts identified in PBMCs. Primers spanning exon-exon junctions in the cysLT1R transcript 1 (exons 1–4; 5’-AAGTTAGCAGGGCGTGGGCT) and the transcript II (exons 1–5; 5’-TTGAGTAATGCACGACCAAAAG) and common primer in the cysLT1R coding region (5’-TTGAGTAATGCACGACCAAGC) were used to amplify specific fragments using Platinum Taq Polymerase High Fidelity (Invitrogen Life Technologies) under the following condition: 94°C for 30 s, 60°C for 40 s, and 68°C for 30 s for 35 cycles, β-actin cDNA amplification was used as control with primers 5’-GCTCACATTAGATGATATCGC and 3′-AGACCTGCCGTCAGCCACCG (Invitrogen Life Technologies).

Real-time PCR

THP-1 cells were cultured and exposed to recombinant human IL-4 (R&D Systems). Total RNA was extracted as above and cysLT1R and GAPDH mRNA expression were measured using a real-time PCR performed on ABI Prism 7900 sequence detection system (Applied Biosystems) using

**FIGURE 1.** Structure of the human cysLT1R gene (A). Exons are shown as boxes. The sizes of exons and introns are shown. Exon numbering is in relation to genomic contig AL445202. The length of exon 1 comes from the longest fragment obtained in 5’-RACE experiments. B, Identified splice variant of the human cysLT1R gene. CDS-coding sequence, 3’-UTR. C. The 617-bp fragment of exon 1 is shown. Numbers refer to the 3’-end of exon 1 as nucleotide –1. Identified TSSs in 5’-RACE experiments are shown with bold letters. Asterisks above the letters represent TSSs found in IL-4 stimulated 5’-RACE experiments. Sequences of 5’-RACE experiments are shown with bold letters. Underline with a dashed line ( _ _ _ ) regions with the most common TSSs are underlined. D, RT-PCR primer localization. Upper and lower panels show control and cysLT1R-specific fragments, respectively, amplified by nested PCR. The obtained cDNA was amplified by PCR (touchdown PCR followed by nested PCR) with gene-specific primers (GSP): 3’-RACE GSP-5’-CATAAACCTTGTCTCTGGCTGAC; 3’-RACE nested GSP-5’TCCACCGTGACTTATGTACCCAG; 5’-RACE GSP-5’-AGACCAACA CGGAGAGAGGACAGTGTGC; 5’-RACE nested GSP-5’-GCGGAAGCT CATCAATAGTGCTGAC; and adaptor GeneRacer primers supplied with the kit. The nested PCR products were gel purified, cloned into pCR4- Topo vector (Invitrogen Life Technologies) and sequenced using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).
commercially available probe and primers sets (Applied Biosystems). Reverse transcription and PCR were performed with an RT kit and TagMan Universal PCR master mix (both Applied Biosystems) according to the manufacturer's manual. Relative gene expression was calculated as a fold induction compared with control.

Plasmid construction

To analyze the active promoter region of the human cysLT1R gene, reporter constructs were generated in the pGL3-Basic plasmid reporter vector (Promega). Regions of interest were amplified from human genomic DNA and cloned into the pGL3 Basic vector after digestion with restriction enzymes using Platinum Taq DNA Polymerase High Fidelity (Invitrogen) under the following conditions: 94°C for 2 min, followed by 35 cycles 94°C for 30 s, 56°C for 30 s, and 68°C for 60 s and 68°C for 10 min. The following pairs of primers were used:

p125 construct: (C1) 5'-GACTAGTTCACGGTCTAAGCTGTGACCT GCCAAC/R1) 5'-GACTAGTTCTCTGACTGAACTTCACGGTAC GAGAC; (C2) 5'-GACTAGTTCACGGTCTAAGCTGTGACCT GCCAAC/R2) 5'-GACTAGTTCTCTGACTGAACTTCACGGTAC GAGAC;

p567: (C1) 5'-GACTAGTTCACGGTCTAAGCTGTGACCT GCCAAC/ R1) 5'-GACTAGTTCTCTGACTGAACTTCACGGTAC GAGAC;

p1570: (C1) 5'-GACTAGTTCACGGTCTAAGCTGTGACCT GCCAAC/R2) 5'-GACTAGTTCTCTGACTGAACTTCACGGTAC GAGAC;

p981: (C6) 5'-GACTAGTTCACGGTCTAAGCTGTGACCT GCCAAC/R1) 5'-GACTAGTTCTCTGACTGAACTTCACGGTAC GAGAC;

p410: (C3) 5'-GACTAGTTCTCTGACTGAACTTCACGGTAC GAGAC/R1) 5'-GACTAGTTCTCTGACTGAACTTCACGGTAC GAGAC;

p344: (C2) 5'-GACTAGTTCTCTGACTGAACTTCACGGTAC GAGAC/R1) 5'-GACTAGTTCTCTGACTGAACTTCACGGTAC GAGAC;

p125 construct: (C1) 5'-GACTAGTTCACGGTCTAAGCTGTGACCT GCCAAC/R1) 5'-GACTAGTTCTCTGACTGAACTTCACGGTAC GAGAC;

p567: (C1) 5'-GACTAGTTCACGGTCTAAGCTGTGACCT GCCAAC/R2) 5'-GACTAGTTCTCTGACTGAACTTCACGGTAC GAGAC;

p1570: (C1) 5'-GACTAGTTCACGGTCTAAGCTGTGACCT GCCAAC/R2) 5'-GACTAGTTCTCTGACTGAACTTCACGGTAC GAGAC;

p981: (C6) 5'-GACTAGTTCACGGTCTAAGCTGTGACCT GCCAAC/R1) 5'-GACTAGTTCTCTGACTGAACTTCACGGTAC GAGAC;

p410: (C3) 5'-GACTAGTTCACGGTCTAAGCTGTGACCT GCCAAC/R1) 5'-GACTAGTTCTCTGACTGAACTTCACGGTAC GAGAC;

p344: (C2) 5'-GACTAGTTCACGGTCTAAGCTGTGACCT GCCAAC/R1) 5'-GACTAGTTCTCTGACTGAACTTCACGGTAC GAGAC;

PCR products were gel purified, cut with the restriction enzymes and ligated into pGL3-Basic vector using LigaFast DNA ligation kit (all obtained from Promega).

Mutations were introduced into the STAT-binding motif contained within the promoter sequence of cysLT1R gene in the p567GL3 construct. The motif was mutared from TTC/N4/GAA to TAT/N4/GAA using the QuikChange II site-directed mutagenesis kit (Stratagene). The mutated oligonucleotide primer was used: 5'-CCACGTCAGCAGCTTATCTAACAGTGAATTCTAT-3'.

All constructs were verified by sequencing. Plasmid DNAs were prepared from these constructs using the EndoFree Plasmid Maxi kit (Qiagen) and quantitated by UV spectroscopy.

Transient transfections and luciferase gene reporter assay

Transient transfections of THP-1 cells were conducted using the Cell Line Nucleofector kit V (Amaxa Biosystems) according to the manufacturer's protocol. THP-1 cells were collected, resuspended in Nucleofector solution V for each individual transfection sample. A total of 0.45 g of pRL-SV40 control vector (Promega) was added, and transfection was performed using the Nucleofector device (Amaxa Biosystems) with V-01 program. Luciferase assays were performed using a dual-luciferase reporter assay system (Promega). Cells were harvested 15 h after transfection, lysed with 200 µl of passive lysis buffer and measured using the commercially available Vector 1420 counter (Promega, Life and Analytical Sciences). Firefly luciferase activity was normalized to Renilla activity to account for transfection efficiency. In IL-4-stimulated experiments cells were transfected, left to recover for 1 h and exposed to IL-4 (10 ng/ml) for 10 h before harvesting.

Flow cytometry

THP-1 cells were washed with PBS and blocked with donkey IgG (Jackson ImmunoResearch) (30 µg of IgG/106 cells) for 15 min at 4°C. The cells then were washed, resuspended in cold PBS, and stained with an isotype control Ab (Southern Biotechnology Associates) or with a polyclonal anti-cysLT1R Ab (Novus Biologicals) directed against the third extracellular loop of the receptor for 30 min at 4°C (1/200 dilution). Cells were washed again with PBS and incubated for 30 min at 4°C with R-PE-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Finally, cells were washed and resuspended in PBS, and single color immunofluorescence analysis of 10,000 cells was performed on FACScan flow cytometer (BD Biosciences).

EMSA

Nuclear extracts from unstimulated and IL-4 stimulated (10 ng/ml for 30 min) THP-1 cells were prepared using Celllytic Nuclear Extraction kit (Sigma-Aldrich). A double-stranded oligonucleotide probe containing a fragment of cysLT1R promoter region with a STAT6 response element (5'-AAATTTTCTTGGAAATTT-3') was used and labeled with biotin using Biotin 3' End DNA Labeling kit (Pierce). STAT6 DNA binding activity was determined using LightShift Chemiluminescent EMSA kit (Pierce). The nucleoprotein binding reaction was performed with 5 µg of nuclear extracts for 30 min in room temperature in the presence or absence of 200-fold excess of unlabeled STAT6 probe (specific competitor) or the control Epstein-Barr Nuclear Ag probe (nonspecific competitor). For supershift experiments, extracts were preincubated with 2 µg of anti-STAT6 polyclonal Ab (Santa Cruz Biotechnology) for 15 min before the labeled probe was added. The protein-DNA complex was run on 6% acrylamide gel and chemiluminescence was measured using charged-coupled device camera system Image Station 440 (Eastman Kodak).

ELISA

For cytokine production, THP-1 cells (1.5 × 10⁶) were incubated with IL-4 (10 ng/ml) (R&D Systems) for 24 h. Then medium was changed, and cells were stimulated with LTD₄, LTC₄, or vehicle control (ethanol) for 6 h in the presence or absence of selective cysLT₁R inhibitor MK571, (all obtained from Cayman Chemicals) or nonselective partial agonist of cysLT₁R, R-(E)4,225-(Z)4,202-(E)4,233-U47,814 (Biomol). Supernatants were stored at −20°C until analysis by commercial QuantiKey ELISA (R&D Systems) for CCL2. Sensitivity of the assay was 5 pg/ml.

Statistical analysis

Data were analyzed by one-way ANOVA or paired and unpaired Student’s t tests, as appropriate. Differences were considered significant when p < 0.05.

Results

CysLT₁R gene structure

To define the human CysLT₁R gene structure 5'-RACE and 3'-RACE were performed on total RNA from U937, THP-1 cells, and PBMCs. The obtained sequences matched to the genomic contig AL445202, which is in reverse complement orientation to a strand encoding cysLT₁R. After alignment with the genomic sequence, five exons were identified (see Fig. 1A), four of them located in the 5'-UTR and the fifth containing the open reading frame for the receptor and the 3'-UTR region. Four different mRNA transcripts of cysLT₁R were found (Fig. 1B). Transcript I was the most abundant in all cells examined, accounting for ~80% of all selected clones. The other splice variants accounted for the remaining 20% of analyzed clones. Transcript II was present in all tested cells; however, transcripts III and IV were detected only in THP-1 cells. All intron/exon junctions conformed to the canonical GT-AG rule. After stimulation of cells with IL-4 (10 ng/ml) for 6 h, all four transcripts were detected in 5'-RACE experiments as in unstimulated cells. Among them, transcript I was again the most abundant. To characterize the tissue distribution of the cysLT₁R transcripts identified in human primary cells RT-PCRs specific for the transcript I and the transcript II were performed on total RNA extracted from different human tissues (Fig. 2). In all studied tissues, transcript I was preferentially expressed, with the highest expression found in blood leukocytes, spleen, thymus, lung, kidney, and heart. Transcript II was found to be weakly expressed compared with transcript I, with the highest expression observed in blood leukocytes, thyroid gland, and lung.

In all stimulated and unstimulated 3'-RACE experiments, the same 3'-UTR fragment was detected, without any intron after alignment with genomic sequence. Five polyadenylation signals (AAUAAA) are located in 3'-UTR, the one located in position 26801–26796 of contig AL445202 is in the correct position to be responsible for polyadenylation in the cells studied.

Thirty transcription start sites (TSSs) (Fig. 1C) were identified in the first exon, which was invariably expressed in all mRNA transcripts analyzed. The most common TSSs were localized to regions: −233/−231 (28%); −225/−221 (19%); and −204/−202 (18%), and they accounted together for 65% of all identified transcripts (numbers refer to the last nucleotide at the 3' end of exon
1 as number −1). To define the region responsible for transcription initiation and to detect rare transcripts, a set of RT-PCRs was conducted (Fig. 1D), showing a region of ∼500 bp as a place of potential transcription initiation. However, in our 5′-RACE experiments most transcripts originated in the region between −150 and −300. All transcripts obtained in IL-4 stimulated experiments had TSSs localized to the above region (Fig. 1C). BLAST search of GenBank human mRNA sequences and expressed sequence tags revealed seven sequences (NM006639, BC035750, BM920299, CD701219, CD692728, CD688768, BX106054) matching the sequence of mRNA transcript I, all originated in the region of transcription initiation defined here.

CysLT1R promoter activity

A luciferase gene reporter approach was used to examine the potential promoter activity of cysLT1R gene in THP-1 cells. Deletion constructs were created covering 3.8 kb of the 5′ upstream region from the 3′ end of exon 1, and promoter activities of these constructs were measured (Fig. 3A). It was found that the putative region promoting basal transcription is situated in the region between −125 and −786 from the 3′ end of exon 1, with a construct p567 showing the highest activity.

The major pathway of IL-4 signaling involves activation of the STAT6 transcription factor. The putative cysLT1R promoter was shown to contain two motifs: motif A (5′-AAATTCTGAGAATT-3′) and motif B (5′-AAATTCCCTGAGAATT-3′). In the presence of IL-4, the promoter activity of the constructs containing motif A was significantly increased compared to the control constructs without motif A. The p567 construct, which contains both motifs, showed the highest activity, followed by the p567-MUT construct, which contains the mutated motif A (5′-AAATTCTGAGAATTC-3′).

Figure 2. Expression of cysLT1R mRNA spliced transcripts in human tissues. Panels represent RT-PCR products specific for spliced cysLT1R transcripts and β-actin. I. cysLT1R transcript I; II. cysLT1R transcript II; III. control β-actin transcripts.

Figure 3. Luciferase reporter activity in THP-1 cells transfected with cysLT1R promoter deletion constructs (A). Luciferase activity in cells transfected with deletion (B) or STAT6 mutated constructs (C) and exposed to IL-4 or vehicle for 10 h. Results are presented as fold values over an empty pGL3 basic vector. The means ± SD from three experiments done in triplicate are shown. *, p < 0.05; **, p < 0.01.
found to contain two STAT6 consensus response elements in positions \(-943/-934\) and \(-459/-450\). To analyze functional activity of putative cysLT1R promoter and STAT6 response elements, THP-1 cells were transfected with deletion constructs, stimulated with IL-4 and gene reporter activity was determined (Fig. 3).

IL-4 stimulation significantly increased luciferase activity in all tested constructs, except for p410, which does not contain a STAT6 motif. The highest increase of activity (1.74 ± 0.06) was observed in a case of construct p567, suggesting that the STAT6 motif localized to position \(-459/-450\) might be responsive to IL-4 stimulation. Mutation of this STAT6 consensus sequence abolished in total the observed IL-4-stimulated increase in luciferase activity (Fig. 3C).

To test whether the identified STAT6 motif can interact with STAT6 protein, EMSA experiments were conducted. Nuclear extract from IL-4-stimulated THP-1 cells but not from unstimulated cells showed strong STAT6 DNA binding activity (Fig. 4). The specificity of IL-4 stimulated STAT6 activity was demonstrated by total inhibition of binding by an excess of unlabeled STAT6 motif-containing probe, whereas the binding was not affected by the same molar excess of an unrelated oligonucleotide. Preincubation of nuclear extracts with Ab directed against STAT6 resulted in disappearance of nucleoprotein complex although a strong supershift band was observed. Taken together, these results indicate that identified putative promoter of cysLT1R gene contains a significant promoter activity that can be further increased by IL-4-induced STAT6 binding to a STAT6 motif present in the proximal region of the cysLT1R promoter region.

**Expression of CysLT1R**

To investigate whether the IL-4-increased cysLT1R promoter activity observed in gene reporter experiments is functional at mRNA and protein levels, expression of cysLT1R mRNA was measured by real-time RT-PCR, and the cysLT1R cell surface expression was determined by flow cytometric analysis. IL-4 stimulation increased cysLT1R mRNA in a time-dependent fashion, with the highest increase observed after 6 h of incubation (Fig. 5A). IL-4 stimulation caused a significant time- and dose-dependent increase in surface receptor expression, with the maximum response observed after 24 h of incubation (Fig. 5, B–D).

**FIGURE 4.** STAT6 binds to a STAT6 motif in the cysLT1R promoter. EMSA with nuclear extracts prepared from unstimulated (line 2–3) and IL-4 stimulated (line 4–7) THP-1 cells was performed with biotin-labeled probe containing the STAT6 motif of the cysLT1R promoter. No protein extract was added in the first line. Cold specific competitors using the original probe and cold nonspecific competitors were added as indicated. The specific DNA-protein complex and supershift (SS) bands are marked. The EMSA shown is representative of three separate experiments.

**FIGURE 5.** CysLT1R mRNA and protein expression. IL-4 increases steady-state levels of cysLT1R mRNA (A). THP-1 cells were stimulated with IL-4 (10 ng/ml) for up to 24 h and cysLT1R mRNA expression was measured by TaqMan analysis. Results are normalized to internal control (GAPDH expression) and presented as fold increase from control values (treated cells vs vehicle). The means ± SD of three different samples are shown. Flow cytometric analysis of cysLT1R surface expression. THP-1 cells were stimulated with IL-4 (10 ng/ml) for 24 h and labeled with anti-cysLT1R Abs or with isotype-matched control. Results of a single experiment, representative of at least three are presented (B). Dotted line represents isotype control Ab. Solid thick and thin lines represent labeling of medium- and IL-4-treated cells, respectively. Data from time-dependent (C) and dose-dependent (D) (ANOVA, \(p < 0.001\)) experiments are shown. Results are presented as mean fluorescence intensity corrected by subtracting isotype control values (MFI cor). The means ± SD of three experiments are shown. *, \(p < 0.01\) and **, \(p < 0.001\) in comparison to unstimulated controls.
Production of CCL2 by THP-1 cells

To determine whether an IL-4-stimulated increase of cysLT1R expression can be functionally relevant for cell activity and whether cysLTs can modulate production of chemokines in human monocytic cells, THP-1 cells were treated with LTD₄ for 6 h and supernatants were assayed for CCL2 expression. No significant CCL2 expression was observed in cells stimulated with LTD₄ (Fig. 6A). To test the hypothesis that IL-4 may increase the responsiveness of human monocytes to cysLTs, THP-1 cells were treated with IL-4 for 24 h and stimulated with various concentrations of LTD₄ for 6 h. IL-4 itself caused a significant increase in CCL2 production. However, THP-1 cells preincubated with IL-4 produced severalfold higher levels of CCL2 after LTD₄ stimulation in a concentration-dependent manner (Fig. 6A). The effect of LTD₄ was effectively blocked by the selective cysLT₁R inhibitor MK571 (Fig. 6B), suggesting a cysLT₁R-mediated mechanism of stimulation. MK571 treatment fully inhibited the LTD₄-mediated increase of CCL2 production in a dose-dependent manner (Fig. 6A). The effect of LTD₄ was also totally blocked by MK571 in a dose-dependent manner (Fig. 7B). BAY-u9773, which is a partial agonist for both cysLT₁R and cysLT₂R, in the presence of LTD₄ or LTC₄ caused only a partial inhibition of increase of CCL2 (Figs. 6B and 7B). These data in all suggest that the induction of CCL2 by cysLTs in IL-4-primed THP-1 cells is mediated through a cysLT₁R pathway.

Discussion

We describe here the genomic organization of human CysLT₁R gene. The results are based on 5′- and 3′-RACE experiments with a modified method that ensures the amplification of only full-length transcripts via elimination of truncated messages from the

![FIGURE 6](http://www.jimmunol.org/Downloaded_from.png)

**FIGURE 6.** Effects of LTD₄ on the production of CCL2 by THP-1 cells. A, Cells were incubated with or without IL-4 (10 ng/ml) for 24 h (t test; *, p < 0.001) and stimulated with various concentrations of LTD₄ for 6 h. LTD₄ stimulation of IL-4-primed cells significantly increased CCL2 expression in a dose-dependent manner (ANOVA; ***, p < 0.001). B, Cells were incubated with IL-4 for 24 h and stimulated with LTD₄ in the absence or presence of inhibitors. Selective cysLT₁R inhibitor MK571 inhibited the LTD₄-induced increase of CCL2 in a dose-dependent manner (***, ANOVA, p < 0.001). Nonselective partial agonist of cysLT receptors BAY-u9773 partially inhibited LTD₄-stimulated CCL2 expression (t test; *, p < 0.002). The results are the mean ± SD; n = 9.

![FIGURE 7](http://www.jimmunol.org/Downloaded_from.png)

**FIGURE 7.** Effects of LTC₄ on the production of CCL2 by THP-1 cells. A, Cells were incubated with or without IL-4 (10 ng/ml) for 24 h (t test; *, p < 0.001) and stimulated with various concentrations of LTC₄ for 6 h. LTC₄ stimulation of IL-4-primed cells significantly increased CCL2 expression in a dose-dependent manner (ANOVA, p < 0.001). B, Cells were incubated with IL-4 for 24 h and stimulated with LTC₄ in the absence or presence of inhibitors. Selective cysLT₁R inhibitor MK571 inhibited the LTC₄-induced increase of CCL2 in a dose-dependent manner (ANOV A, p < 0.001). Nonselective partial agonist of cysLT receptors BAY-u9773 partially inhibited LTC₄-stimulated CCL2 expression (t test, p = NS). The results are the mean ± SD; n = 9.
amplification process by removing 5'-phosphates. The total length of the identified cysLT1R transcripts (~2.7 kb) is in agreement with data obtained in Northern blot experiments (5, 6). The gene consists of five exons variably spliced. Transcript I, composed of exons 1, 4, and 5, represents the major transcript present in human leukocytes, smooth muscle cells as well as in other studied human tissues. A similar structure of the human cysLT1R 5'-UTR was found by Sarau et al. (6) in a PBMC cDNA library and by Maekawa et al. (22) in a human lung cDNA library. Similarly, BLAST search of GenBank sequences revealed only sequences representing the 5'-UTR sequence of transcript I. The widespread distribution of cysLT1R transcripts found in our study is similar to the data reported by others (5, 6). Transcript II, although less abundant, was also found in blood leukocytes, smooth muscle cells, and tissues showing high cysLT1R mRNA expression, such as heart, lung, and spleen. The tissue distribution of the mouse cysLT1R mRNA was reported (22), showing a similar pattern with the exception of lack of cysLT1R expression in the murine spleen. All identified transcripts originated in the same region of exon 1, suggesting that the region between −150 and −300 (−1 corresponds to the first nucleotide at 3' end of exon 1) is the primary site of cysLT1R transcription initiation. Reporter gene expression analysis performed in THP-1 cells provided data that the sequence immediately upstream of TSSs region possesses a significant promotor activity. Deletion construct reporter gene data pointed to a region −125 and −786, as containing a putative basal promoter. Analysis of that region shows that like other G-protein-coupled receptor promoters (23, 24), the human cysLT1R promotor is TATA-less and contains several binding sites for transcription factors, such as AP-1 and GATAA. Interestingly, we found very high homology (80%) between the human putative basal promoter region and the mouse equivalent region, which was predicted by using the sequence of mouse cysLT1R exon 1 (AF329271) (22). High homology between the putative mouse and human cysLT1R 5'-UTR sequence was reported, too (22). Unfortunately, the promotor activity of the mouse sequence has not been reported yet, but we assume based on sequence homology that it should be localized to a similar region. The role of alternative splice variants of cysLT1R is not known. The rare cDNAs represented ~20% of all cysLT1R transcripts in our study and the ratio between expression of transcript I and other transcripts did not change after IL-4 stimulation. There is evidence that the structure of the 5'-UTR region may regulate gene expression (25), or it can be related to cell-specific gene expression. In our study, we did not observe any significant differences related to expression of these rare transcripts. Comparison of the human and the mouse cysLT1R cDNAs shows that human transcript II contains sequences equivalent to the mouse short isoform. Both cDNAs, the human transcript II and the mouse short isoform, are less preferentially expressed. However, all human transcripts lack the equivalent of mouse exon 3. In addition, rare human transcripts III and IV contain exons 2 and 3, equivalents of which have not been identified in mice. Comparison of human and mouse (22) 3'-UTR regions revealed similar length (1259 bp in human and 1110 bp in mouse) and intronless fragments with high homology (60–80%) at the sequence level, showing, apart from some differences in 5'-UTR structure, high homology between the human and mouse cysLT1R gene structures.

The mechanism of IL-4-mediated priming of monocytes may involve several pathways. It has been reported that IL-4 regulates cysLT1R expression in human monocytes and macrophages (21). In our study, THP-1 cells stimulated with IL-4 demonstrated a 2-fold increase of cysLT1R mRNA expression, followed by a significant change in the surface cysLT1R protein expression, consistent with data obtained by Thivierge et al. (21). The maximum effect of IL-4 on cysLT1R mRNA was observed after 6 h of incubation. However, the change of cell surface receptor numbers was delayed, showing the maximum expression after 24 h. These data in addition to transcriptional assays reported here suggest that at least part of the observed increase in receptor surface expression is transcriptionally regulated at the gene promoter level. The primary pathway of IL-4R signaling relates to the activation of cytoplasmic Janus kinases and the subsequent phosphorylation of several molecules, in particular STAT6, which dimerizes and is translocated to nucleus (26). In the nucleus, it binds preferentially to the consensus sequence TTC(N4)GAA (27) and drives expression of many IL-4-responsive genes (28, 29). There are two putative STAT6 response elements in the cysLT1R promotor. Here we provide the evidence that the proximal STAT6 response element is necessary for IL-4-induced up-regulation of cysLT1R expression in THP-1 cells. Altogether, these data show that IL-4 stimulation induces cysLT1R expression in THP-1 cells by a transcriptionally regulated, STAT6-dependent mechanism.

To address a question whether the IL-4-stimulated increase of cysLT1R expression may influence functions of monocytes, we analyzed CC chemokine (CCL2) expression after cysLTs stimulation. CysLTs stimulation of nonstimulated THP-1 cells did not induce significant CCL2 expression, as in earlier studies (17). However, THP-1 cells primed with IL-4 responded to LTD4 and LTC4 with a several-fold increase in CCL2 synthesis. Pretreatment of THP-1 cells with MK571, a cysLT1R-specific antagonist, effectively inhibited increased CCL2 production in response to cysLTs, implying a direct cysLT1R-mediated mechanism. A similar effect of IL-4 priming on CCL2-induced proinflammatory mediator expression was observed in human mast cells. A 5-day incubation of human mast cells with IL-4 caused CCL2-induced up-regulation of IL-5, TNFα production, and very potent increase of MIP-1β expression (30, 31). All these effects were inhibited by cysLT1R antagonists. However, no increased expression of cysLT1R was observed. The mechanism by which cysLTs stimulate CCL2 expression in IL-4-primed cells is not known. It has been shown that the LTD4 signaling pathway in THP-1 cells involves activation of MAPK (Erk-2) through a protein kinase Cε and Raf-1-dependent pathway (32), but no data is available on regulation of gene expression by cysLTs at the level of interaction of transcription factors. Increased expression of cysLT1R could result in enhanced signaling through the receptor and CCL2 production. However, because non-IL-4-stimulated THP-1 cells did not produce CCL2, it cannot be ruled out that this could be a cross-talk between activated STAT6 and cysLT-activated pathway, which is responsible for observed production of CCL2. It has been reported that IL-4 stimulation can induce CCL2 expression through a STAT6-mediated mechanism in human endothelial cells (33). Similarly in our study, IL-4 stimulation caused increased production of CCL2 that was further up-regulated by cysLTs stimulation. In murine fibroblasts, IL-4 stimulation also induced CCL2 expression that was markedly reduced in STAT6−/− cells (34), suggesting that an IL-4/STAT6 pathway is responsible for CCL2 induction, and cysLTs signaling may up-regulate this mechanism. 5-LO pathway products, cysLTs and LTβP, play an important role in the pathogenesis of chronic inflammatory disorders, such as bronchial asthma (35), atherosclerosis (36), or hyperlipidemia-dependent aortic aneurysm formation (15). We provide here evidence that apart from already known effects of cysLT1R in pathogenesis of chronic inflammatory diseases, cysLTs might potentiate inflammatory reactions by enhancing CCL2 production in IL-4-stimulated cells. CCL2 is a potent chemoattractant for monocytes (37, 38). Increased expression of CCL2 in asthmatic bronchial tissue as well as in bronchoalveolar lavage fluid is described (39, 40), suggesting that CCL2 may effectively regulate cell trafficking in asthma in response to allergen exposure. It has been shown in a murine model that blockage of CCL2 reduced...
both lung leukocyte infiltration and bronchial hyperreactivity (41). In addition, a recent study showed that corticosteroids had no effects on CCL2 mRNA expression in bronchial epithelium and submucosa in asthmatic patients, suggesting that a part of this inflammatory pathway related to cystkTs may not be fully inhibited by corticosteroid treatment (42). Similarly, among chemokines involved in the pathogenesis of atherosclerosis, CCL2 seems to play one of the major roles (43). Up to now, it has been shown that LTB₄, not cystkTs, might directly increase CCL2 expression in human monocytes (17). Our preliminary observation that cystkTs may synergize with the IL-4/IL-STAT6 pathway to enhance CCL2 production in monocytic cells, when confirmed in primary cell systems, might be significant for studying pathomechanisms of chronic inflammatory reactions, such as in bronchial asthma and atherosclerosis.

In conclusion, we have characterized the structure of the human cysteLT-R gene and demonstrated that the expression of cysteLT-R is functionally regulated by IL-4 through a STAT6 element localized to the cysteLT-R promoter region. In addition, we have shown that cysteLTs generated by the 5-LO pathway and IL-4 might contribute to inflammatory reactions by inducing CCL2 production in human monocytic cells through a STAT6-dependent mechanism.

Disclosures

The authors have no financial conflict of interest.

References