Concordant Modulation of Cysteinyl Leukotriene Receptor Expression by IL-4 and IFN- γ on Peripheral Immune Cells

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Arachidonic acid can be metabolized to form a group of compounds known as the cysteinyl leukotrienes (CysLT) that bind to one of two receptors to mediate their actions. On circulating cells, expression of the leukotriene receptors is low, but in inflamed tissue the receptor number is dramatically increased. We hypothesized that the cytokine milieu present during inflammation can increase receptor expression on infiltrating immune cells. Various cell populations were purified from peripheral blood and stimulated *in vitro* with cytokines characteristic of allergic inflammatory disorders, and CysLT receptor expression was measured using quantitative PCR analysis, Western blot, and flow cytometry. IL-4, but not IL-13, was able to significantly induce mRNA and protein levels for both CysLT receptor 1 and 2 from T cells and B cells. CysLT2 receptor expression was also significantly increased in monocytes and eosinophils after IL-4 stimulation. Surprisingly, CysLT2 receptor expression was increased in monocytes, T cells, and B cells when IFN- γ was used as the stimulus. Factors involved in eosinophil growth and survival were tested for their ability to alter CysLT receptor expression. These results support the concept that cytokines increase expression of both receptors on lymphocytes and granulocytes, allowing these cells to be more responsive to secreted leukotrienes at sites of inflammation.

Keywords: cysteinyl leukotriene receptor; T cell; B cell; eosinophil; cytokine

In 1937, the slow-reacting substance of anaphylaxis was described based on the identification of a mediator derived from activated mast cells that, in an antihistamine-resistant fashion, mediated the sustained, prolonged contraction of smooth muscle. This mediator was later identified as a group of compounds known as the cysteinyl leukotrienes (CysLTs). Leukotrienes (LTs) are derived from the metabolism of arachidonic acid. Membrane phospholipids are converted into arachidonic acid by the action of a family of enzymes termed phospholipase A₂. With cell activation, the enzyme 5-lipoxygenase (5-LO) translocates from the cytosol to the inner nuclear membrane, where in association with 5-lipoxygenase-activating protein (FLAP), it oxygenates and then dehydrates arachidonic acid to generate LTA₄ (1). LTA₄ can be conjugated with the tripeptide glutathione into the cysteinyl leukotriene LTC₄ by the enzyme LTC₄ synthase (LTC₄S) (or the related enzyme microsomal glutathione

CLINICAL RELEVANCE

These results show that the inflammatory milieu in asthma can increase both type 1 and 2 cysteinyl leukotriene receptor expression. Increased receptor expression will make these cells more responsive to leukotriene expression and increase the severity of disease.

transferase II). LTC₄ is exported from the cell where removal of the amino acid glutamate by serum γ glutamyl transpeptidase generates LTD₄. LTE₄ is produced after cleavage of a glycine residue from LTD₄ by the enzyme dipeptidase, leaving behind the single amino acid cysteine from which this family derives its name (2). Together, LTC₄, D₄, and E₄ comprise the CysLTs.

Actions of the CysLTs are mediated through their high-affinity interactions with the CysLT1 and CysLT2 receptors. Both receptors are seven transmembrane domain G protein-coupled receptors that in part use calcium as a second messenger (3, 4). The two receptors can be distinguished by their relative potency for the CysLTs: CysLT1 receptor LTD₄ > LTC₄ >> LTE₄ and CysLT2 receptor LTD₄ = LTC₄ >> LTE₄. Thus, the CysLT1 receptor has an affinity for LTD₄ ~ 50-fold greater than that for LTC₄. There is varied distribution of the CysLT receptors on peripheral blood leukocytes (4–6). Both receptors are widely expressed on eosinophils and mast cells, whereas only CysLT1 receptors are expressed on neutrophils. Very few circulating T lymphocytes are reported to express either class of receptor (~ 4–8%) (4, 5). In addition to these immune cells, the CysLT1 receptor has been found on smooth muscle cells and lung fibroblasts and the CysLT2 receptor is expressed on heart Purkinje fiber cells, adrenal chromaffin cells, brain, and endothelial cells (5, 7). In contrast to lung fibroblasts, nasal polyp-derived fibroblasts do not express either the CysLT1 or -2 receptors (8).

Asthma, allergic rhinitis, and chronic hyperplastic eosinophilic sinusitis (CHES) with or without nasal polyposis (NP) are allergic inflammatory diseases characterized by the influx of multiple cell types to affected tissue sites. These diseases are associated with a complex cytokine milieu described mainly as Th2 in nature (IL-4, IL-13, and IL-9), but including Th1 cytokines (IFN- γ) as well (9, 10). Several studies have shown markedly increased CysLT receptor expression on cells at sites of inflammation in these disorders as compared with circulating cells or those present in noninflamed tissue (5, 6, 11). We therefore tested the hypothesis that cytokines present at sites of allergic airway inflammation can modulate CysLT receptor expression on immune cells.

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MATERIALS AND METHODS

Subjects

Heparinized venous blood was obtained with informed consent from healthy human volunteers (18–55 yr old) using a protocol approved by the Human Investigation Committee at the University of Virginia.

Cell Culture

Peripheral blood mononuclear cells (PBMCs) were isolated through Ficoll-Hypaque (Sigma, St. Louis, MO) density centrifugation. CD14⁺ monocytes were enriched from PBMCs using positive selection magnetic affinity column purification (CD14⁺; Miltenyi Biotec, Auburn, CA). CD4⁺ T cells were enriched from PBMCs using positive magnetic affinity column purification (CD4⁺; Miltenyi Biotec). CD19⁺ B cells were enriched from PBMCs using positive magnetic affinity column purification (CD19⁺; Miltenyi Biotec). Granulocytes were isolated through dextran sedimentation and hypotonic lysis. Eosinophils were enriched from granulocytes using negative magnetic affinity column purification (CD16⁺; Miltenyi Biotec). The purity of each cell population as measured by flow cytometry was as follows: CD4⁺ T cells, 99.2%; monocytes, 91.3%; B cells, 86.8; and eosinophils, 98.9%.

The cells were washed and resuspended in complete RPMI-1640 medium containing 0.01 mol/liter HEPES (Invitrogen, Carlsbad, CA) and 10,000 U/ml penicillin and 10 µg/ml streptomycin supplemented with 10% autologous serum and maintained at 37°C in 5% CO₂. Cells were stimulated with IL-4 (20 ng/ml), IFN-γ (20 ng/ml), IL-5 (10 ng/ml), IL-3 (10 ng/ml), GM-CSF (50 ng/ml) (BD Pharmingen, San Diego, CA), or IL-13 (20 ng/ml; Biosource, Camarillo, CA). These concentrations were chosen from our studies involving dose-response curves that demonstrated optimal activity or from other published studies (8, 12, 13). For studies involving changes in mRNA expression, cells were stimulated for 16 h (determined to be time point of maximal effect from time course induction studies) before RNA was harvested. For studies involving protein expression, cells were stimulated for 72 h with cytokines for Western blot and 24 h for flow cytometry.

Reverse Transcription of mRNA

Total RNA was extracted from cells using a SV Total RNA Isolation kit (Promega, Madison, WI). Conversion of the mRNA to cDNA was performed using a Taqman Reverse Transcription kit (Roche, Branchburg, NJ) as previously described (8). Briefly, 200 ng of RNA were added to each reaction along with oligo dT primers, 5.5 mM MgCl₂, 2 mM dNTPs, RNasin, and reverse transcriptase. Reactions went through one cycle of 10 min at 25°C, 30 min at 48°C, and 5 min at 95°C in a Bio-Rad iCycler thermocycler (Bio-Rad, Hercules, CA). The cDNA was amplified by polymerase chain reaction (PCR) using appropriate primer pairs and the resulting products analyzed by quantitative PCR for the CysLT1 and CysLT2 receptors (8). Primers for β-actin, CysLT1 receptor and CysLT2 receptor have been previously described (8). Probes used for detection of CysLT1 and CysLT2 receptors are as follows: CysLTR1 probe, 5'-FAM-CACTGCCCTCTCCGTGTGGTC-BHQ1-3' and CysLTR2 probe, 5'-FAM-CTTGGTGGTGGGCTGCCTGCT-BHQ1-3'. Quantification of changes in receptor expression induced by cytokines was performed using the comparative Cr method. Briefly, the amount of target, normalized to an endogenous reference and relative to a calibrator, was calculated by $2^{-\Delta\Delta C_t}$ with $\Delta\Delta C_t = (\text{threshold cycle unstimulated gene of interest} - \text{threshold cycle unstimulated housekeeping gene}) - (\text{threshold cycle stimulated gene of interest} - \text{threshold cycle stimulated housekeeping gene})$. The comparative Cr method was validated by showing that the efficiencies of target and reference amplification were equal. For Figure 1, data were analyzed by $1/(\Delta C_t(\text{receptor}) - \Delta C_t(\beta\text{-actin})) \times 100$, allowing a semi-quantitative comparison of receptor expression between unstimulated cells in each population. Primer pairs and probes for each reaction were synthesized by Integrated DNA Technologies (Coralville, IA).

Flow Cytometry

Cell surface expression of CysLT1 and CysLT2 receptors were evaluated using rabbit polyclonal anti-CysLTR1 (Novus Biologics, Littleton, CO) and anti-CysLTR2 (directed against the N terminus #120560) (Cayman Chemical Co., Ann Arbor, MI) respectively, followed by

labeling with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Caltag Laboratories, Burlingame, CA). Rabbit IgG was used as the isotype control. Goat IgG (30 min at 4 degrees) was used to block the cells to prevent nonspecific binding of the rabbit IgG to cell surface Fc receptors. Data were analyzed by comparing cells labeled for each receptor to cells labeled with the isotype control and the percentage of cells positive and mean fluorescence was determined (Flowjo, San Carlos, CA).

Western Blots

Cell pellets were collected and lysed in 300 µl of lysis buffer (20 mM Tris-HCl [pH 7.6], 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 10% glycerol, 1% Triton X-100, 1 mM PMSF). Protein concentration was determined for each sample and equal amounts of protein were added to 2× Laemmli Sample Buffer (Bio-Rad) and heated for 3 min at 95°C. Samples were run on a 10% SDS polyacrylamide gel (ISC BioExpress, Kaysville, UT) and transferred to a nitrocellulose membrane for 18 h at 40 V. The membrane was blocked with TBST buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.05% Tween 20) with 5% nonfat dry milk before addition of anti-CysLTR1 or anti-CysLTR2 (Cayman). The membrane was washed before addition of the goat anti-rabbit horseradish peroxidase secondary antibody (Bio-Rad). Blots were developed using the Supersignal West Pico Chemiluminescent kit (Pierce, Rockford, IL).

Calcium Flux Assay

Individual peripheral blood cell populations were purified as described above and plated in a 96-well dark-walled plate at 150,000 cells per well in 100 µl. Cells were either left untreated or stimulated with either IL-4 (20 ng/ml) or IFN-γ (20 ng/ml) for 16 h at 37°C. The calcium flux assay was performed using the FLIPR calcium assay kit following manufacturer's instructions (Molecular Devices, Sunnyvale, CA). Briefly, 100 µl of loading buffer supplemented with 2.5 mM probenecid (Sigma) was added to each well and incubated at 37°C for 1 h. Cells were stimulated with LTC₄ or LTD₄ (Cayman) at concentrations ranging from 1×10^{-13} to 1×10^{-6} M to generate dose-response curves. MK571 (BIOMOL International, Plymouth Meeting, PA) at 1×10^{-5} M was used to antagonize the CysLT1 receptor. Cytoplasmic $[Ca^{2+}]_i$ was determined at an extinction wavelength of 485 nm and an emission wavelength of 525 nm using a Flexstation fluorescence spectrophotometer (Molecular Devices). Each condition was set up in triplicate and the average of the three measurements used for analysis. Data analysis was performed using GraphPad Prism 3 (GraphPad Software Inc, San Diego, CA).

Statistical Analyses

Data were contrasted between unstimulated and stimulated cells by Wilcoxon Rank Sum for nonparametric data analyses or paired *t* test for parametric data using JMP 3.2 software (Cary, NC).

RESULTS

Ability of Cytokines to Alter CysLT Receptor mRNA Levels on Circulating Immune Cells

On circulating immune cells, it has been reported that the expression of CysLT receptors is low; however, several reports have shown high levels of receptor expression at sites of allergic airway inflammation. We hypothesized that the cytokine milieu present under these inflamed conditions can induce expression of the receptors, thus making them more responsive to LTs present in the tissue. Monocytes, T and B lymphocytes, and eosinophils were enriched from peripheral blood using magnetic bead separation and cultured with either IL-4, IL-13, IL-9 (Th2 cytokines), or IFN-γ (Th1 cytokine). RNA was extracted from the cells and CysLT receptor message was measured using qPCR (Table 1). IL-4, but not IL-13, was able to significantly induce mRNA levels for both CysLT receptor 1 and 2 from T cells and B cells ($P < 0.01$). CysLT1 and CysLT2 receptor mRNA expression was increased for monocytes with IL-4 stimulation, but did not reach statistical significance. To our surprise, CysLT2 receptor

TABLE 1. CYTOKINE MODULATION OF CYSTEINYL LEUKOTRIENE RECEPTOR EXPRESSION

	IL-4		IL-13		IFN- γ	
	CysLT1R	CysLT2R	CysLT1R	CysLT2R	CysLT1R	CysLT2R
Monocytes ($n = 10$)	2.5 $\pm$ 1.0	2.9 $\pm$ 1.3	0.6 $\pm$ 0.2*	3.3 $\pm$ 2.0	2.1 $\pm$ 0.9	10.1 $\pm$ 3.8 [†]
T lymphocytes ($n = 8$)	4.3 $\pm$ 2.1*	18.9 $\pm$ 10.1 [§]	1.1 $\pm$ 0.3	3.7 $\pm$ 1.5	1.1 $\pm$ 0.4	3.7 $\pm$ 1.9 [†]
B lymphocytes ($n = 6$)	3.5 $\pm$ 0.6*	11.1 $\pm$ 2.7*	1.4 $\pm$ 0.4	1.5 $\pm$ 0.3	1.1 $\pm$ 0.3	4.8 $\pm$ 1.8 [†]
Eosinophils ($n = 10$)	2.4 $\pm$ 0.9	4.2 $\pm$ 1.5 [†]	1.9 $\pm$ 0.4 [†]	3.0 $\pm$ 1.2 [†]	3.4 $\pm$ 1.8	2.0 $\pm$ 1.0
	IL-5		GM-CSF		IL-3	
	CysLT1R	CysLT2R	CysLT1R	CysLT2R	CysLT1R	CysLT2R
Eosinophils ($n = 8$)	110.9 $\pm$ 85.8	35.7 $\pm$ 24.7	31.8 $\pm$ 25.3	12.8 $\pm$ 11.1	29.9 $\pm$ 25.5	9.3 $\pm$ 5.2

Data presented as a fold change in comparison to unstimulated cells which was set at 1.

[†] $P < 0.05$.

* $P < 0.01$.

[§] $P < 0.001$.

mRNA expression was increased in monocytes, T cells, and B cells when IFN- γ was used as a stimulus ($P < 0.05$). Similar to mononuclear cells, circulating eosinophils express low levels of both the CysLT1 and CysLT2 receptors, but increased expression is observed in allergic inflammatory disorders of the airways. Stimulation with IL-4 significantly ($P < 0.05$) increased expression of CysLT2 receptor mRNA, and IL-13 significantly ($P < 0.05$) increased both CysLT1 and CysLT2 receptor expression (Table 1). A trend toward increased CysLT1 and CysLT2 receptor expression was observed when eosinophils were stimulated with IFN- γ . Other cytokines, including IL-3, IL-5, and GM-CSF, are critically involved in eosinophil growth, maturation, and delay in apoptosis in allergic diseases. We therefore also tested whether the addition of these cytokines to eosinophil cultures would lead to increases in CysLT receptor expression. Both CysLT1 and CysLT2 receptor expression were increased when IL-3, IL-5, or GM-CSF was added to the cultures, but none were statistically significant (Table 1). The high degree of variation observed with IL-3, IL-5, and GM-CSF reflects large intra-individual differences regarding both the tendency and degree of responsiveness to eosinophil activation and the variable anti-apoptotic effects of these cytokines.

In general, the effect of cytokines on CysLTR2 expression was more pronounced than on CysLTR1 expression. We examined the relative levels of both CysLT receptors in each cell population to see if differences in basal expression could, in part, explain the stronger CysLTR2 induction (Figure 1). The basal levels of CysLTR1 expression were highest on T cells (64.9 $\pm$ 12.7) and B cells (59.3 $\pm$ 7.8), though of all cell types, T cells had the widest variation in CysLTR1 receptor expression. Monocytes and eosinophils had the lowest levels of CysLTR1 expression: 20.9 $\pm$ 4.5 and 13.2 $\pm$ 0.8, respectively. All of the cell types tested had low basal levels of CysLTR2; T cells 13.3 $\pm$ 1.8, B cells 16.1 $\pm$ 1.9, monocytes 12.4 $\pm$ 2.1, and eosinophils 12.8 $\pm$ 1.0. When compared with each other, there were significant differences ($P < 0.05$) in the levels of CysLTR1 compared with CysLTR2 for T cells, B cells, and monocytes.

Modulation of CysLT Receptor Expression

We confirmed that increased mRNA expression for the CysLT receptors resulted in increased protein production by Western blot analysis (data not shown) and flow cytometry. As demonstrated in Figure 2 using flow cytometry, T cells stimulated with IL-4 displayed increased levels of CysLT1 (Figure 2A) and 2 (Figure 2B) receptor protein on the cell surface. When B cells were stimulated with IL-4, again both the CysLT1 (Figure 2C) and CysLT2 (Figure 2D) receptor numbers were increased. Similar results were observed on monocytes and eosinophils (data

not shown). In agreement with the mRNA data in Table 1, IFN- γ increased cell surface expression of the CysLT2 receptor on monocytes (Figure 3A) and B cells (Figure 3B). Similar results were observed on T cells and eosinophils (data not shown). Even though the mRNA induction was not significant, we tested the ability of IL-5 to induce the CysLT receptors on eosinophils. After 24 h stimulation with IL-5, there was a large increase in the number of CysLT1 receptors and a small increase in the number of CysLT2 receptors on the surface of eosinophils (see the online supplement for details).

Functional Analysis of Increased Leukotriene Receptor Expression

Having demonstrated that cytokines can increase both mRNA and protein expression for the CysLT receptors, we wanted to

Figure 1. Relative expression of CysLT receptor mRNA expression on unstimulated peripheral leukocyte populations. Cells were separated from blood using magnetic bead affinity chromatography, and RNA was harvested. Real-time PCR was used to detect the CysLTR1 and CysLTR2 receptors and the housekeeping gene β -actin. Relative receptor expression in each cell population was determined using the equation $1/(\Delta C_t(\text{receptor}) - \Delta C_t(\beta\text{-actin})) \times 100$. Each dot represents an individual data point, and the line represents the mean of all data points for a particular receptor and cell type.

Figure 2. Flow cytometric analysis of CysLT receptor expression on IL-4-stimulated cells. T cells (A, B) and B cells (C, D) were separated from blood using magnetic bead affinity chromatography and stimulated with 20 ng/ml IL-4 for 16 h before cells were collected for analysis. Cell surface expression of CysLTR1 (A, C) and CysLTR2 (B, D) receptors were evaluated using rabbit polyclonal anti-CysLTR1 and anti-CysLTR2, respectively, followed by labeling with FITC-conjugated goat anti-rabbit IgG. *Open histogram*, isotype control; *solid histogram*, unstimulated; *shaded histogram*, IL-4 stimulated. The data are representative of three independent experiments.

determine whether or not the increased levels we observed led to increased functionality of the receptors on the cells. To address this, we performed calcium mobilization assays. T cells were plated and left untreated or treated with IL-4 for 24 h, after which cells were loaded with a fluorescent dye and calcium mobilization measured in response to increasing concentrations of LTD₄. As shown in Figure 4A, buffer did not alter calcium levels as evidenced by the flat line. Unstimulated cells had low levels signaling when LTD₄ was added to the cultures. The level of signaling was further increased in the cells that had been treated with IL-4 for 24 h. To show that this increase was due to an increase in the number of leukotriene receptors, the CysLT1 antagonist MK571 was added to the cultures at 1×10^{-5} M. Addition of MK571 prevented any calcium mobilization (Figure 4B), as the dose-response curve for LTD₄ was similar to the buffer-only condition.

DISCUSSION

CysLTs function through their ability to interact with two homologous receptors. The CysLT type 1 receptor is prominently

expressed on airway smooth muscle, eosinophils, and other immune cells, and these receptors mediate CysLT-induced bronchospasm (3). CysLT2 receptors (4) are prominently expressed in the heart, prostate, brain, adrenal cells, endothelium, and lung but are expressed at lower levels on eosinophils, monocytes, T and B lymphocytes, and mast cells (7). The precise function of CysLT2 receptors in allergic disease and immunity is not known, although the CysLT2 receptor is thought to play a greater role in remodeling and fibrotic processes (14). Both papers describing the cloning of the CysLT1 and CysLT2 receptors (3, 4) suggest limited expression of these receptors on circulating immune cells. In contrast, the increased expression of these receptors in asthma has been demonstrated (5), and we have reported their increased expression in allergic rhinitis (6). CysLT1 receptors were expressed on the majority of eosinophils and in subsets of mast cells, monocytes, macrophages, and neutrophils. CysLT2 receptors were expressed on most eosinophils, mast cells, and monocytes/macrophages, but not on neutrophils. The increased expression was observed both at the mRNA and protein levels. Similarly, in aspirin-exacerbated respiratory

Figure 3. Flow cytometric analysis of CysLT receptor expression on IFN- γ -stimulated cells. Cells were separated from blood using magnetic bead affinity chromatography and stimulated with 20 ng/ml IFN- γ for 16 h before cells were collected for analysis. Cell surface expression of CysLTR2 receptors were evaluated using rabbit polyclonal anti-CysLTR2 followed by labeling with FITC-conjugated goat anti-rabbit IgG. Data are shown for monocytes (A) and B cells (B). *Open histogram*, isotype control; *solid histogram*, unstimulated; *shaded histogram*, IFN- γ stimulated. The data are representative of three independent experiments.

Figure 4. Functional analysis of increased cysteinyl leukotriene receptor expression. Calcium mobilization assays were performed on T cells by plating 150,000 cells/well in a 96-well plate and incubating the cells with 20 ng/ml IL-4 for 24 h. Cells were then incubated with reagent from the FLIPR calcium assay kit supplemented with 2.5 mM probenecid for 1 h. (A) Dose-response curves were generated by incubation of the cells with 1×10^{-13} to 1×10^{-6} M LTD₄ and measuring cytoplasmic calcium flux. Triangles, buffer; diamonds, LTD₄ unstimulated; squares, LTD₄ + IL-4. (B) Demonstration that the increased calcium mobilization was mediated by the leukotriene receptors was determined by blocking the LTD₄-mediated response with the CysLT1 receptor antagonist MK571 (1×10^{-5} M). Triangles, buffer; squares, LTD₄ + IL-4; diamonds, LTD₄ + IL-4 + MK571.

disease (AERD), 31% of the T cells in the nasal mucosa were positive for CysLT1 receptors (11). Together, these studies suggest that during an allergic inflammatory insult, T cells can up-regulate their CysLT receptor expression. Alternatively, it is plausible that CysLT receptor-expressing T cells may either be selectively recruited into the nasal and sinus tissue or may selectively expand or survive under the influence of locally produced CysLTs (15).

Previous studies have indicated that CysLT receptor expression can be altered depending on cell type and cytokine stimulus. IL-4 up-regulates cell surface expression of both CysLT1 and CysLT2 receptors on mast cells reportedly without altering mRNA or protein levels in the cells (16, 17). In contrast, IL-4 stimulated CysLT2 receptor mRNA production in endothelial cells, and both IL-4 and IL-13 stimulated mRNA and cell surface expression of the CysLT1 receptor in monocytes (13, 18). In the latter study, IFN- γ decreased CysLT1 receptor mRNA expression in monocytes, while IFN- γ stimulated CysLT1 and CysLT2 receptor mRNA production and CysLT1 receptor surface expression on smooth muscle cells (13). IL-5 increased CysLT1

receptor mRNA and cell surface expression on a human eosinophil cell line (19), and recently it has been reported that IFN- γ increases CysLT2 receptor expression on eosinophils from patients with asthma (20). Together these studies broadly suggest the ability of cytokines associated with allergic inflammation to up-regulate expression of CysLT receptors. Our data extend these previous studies and examine a broader range of cytokines and expression on multiple immune cell types. The most profound results were observed for IL-4 stimulation of the CysLT2 receptor. Significant increases in expression of the CysLT2 receptor were seen on T and B lymphocytes and eosinophils (Table 1, Figures 2B and 2D). IL-4 also up-regulated CysLT1 receptor mRNA expression on T and B cells with trends toward increased expression on monocytes and eosinophils (Table 1, Figures 2A and 2C). This increase in CysLT1 receptor expression is consistent with the recent identification of a STAT6 response element in the CysLT1 receptor promoter that mediates responses to IL-4 stimulation (21). The increase in receptor number after IL-4 stimulation resulted in increased responsiveness of T lymphocytes to LTD₄ as measured by higher levels of calcium mobilization (Figure 4).

Surprisingly, in addition to IL-4, IFN- γ significantly increased expression of the CysLT2 receptor on monocytes and T and B lymphocytes (Table 1, Figures 3A and 3B). Asthma has traditionally been defined as a Th2 cytokine-mediated disease; however, recent evidence indicates that severe asthma is associated with increased expression of IFN- γ (22, 23). Our data suggest that under these conditions, the expression of CysLT2 receptors will preferentially be increased. This may have important implications for remodeling. CysLT1 receptors on smooth muscle mediate the bronchospastic effects of CysLT, whereas CysLT2 receptors may have a complementary or even a predominant role in remodeling and fibrosis pathways (14). Thus, the proliferative effects mediated by CysLT on myofibroblasts and smooth muscle are blocked by less specific CysLT receptor antagonists but not by an antagonist specific for CysLT1 receptors (24). CysLT2 receptors are prominently expressed in cardiac myocytes and, as such, support for a role for CysLT2 receptors in fibrosis is derived from observations that conditions associated with intravascular activation of eosinophils, such as hypereosinophil and Churg-Strauss syndromes, can lead to the development of endomyocardial fibrosis. In a bleomycin-induced model of pulmonary fibrosis, fibrosis does not develop in either 5-LO or LTC₄S knockout mice (25, 26). Surprisingly, when these studies were performed in the CysLT1 receptor knockout mouse, enhanced fibrosis was observed (27). In contrast, the CysLT2 receptor knockout mouse had markedly reduced inflammation and fibrosis (28). Conflicting results observed in a CysLT1 receptor antagonist study (29) may reflect the different models, activity of montelukast on CysLT secretion and, at higher concentrations, directly upon CysLT2 receptors, or synergism between the two receptors. These data support the concept that severe persistent asthma with remodeling, which is characterized by high levels of IFN- γ , may be driven in part by increased expression of the CysLT2 receptor and CysLT2 receptor-mediated pro-fibrotic pathways.

Although beneficial effects of CysLT1 receptor antagonists have been anecdotally reported in chronic hyperplastic eosinophilic sinusitis/nasal polyposis (30), the only successful controlled trial of a leukotriene modifier in this condition was reported with the 5-LO inhibitor zileuton (31). Inhibition of 5-LO has broader implications, insofar as in addition to blockade of LTC₄ and LTD₄, zileuton will block production of other proinflammatory lipids including LTB₄ and 5(S)HETE (hydroxyecosateraenoic acid). However, it is plausible that it is the ability of this agent to block CysLT production and therefore to inhibit activation

mediated through both CysLT1 and CysLT2 receptors that is responsible for its clinical efficacy. Although combined CysLT1 and CysLT2 antagonists provide no superior benefit to selective CysLT1 antagonists in short-term studies evaluating lung function and rescue albuterol use, no studies have addressed inhibition of remodeling or fibrotic pathways.

In conclusion, we have demonstrated the ability of both Th1- and Th2-type cytokines to increase expression of both the CysLT1 and -2 receptors at the mRNA and protein levels in many cell types. Combined with previous studies showing increased numbers of both CysLT1 and -2 receptor-expressing cells in inflamed tissue, our results are consistent with the concept that increased cytokine production increases expression of both receptors on mononuclear cells and granulocytes, allowing these cells to be more responsive to secreted leukotrienes.

Conflict of Interest Statement: S.B.E. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. E.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. L.B. currently has a \$67,000 research grant from GlaxoSmithKline not related to this study. He is also on an Advisory Board for Critical Therapeutics for \$2,500/yr to discuss marketing of ziluton for AERD, and also received lecture fees from Merck for \$15,000/yr for lectures on leukotriene modifiers. J.W.S. had a research grant for \$32,000 from Merck to study modulation of leukotriene receptors on immune cells. The grant supported the research in this paper.

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