

Corticosteroids as inhibitors of cysteinyl leukotriene metabolic and signaling pathways

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Background: Corticosteroids (CCSs) do not influence secretion of cysteinyl leukotrienes (CysLTs) that occurs on cellular activation during allergic reactions nor do they modulate bronchospastic responses to inhalation challenges with leukotrienes (LTs).

Objectives: We speculated that CCSs might modulate pathways responsible for CysLT production and diminish the ability of cellular activation to cause their release. Similarly, CCSs could reduce expression of CysLT receptor 1 (CysLTR1) and CysLT receptor 2 (CysLT2R) and modulate their responsiveness.

Methods: We investigated influences of fluticasone on expression of mRNA for LTC₄ synthase (LTC₄S), CysLT1R, and CysLT2R within T lymphocytes, monocytes, and eosinophils by means of quantitative PCR. Effects on receptor protein expression were evaluated by means of flow cytometry.

Results: Circulating immune cells (T cells, monocytes, and eosinophils) express low levels of LTC₄S mRNA, and this was not influenced by CCSs. However, IL-4 induced transcripts in T lymphocytes, and this was prevented by fluticasone.

Paradoxically, CCSs synergized with IL-4 to increase LTC₄S expression in monocytes. Although not influencing basal or IL-4-stimulated CysLT1R expression, fluticasone inhibited basal CysLT2R transcript expression on monocytes and IL-4-induced expression in all 3 cell types.

Conclusions: In addition to not blocking the acute release of CysLTs on cellular activation, CCSs do not diminish the capacity of cells to synthesize these compounds. CCSs do not diminish CysLT1R expression, consistent with their lack of influence on bronchospasm, which is mediated through this receptor. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Cysteinyl leukotrienes, corticosteroids, IL-4

Cysteinyl leukotrienes (CysLTs) are important mediators of allergic inflammation and contribute to the presence and severity of asthma. In addition to mediating robust and sustained bronchospasm, they also contribute to inflammation and remodeling

Abbreviations used

CCS: Corticosteroid
CysLT: Cysteinyl leukotriene
CysLT1R: Cysteinyl leukotriene receptor 1
CysLT2R: Cysteinyl leukotriene receptor 2
5-LO: 5-Lipoxygenase
LT: Leukotriene
LTC₄S: Leukotriene C₄ synthase

through their influences on eosinophil production, recruitment, and activation; mucus secretion; vascular permeability; smooth muscle proliferation; and lymphocyte activation.¹ The contribution of these activities is highlighted by the efficacy of leukotriene (LT) modifiers as therapeutic agents for asthma.^{1,2} Although considered the most effective class of drugs used to treat asthma,^{3,4} corticosteroids (CCSs) are generally thought to have little or no influence either on the production or activity of CysLTs. This is based on observations that neither systemic nor topical CCSs influence the production of CysLTs⁵⁻¹¹ and, similarly, the failure of CCSs to influence the bronchospastic response to challenge with CysLTs.^{12,13} Thus LT receptor antagonists are often used in combination with CCSs through their capacities to provide additive or even synergistic therapeutic benefits in asthma.^{10,14-17}

However, these studies showing little or no influence of CCSs on CysLT production or responsiveness might have incompletely addressed the full spectrum of pathways responsible for these processes. CysLTs are synthesized by mast cells, basophils, eosinophils, and mononuclear phagocytic cells and, to a lesser extent, T lymphocytes.^{18,19} Secretion of CysLTs by these cell types after cell activation is not modulated by CCSs.^{5-11,13} However, the capacity for CysLT synthesis is tightly regulated, particularly through the expression of the enzymes 5-lipoxygenase (5-LO) and LTC₄ synthase (LTC₄S).^{20,21} A previous study demonstrated that CCSs might paradoxically enhance the LT pathway through their ability to induce 5-LO expression.⁹ LTC₄S expression is modulated by cytokines; for example, IL-4 was shown to be uniquely responsible for its expression by mast cells.²⁰ Therefore it is plausible that although unable to modulate CysLT secretion by acutely activated cells, CCSs could indirectly modulate CysLT secretion by influencing their expression of LTC₄S.

The biologic responses to CysLTs are also tightly regulated through modulation of the expression of their receptors. At least 2 classes of CysLT receptors have been defined: CysLT receptor 1 (CysLT1R) and CysLT receptor 2 (CysLT2R). CysLT1Rs are widely expressed on smooth muscle and inflammatory cells and are largely responsible for bronchospasm. The function of CysLT2Rs has not been fully elucidated.¹⁹ Several observations

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suggest that CysLT2Rs might be more important than CysLT1Rs in mediating airway remodeling and fibrosis, including observations showing enhanced fibrosis in CysLT1R knockout mice.^{19,22} Similar to LTC₄S, these receptors are upregulated in allergic conditions, such as asthma, and especially in aspirin-exacerbated respiratory disease.²³ This again seems to primarily reflect the biologic activities of T_H2-derived cytokines, including IL-5, IL-9, and IL-13, but IL-4 is singularly effective.²⁴⁻²⁶ Given the anti-inflammatory influences of CCSs on cytokine production and actions, these observations suggest a mechanism by which CCSs could indirectly modulate the biologic activity of CysLTs by influencing expression of their receptors. We therefore investigated the hypothesis that these 2 complementary pathways necessary for the synthesis and biologic effects of CysLTs, respectively, could be regulated by CCSs and that CCSs might thereby influence the role of CysLTs in allergic inflammation. Fluticasone is currently the most widely used topical CCS for the treatment of asthma. These studies therefore investigated the influence of fluticasone on basal and IL-4-stimulated expression of LTC₄S and the CysLT1Rs and CysLT2Rs within T cells, monocytes, and eosinophils.

METHODS

Subjects

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

Cell isolation and culture

T_H lymphocytes (CD4⁺) and monocytes (CD14⁺) were enriched from Ficoll-Hypaque (Sigma, St Louis, Mo)-derived PBMCs by using magnetic affinity purification (Miltenyi Biotec, Auburn, Calif). Granulocytes were isolated through sequential application of Ficoll-Hypaque centrifugation, followed by dextran sedimentation (Amersham Biosciences, Piscataway, NJ) and hypotonic lysis. Eosinophils were enriched from granulocytes by using negative magnetic affinity column purification (CD16⁻; Miltenyi Biotec). Enriched populations were screened for purity by means of flow cytometry with CD4 (T lymphocytes; Sigma-Aldrich), CD14 (monocytes; BD Biosciences, San Diego, Calif), and CCR3 (eosinophils; BD Biosciences). Reflecting their distribution among leukocytes and vagaries of the enrichment procedure, the relative purity of each of these populations was as follows: T lymphocytes, greater than 99% pure; monocytes, greater than 90% pure; and eosinophils, greater than 95% pure (data not shown). Cells were cultured in complete RPMI medium supplemented with 10% autologous serum with or without IL-4 (10 ng/mL; BD Biosciences). IL-4 was used at this concentration, as previously shown to be optimal for induction of LTC₄S and CysLT receptor expression.^{20,25,26} Similarly, as shown to be optimal in these previous studies,²⁶ mRNA expression was investigated after 16 hours of culture, and surface expression of receptors was investigated at 48 hours. Cultures were performed with or without the additional presence of fluticasone (10⁻⁹ and 10⁻⁷ mol/L; GlaxoSmithKline, Research Triangle Park, NC).

CysLT production

In additional studies, monocytes prepared under conditions as described were cultured for 48 hours and then stimulated with LPS (1 μg/mL; Sigma) for 1 hour. Supernatants were collected for assay of CysLTs by using a commercial ELISA kit that quantifies all 3 CysLTs (LTC₄, LTD₄, and LTE₄; Cayman Chemical, Ann Arbor, Mich), according to the manufacturer's instructions. The sensitivity of this assay was approximately 7.8 pg/mL. Concentrations were calculated on the basis of standard curves with the KC4 Kineticalc for Windows software (Bio-Tek, Winooski, Vt).

Real-time quantitative RT-PCR

Initially, we investigated the ability of CCSs to modulate LTC₄S, CysLT1R, and CysLT2R mRNA transcript expression by resting and IL-4-treated cells. Total RNA was extracted from cells with an SV Total RNA Isolation kit (Promega, Madison, Wis). Conversion of the mRNA to cDNA was performed by using a Taqman Reverse Transcription kit (Roche, Branchburg, NJ), as previously described.²⁷ Briefly, 200 ng of RNA was added to each reaction, along with oligo dT primers, 5.5 mmol/L MgCl₂, 2 mmol/L deoxyribonucleoside triphosphates, RNasin, and reverse transcriptase. Reactions went through 1 cycle of 10 minutes at 25°C, 30 minutes at 48°C, and 5 minutes at 95°C in a Bio-Rad iCycler thermocycler (Bio-Rad, Hercules, Calif). The cDNA was amplified by means of PCR by using appropriate primer pairs, and the resulting products were analyzed by means of quantitative PCR for LTC₄S and the CysLT1R and CysLT2R. Primers for β-actin have been previously described.²⁷ Probes used for detection of CysLT1R and CysLT2R were as follows: CysLTR1 probe 5'-FAM-CACTGCCTCTCCGTGTGGTC-BHQ1-3' and CysLTR2 probe 5'-FAM-CTTGGTGGTGGGCTGCCTGCT-BHQ1-3'. Primers for LTC₄S were as follows: LTC₄S forward primer 5'-GCTGTTCTCGCCAC-3' and LTC₄S reverse primer 5'-TAGCCCTGGAAGTAGCG-3'. For detection of LTC₄S, Syber Green Supermix (Bio-Rad) was used. Quantification of changes in receptor expression induced by cytokines was performed by using the comparative threshold cycle (C_T) method. Briefly, the amount of target normalized to an endogenous reference and relative to a calibrator was calculated by 2^{-ΔΔCT}, with ΔΔCT defined as follows: (Threshold cycle unstimulated gene of interest - Threshold cycle unstimulated housekeeping gene) - (Threshold cycle stimulated gene of interest - Threshold cycle stimulated housekeeping gene). The comparative C_T method was validated by showing that the efficiencies of target and reference amplification were equal. Primer pairs and probes for each reaction were synthesized by Integrated DNA Technologies (Coralville, Iowa).

Flow cytometry

Cell-surface expression of CysLT1R and CysLT2R was evaluated by using rabbit polyclonal anti-CysLT1R (Novus Biologics, Littleton, Colo) and anti-CysLT2R (Cayman Chemical Co, Ann Arbor, Mich), respectively, followed by labeling with phycoerythrin-conjugated goat anti-rabbit IgG (Caltag Laboratories, Burlingame, Calif). Rabbit IgG was used as an isotype control. Data were analyzed as the percentage of positive cells and mean fluorescence intensity (Flowjo, San Carlos, Calif).

Statistical analysis

Quantitative RT-PCR data were contrasted between resting and IL-4-stimulated cells with and without fluticasone by means of the Wilcoxon rank sum test, and CysLT synthesis data were analyzed by using the *t* test (JMP 6.0 software; SAS Institute, Inc, Cary, NC). Data regarding expression of CysLT receptors on immune cells were expressed as both percentage of cells expressing each receptor and as mean fluorescence intensity.

RESULTS

Regulation of CysLT synthesis pathways

Previous studies have compellingly demonstrated that CCSs do not influence CysLT release either when administered *in vivo* or when applied to immune cells *ex vivo*. We were interested, however, in discerning whether CCSs might still indirectly modulate CysLT production through an influence on the inherent capacity of immune cells to synthesize CysLTs. However, consistent with these previous studies, no influence on basal LTC₄S expression by T cells, monocytes, or eosinophils was observed (Table I). Because it has previously been shown to induce expression of LTC₄S in mast cells,²⁰ we also investigated the influence of fluticasone on IL-4-modulated expression of this enzyme in these cell types. Our studies extend to T cells an influence of

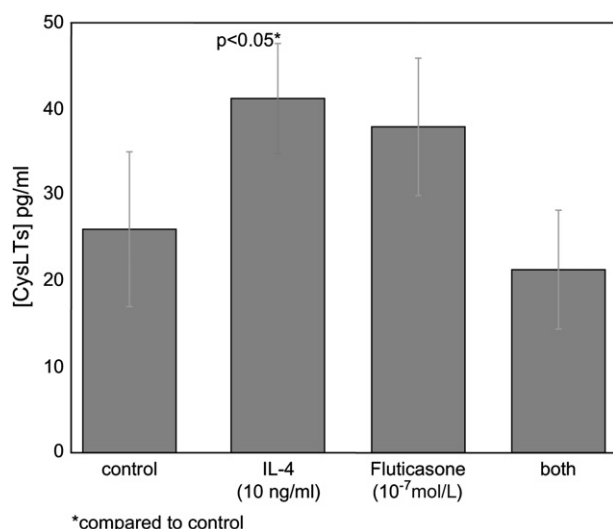
TABLE I. CysLT1R, CysLT2R, and LTC₄S mRNA expression in T lymphocytes, monocytes, and eosinophils by means of quantitative RT-PCR

Fluticasone:	Control			IL-4 (10 ng/mL)		
	None	10 ⁻⁹	10 ⁻⁷	None	10 ⁻⁹	10 ⁻⁷
T lymphocytes						
CysLT1R	1	1.05 ± 0.70*	0.85 ± 0.17*	2.37 ± 0.38‡	2.67 ± 0.64†	1.80 ± 0.58*
CysLT2R	1	1.02 ± 0.38*	0.71 ± 0.17*	7.62 ± 1.09§	5.46 ± 1.20†	3.74 ± 1.54*
LTC ₄ S	1	2.87 ± 2.11*	1.73 ± 0.26*	6.56 ± 3.79†	7.02 ± 3.44**	2.25 ± 2.26**
Monocytes						
CysLT1R	1	1.24 ± 0.55*	1.06 ± 0.40*	4.44 ± 1.45†	1.26 ± 0.96**	1.65 ± 0.91*
CysLT2R	1	0.74 ± 0.23*	0.27 ± 0.20‡	5.45 ± 1.32‡	2.34 ± 1.22**	4.83 ± 2.47*
LTC ₄ S	1	1.83 ± 0.64*	1.61 ± 0.83*	1.96 ± 0.72*	10.73 ± 7.92**	11.62 ± 4.09†
Eosinophils						
CysLT1R	1	1.72 ± 0.99*	0.75 ± 0.28*	2.81 ± 0.83†	5.41 ± 3.14**	1.45 ± 0.37*
CysLT2R	1	2.50 ± 0.89*	1.54 ± 0.66*	4.90 ± 1.26‡	3.25 ± 1.34**	1.37 ± 0.68*
LTC ₄ S	1	2.84 ± 1.95*	1.03 ± 0.37*	4.53 ± 2.68*	2.59 ± 2.10**	8.63 ± 4.82*

Data are generated by means of quantitative real-time PCR and normalized to expression of the gene for the housekeeping protein β -actin. Data represent means $\pm$ SEMs of 10 replicate experiments. Data are expressed as the fold increase of expression of receptors over that of resting cells.

*Not significant, † $P < .05$, ‡ $P < .01$, and § $P < .001$ compared with untreated cells (without fluticasone or IL-4).

||Not significant compared with cells without IL-4 (with fluticasone).

**FIG 1.** Modulation of monocyte CysLT secretion by IL-4 and fluticasone. Enriched monocytes were cultured with or without IL-4 (10 ng/mL) and with or without fluticasone (10⁻⁷ mol/L) for 48 hours and stimulated with LPS for 1 hour. Supernatants were collected and assayed for CysLT secretion by means of ELISA.

IL-4 on LTC₄S expression (Table I). This increased expression was abrogated by the additional presence of the CCS. Although trends toward increased expression were observed with monocytes and eosinophils, because of large intraindividual variability, these differences did not achieve statistical significance (Table I). Paradoxically, a further increase in LTC₄S mRNA transcript expression was observed for monocytes after the addition of CCSs, which did achieve statistical significance.

Influence on CysLT synthesis

To discern whether the influence of IL-4 and CCSs on LTC₄S expression correlated with changes in the ability to secrete CysLTs, enriched monocytes were cultured for 48 hours with IL-4 and fluticasone and stimulated for 1 hour with LPS, and

secreted CysLTs were quantified by means of ELISA (Fig 1). IL-4 treatment was associated with a significant increase in the ability of these cells to secrete CysLTs. However, the combination of fluticasone with IL-4 abrogated increased CysLT secretion.

Regulation of CysLT receptors

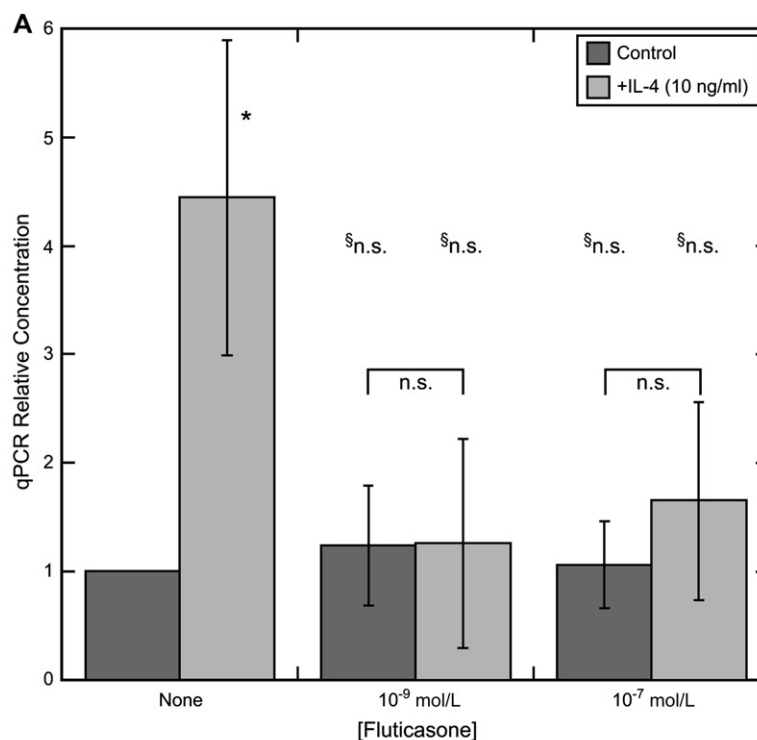
Although not having an influence on basal expression of CysLT1R, fluticasone significantly inhibited basal CysLT2R expression on monocytes (Fig 2, B, and Table I). This was not observed on the other cell types. These studies confirm our published observations²⁶ regarding the ability of IL-4 to induce CysLT1R and CysLT2R mRNA transcripts on monocytes (Fig 2), T lymphocytes, and eosinophils (Table I). The combination of a CCS and IL-4 precluded significant induction of CysLT1R and CysLT2R on all 3 cell types, such that in the additional presence of a CCS, IL-4 no longer increased receptor mRNA expression (Table I and Fig 2).

Flow cytometry

To confirm that our observations regarding mRNA transcripts extended to surface expression of protein, we performed flow cytometry to quantify the expression of CysLT1R and CysLT2R. These studies confirmed a lack of influence of fluticasone on CysLT1R on CD4⁺ T cells (Fig 3, A), monocytes, and eosinophils (data not shown). In contrast, CCSs diminished constitutive CysLT2R expression. One representative experiment for T lymphocytes derived from 6 separate experiments is displayed (Fig 3).

DISCUSSION

The ability of a cell to secrete CysLTs is regulated through its capacity to respond to the activating signal and whether the cell expresses the requisite metabolic enzymes. Previous studies^{5-11,13} have compellingly demonstrated the lack of influence of CCSs on activation of the arachidonate-CysLT cascade and on subsequent CysLT secretion. It is plausible that CCSs could still influence constitutive or inducible LTC₄S expression. Our studies demonstrate contrasting results on different cell types, with CCSs



* $p < .05$, ** $p < .01$ compared to without fluticasone;
 §N.S. = not significant compared to without fluticasone

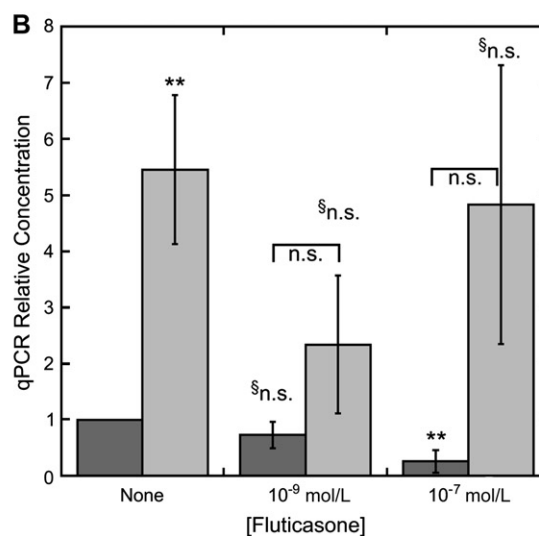


FIG 2. Modulation of CysLT receptor mRNA expression in monocytes by IL-4 and fluticasone. Monocytes were cultured with or without IL-4 (10 ng/mL) and with or without the additional presence of fluticasone (10^{-9} and 10^{-7} mol/L). CysLT1R (**A**) and CysLT2R (**B**) mRNA expression was determined by means of quantitative PCR (qPCR), and data are expressed in comparison with that for monocytes not exposed to IL-4 or fluticasone. Data represent means $\pm$ SEMs of 10 replicate experiments.

inhibiting IL-4-induced LTC₄S expression in T cells but synergizing with IL-4 to enhance its expression within monocytes. These latter results are consistent with previous studies demonstrating enhanced expression of 5-LO in monocytes exposed to dexamethasone,⁹ as well as studies reporting a paradoxical increase in CysLT secretion by CCS-treated bronchoalveolar lavage cells *ex vivo*.⁸ Although increasing LTC₄S expression, the combination of the CCSs with IL-4 did not enhance CysLT secretion, suggesting that the anti-inflammatory influences of CCSs superseded this

modulation of LTC₄S. The inhibition of IL-4-induced LTC₄S in T cells might reflect the unique susceptibility of these cells to CCSs, but in general, T lymphocytes are not an important source for CysLTs. As such, our results largely agree with previous conclusions that even prolonged treatment with CCSs is not likely to modulate CysLT secretion.

IL-4 increased the secretion of CysLTs by monocytes, possibly reflecting their increased LTC₄S expression, although we cannot rule out influences of IL-4 on other facets of the pathway linking

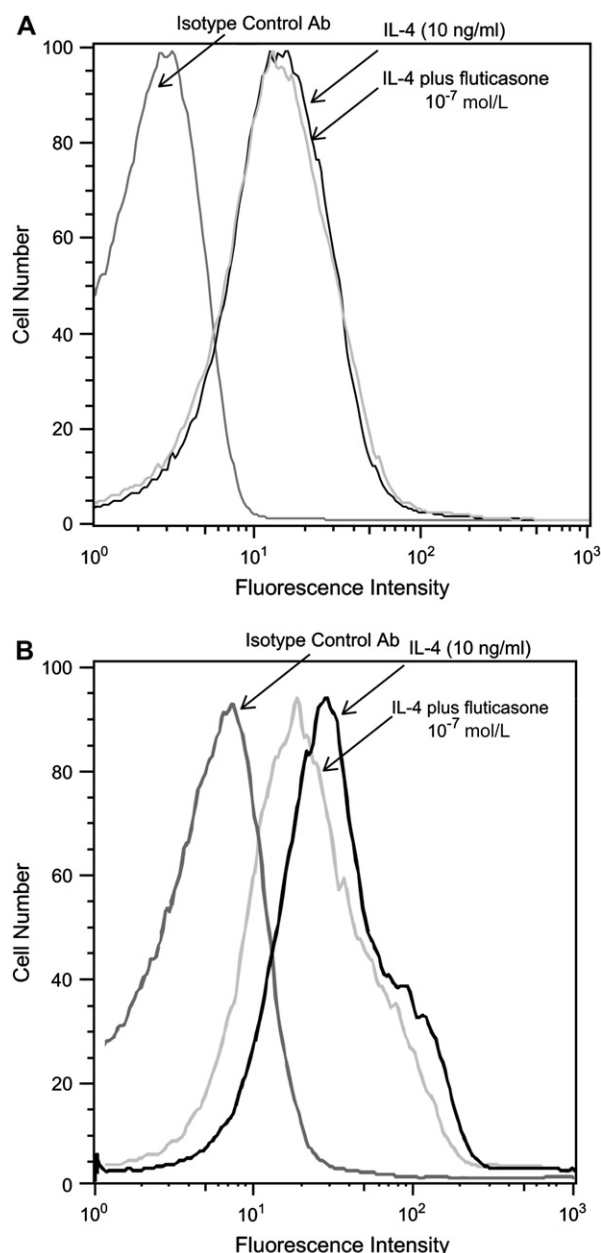


FIG 3. Modulation of CysLT1R (A) and CysLT2R (B) expression on T lymphocytes by IL-4 and fluticasone. T_H lymphocytes were cultured with IL-4 (10 ng/mL) with or without fluticasone (10^{-7} mol/L). Cells were labeled with rabbit polyclonal anti-CysLT receptor antibodies followed by phycoerythrin-conjugated goat anti-rabbit IgG and evaluated by means of flow cytometry. No inhibition of IL-4-induced CysLT1R expression was observed. Fluticasone decreased mean fluorescence of CysLT2R from 40.5 to 30.7. Ab, Antibody.

LPS activation to CysLT synthesis. However, despite the dramatically increased expression of LTC₄S observed with the concomitant presence of IL-4 and CCSs, the additional presence of the CCSs paradoxically inhibited this enhanced secretion of CysLTs. LPS-induced CysLT secretion presumably reflects activation of the Toll-like receptor pathway, and inhibition of components of this pathway, in particular nuclear factor κ B, is known to be an anti-inflammatory target of CCSs, possibly explaining this phenomenon. Although not influencing allergen-induced CysLT

secretion,^{5-11,13} inhibition by CCSs of innate immune signal-induced CysLT secretion could have clinical significance in allergic disorders.

Previous studies demonstrated the importance of IL-4 to LTC₄S expression by mast cells,²⁰ and the current studies extend that observation to T cells. However, our studies failed to demonstrate a conclusive influence of IL-4 on either monocyte or eosinophil expression of LTC₄S. Asthma and, in particular, aspirin-exacerbated respiratory disease is associated with robust upregulation of LTC₄S expression, especially within eosinophils, and the immune mechanism underlying this dysregulation remains unclear, although this could occur through an action of IL-4 (or other cytokines) on hematopoietic precursors.

Bronchoconstriction mediated by CysLTs is mediated primarily through the CysLT1R and not the CysLT2R.¹⁹ Our inability to demonstrate an influence of CCSs on CysLT1R is therefore consistent with findings that neither treatment with 800 μ g of inhaled budesonide daily for 6 days¹² or 500 μ g of fluticasone twice daily for 2 weeks¹³ influenced the dose response to subsequent inhalation challenge with LTD₄. In contrast, we did observe a decrease in CysLT2R expression, both as mRNA transcripts and surface-expressed protein. This was only observed on monocytes, possibly reflecting the low level of expression of CysLT2R on other circulating immune cells.^{26,28} As with LTC₄S, CysLT receptor expression is upregulated in allergic rhinitis, asthma, and especially in aspirin-exacerbated respiratory disease.^{23,28-31} This upregulation is controlled, at least in part, by cytokines,²⁴⁻²⁶ and this cytokine-induced effect was significantly blocked by CCSs on all 3 immune cell types. Given the proposed importance of CysLT2R to inflammation and remodeling, this suggests an important influence of CCSs on these facets of CysLT function in asthma and allergies.^{1,19,22} These published studies do not suggest which cell type is responsible for CysLT2R-mediated remodeling; however, it seems likely that this would largely involve nonimmune cells, such as airway smooth muscle cells, myofibroblasts, fibrocytes, and fibroblasts. *Ex vivo* studies, including lung biopsy, would be required to prove whether CCSs modulate CysLT2R on these cell types within the asthmatic lung.

In summary, our studies confirm the inability of CCSs to influence the capacity of immune cells to synthesize and secrete CysLTs. Similarly, CCSs had no influence on CysLT1R expression, which is consistent with the inability of CCSs to modulate bronchospasm in response to an inhalation challenge with CysLTs. However, CCSs did downregulate constitutive CysLT2R expression on monocytes and their IL-4-induced expression on monocytes, T cells, and eosinophils. Thus in contrast to the currently available LT receptor antagonists, which selectively block only the CysLT1R, CCSs could attenuate inflammatory and remodeling pathways mediated through the CysLT2R.

Clinical implications: By reducing CysLT2 receptors, CCSs could modulate the profibrotic and remodeling influences mediated through these receptors.

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