Human T_H2 cells respond to cysteinyl leukotrienes through selective expression of cysteinyl leukotriene receptor 1

Celine N. Parmentier, MSc,[a,b] Elisabeth Fuerst, PhD,[a,b] Joanne McDonald, PhD,[a,b] Holly Bowen, PhD,[a,b] Tak H. Lee, MD, ScD,[a,b] James E. Pease, PhD,[a,c] Grzegorz Woszczek, MD, PhD,[a,b] and David J. Cousins, PhD[a,b] *London, United Kingdom*

Background: Allergic asthma is characterized by reversible airway obstruction and bronchial hyperresponsiveness associated with T_H2 cell–mediated inflammation. Cysteinyl leukotrienes (CysLTs) are potent lipid mediators involved in bronchoconstriction, mucus secretion, and cell trafficking in asthmatic patients. Recent data have implicated CysLTs in the establishment and amplification of T_H2 responses in murine models, although the precise mechanisms are unresolved.
Objectives: Preliminary microarray studies suggested that human T_H2 cells might selectively express cysteinyl leukotriene receptor 1 *(CYSLTR1)* mRNA. We sought to establish whether human T_H2 cells are indeed a CysLT target cell type.
Methods: We examined the expression of *CYSLTR1* using real-time PCR in human T_H1 and T_H2 cells. We functionally assessed cysteinyl leukotriene receptor 1 protein ($CysLT_1$) expression using calcium flux, cyclic AMP, and chemotaxis assays.
Results: We show that human T_H2 cells selectively express *CYSLTR1* mRNA at high levels compared with T_H1 cells after *in vitro* differentiation from naive precursors. Human T_H2 cells are selectively responsive to CysLTs in a calcium flux assay when compared with T_H1 cells with a rank order of potency similar to that described for $CysLT_1$ (leukotriene [LT] $D_4 > LTC_4 > LTE_4$). We also show that LTD_4-induced signaling in T_H2 cells is mediated through $CysLT_1$ coupled to $G_\alpha q$ and $G_\alpha i$ proteins, and both pathways can be completely inhibited by selective $CysLT_1$ antagonists. LTD_4 is also found to possess potent chemotactic activity for T_H2 cells at low nanomolar concentrations.
Conclusions: These findings suggest a novel mechanism of action for CysLTs in the pathogenesis of asthma and provide a potential explanation for the anti-inflammatory effects of $CysLT_1$ antagonists. (J Allergy Clin Immunol 2012;129:1136-42.)

Key words: *Human, T_H1, T_H2, cysteinyl leukotrienes, chemotaxis,* CYSLTR1*, cysteinyl leukotriene receptor 1 protein, LTD_4*

Allergic asthma is characterized by reversible airway obstruction and bronchial hyperresponsiveness to otherwise innocuous environmental antigens and is associated with inflammation of the airways involving numerous immune cell types and inflammatory mediators.[1,2] In particular, the immune pathology observed is dominated by a T_H2-type response with increased expression of the classical T_H2 cytokines IL-4, IL-5, and IL-13, which might cause many of the hallmark features of allergic airways disease.[3] Central to this T_H2 immune pathology is the allergen-specific T_H2 cell, which is the predominant source of T_H2 cytokines in asthmatic subjects, although the precise mechanisms by which the T_H2 phenotype develops in asthmatic subjects remain to be determined.[4]

Cysteinyl leukotrienes (CysLTs) are potent lipid mediators involved in the pathogenesis of a wide range of chronic inflammatory and immune disorders, including asthma, allergic rhinitis, atherosclerosis, and inflammatory bowel disease.[5,6] The CysLT leukotriene (LT) C_4 is derived from arachidonic acid through cytosolic phospholipase A_2, 5-lipoxygenase, and LTC_4 synthase. LTC_4 is transported out of the cell and converted sequentially to LTD_4 and LTE_4 by the ubiquitous enzymes γ-glutamyl transpeptidase and aminopeptidase, respectively. The biological activities of the CysLTs are mediated through at least 4 G protein–coupled receptors, cysteinyl leukotriene receptor 1 protein ($CysLT_1$), CysLT receptor 2 protein, G protein-coupled receptor 17 (GPR17), and purinergic receptor $P2Y_{12}$ protein ($P2Y_{12}$), although the role of each of these receptors in CysLT action is incompletely understood.[6-8] In asthmatic subjects the CysLTs are believed to be involved in bronchoconstriction, mucus secretion, and cell trafficking and have been shown to be chemotactic agents for eosinophils, hematopoietic progenitor cells, and monocytes.[9-11] In addition to their role in bronchoconstriction and innate cell recruitment to the lungs of asthmatic subjects, CysLTs play a role in the establishment and amplification of antigen-specific T_H2 cell–dependent immune responses in murine models of allergic airways disease.[12] Although the precise mechanistic role of CysLTs in the development of the adaptive T_H2 response is unclear, prior studies have focused on the role of CysLTs in pulmonary dendritic cell migration and maturation.[13-15] Here we report that human T_H2 cells selectively express functional $CysLT_1$ and that LTD_4 is a potent chemotactic agonist for T_H2 cells. T_H2 cells exhibit robust calcium signaling in response to all 3 CysLTs,

From [a]the MRC & Asthma UK Centre in Allergic Mechanisms of Asthma and [b]the Division of Asthma, Allergy & Lung Biology, King's College London, and [c]the Leukocyte Biology Section, National Heart and Lung Institute, Imperial College London.

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Corresponding author: David J. Cousins, PhD, MRC & Asthma UK Centre in Allergic Mechanisms of Asthma, King's College London, Division of Asthma, Allergy & Lung Biology, 5th Floor Tower Wing, Guy's Hospital, London SE1 9RT, United Kingdom. E-mail: david.cousins@kcl.ac.uk.

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Abbreviations used
cAMP: Cyclic AMP
CysLT: Cysteinyl leukotriene
$CysLT_1$: Cysteinyl leukotriene receptor 1 protein
CYSLTR1: Cysteinyl leukotriene receptor 1 gene/mRNA
GPR17: G protein-coupled receptor 17
LT: Leukotriene
P2RY12: Purinergic receptor $P2Y_{12}$ gene/mRNA
$P2Y_{12}$: Purinergic receptor $P2Y_{12}$

with the most potent response observed with LTD_4 followed by LTC_4 and LTE_4, which is consistent with previous studies on $CysLT_1$.[16,17] The calcium signaling in response to LTD_4 was completely blocked with the selective $CysLT_1$ antagonists MK571, montelukast, and zafirlukast. These findings suggest a novel mechanism of action for CysLTs in the pathogenesis of asthma and provide a potential explanation for the anti-inflammatory effects of $CysLT_1$ antagonists.

METHODS

Reagents

Leukotrienes (LTC_4, LTD_4, and LTE_4) and $CysLT_1$ antagonists (MK571, montelukast, and zafirlukast) were purchased from Cayman Chemical (Ann Arbor, Mich). Recombinant chemokines (CXCL12 and CCL18) were purchased from R&D Systems (Abingdon, United Kingdom). Pertussis toxin, thapsigargin, and forskolin were purchased from Sigma-Aldrich (Dorset, United Kingdom). EDTA was purchased from Life Technologies (Paisley, United Kingdom).

Human T-cell isolation and differentiation

These studies were approved by the Research Ethics Committee of Guy's Hospital with informed consent. The donors used in this study were nonatopic, nonasthmatic male subjects (25-40 years) with no other chronic or acute illnesses at the time of venipuncture. Naive $CD4^+$ T cells were isolated from peripheral blood by using magnetic positive selection of $CD4^+$ cells (Dynal CD4 Positive Isolation kit; Invitrogen, Paisley, United Kingdom) followed by depletion of $CD45RO^+$ memory cells, as described in detail previously.[18] Naive T cells were stimulated with anti-CD3 (clone OKT3, ECACC, prepared in house) and anti-CD28 (clone 15E8; Sanquin Reagents, Amsterdam, The Netherlands) in the presence of cocktails of cytokines and antibodies to generate T_H1 and T_H2 cells, as described in detail previously.[18] Every 7 days, T_H1/T_H2 differentiation was assessed by using intracellular cytokine staining, as described previously.[18]

RNA isolation, microarrays, and real-time reverse transcription PCR

Cells for RNA isolation were snap-frozen in liquid nitrogen and stored at −80°C. Total cellular RNA was isolated with the miRNeasy mini kit (Qiagen, Crawley, United Kingdom), according to the manufacturer's instructions. cRNA samples were prepared for microarray hybridization to GeneChip U133 plus 2 arrays, according to the manufacturer's instructions (Affymetrix, Santa Clara, Calif). Fragmented cRNA was hybridized to GeneChip arrays at 45°C for 18 hours. Arrays were washed and stained with streptavidin-phycoerythrin, according to the manufacturer's instructions, on the GeneChip fluidics station 450 (Affymetrix). Fluorescent signals were detected with the GeneChip scanner 3000. Images were analyzed with the GeneChip operating software (Affymetrix) to generate raw data as .cel files. Further analysis was performed with the Partek Genomics Suite (Partek, St Louis, Mo) by using the gene expression workflow to identify differentially expressed genes. Briefly, robust multichip average preprocessing was performed, and genes differentially expressed between T_H1 and T_H2 cells were identified by using the Partek ANOVA model. One thousand seven hundred ninety-four genes were identified as differentially expressed with a P value of less than .05, including Benjamini-Hochberg step-up for false discovery rate. Data shown in Table I are the mean probe-set signals from 3 independent biological replicates for each condition. cDNA synthesis and real-time PCR were performed with TaqMan MGB probes (Applied Biosystems, Invitrogen, Paisley, United Kingdom), as described in detail previously.[19] TaqMan probe sets used in this study were as follows: cysteinyl leukotriene receptor 1 *(CYSLTR1)*, Hs00272624_s1; cysteinyl leukotriene receptor 2 *(CYSLTR2)*, Hs00272624_s1; *IL5*, Hs00174200_m1; *IFNG*, Hs00174143_m1; and *18s* rRNA, 4319413E. Statistical analyses were performed by comparing gene expression levels relative to *18s* rRNA levels by using a 2-way repeated-measures ANOVA with Bonferroni post tests in GraphPad Prism version 5.0a software (GraphPad, Inc, La Jolla, Calif).

Calcium-signaling assay

Cultured human T_H1 and T_H2 cells were tested for calcium signaling when fully differentiated at days 21 and 28 in response to LTB_4, LTC_4, LTD_4, and LTE_4. Cells were resuspended at 200,000 cells/well in 100 μL of RPMI plus 20 mmol/L HEPES and plated onto a 96-well, black-wall, clear, flat-bottom assay plate (Costar), and an equal volume of loading buffer (Component A with 1X HBSS Buffer; FLIPR Calcium 4 Assay Kit; Molecular Devices, Eugene, Ore) was added. The plate was incubated for 45 minutes at 37°C and 5% CO_2. After incubation, the plate was centrifuged at 200 *g* for 5 minutes and transferred directly to a FlexStation 3 Microplate Reader (Molecular Devices) at 37°C. Varying concentrations of CysLTs were added as indicated. Where indicated, the selective $CysLT_1$ antagonist MK571 (100 nmol/L), montelukast (100 nmol/L), or zafirlukast (100 nmol/L) was added 5 minutes before adding ligand. In some experiments pertussis toxin (100 ng/mL) was added 16 hours before the assay was performed to inhibit $G_\alpha i$. Where indicated, thapsigargin (1 μmol/L) or EDTA (2.5 mmol/L) was added 5 minutes before adding ligand. Results were analyzed with SoftMax Pro Software (Molecular Devices), and data are shown as a percentage of maximal response. Controls included a negative medium control of RPMI plus 20 mmol/L HEPES and a positive control of stromal cell–derived factor 1/CXCL12, and all experiments were performed with triplicate wells.

Cyclic AMP assay

Intracellular cyclic AMP (cAMP) levels were measured by luminescence with the HitHunter cAMP XS+ assay (DiscoverRX, Birmingham, United Kingdom). The assay was carried out according to the manufacturer's protocol for a 3-reagent addition. T_H2 cells were suspended in PBS at a concentration of 10×10^6/mL and warmed to 37°C, and 1 nmol/L 3-isobutyl-1-methylxanthine (Sigma) was added to inhibit cAMP phosphodiesterases. The T_H2 cell suspension (2×10^5 cells/well) was loaded into a 96-well plate, and 20 μmol/L forskolin was added to stimulate cAMP induction along with varying concentrations of LTD_4, as indicated. Where indicated, 100 nmol/L MK571 was added 5 minutes before adding ligand. Plates were incubated at 37°C for 15 minutes before cells were processed according to the kit's instructions, and cAMP signal was detected by means of luminescence measured 4 hours after cell lysis. Luminescence was detected by using a Flexstation 3 (Molecular Devices), and analysis of cAMP concentrations was performed with SOFTMax Pro software (Molecular Devices). Data are shown as a percentage of maximal response.

Chemotaxis assay

Chemotaxis assays were carried out by using a modification of a protocol[20] obtained from Andrew Luster (Massachusetts General Hospital, Harvard, Boston, Mass) by using 24-well 6.5-mm Transwell plates with a polycarbonate membrane insert filter and a pore size of 5.0 μm (3421; Corning, Corning, NY). *In vitro* cultured human T_H2 cells were resuspended at 1×10^6/mL in RPMI buffer; rested for 4 hours in a low rIL-2 concentration (25 U/mL;