

**Title: Leukotriene E<sub>4</sub> is a full functional agonist for human cysteinyl leukotriene type 1 receptor-dependent gene expression**

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## Abstract

Leukotriene E<sub>4</sub> (LTE<sub>4</sub>) the most stable of the cysteinyl leukotrienes (cysLTs) binds poorly to classical type 1 (CysLT<sub>1</sub>) and 2 (CysLT<sub>2</sub>) receptors although it induces potent responses in human airways *in vivo*, such as bronchoconstriction, airway hyperresponsiveness and inflammatory cell influx suggesting the presence of a novel receptor that preferentially responds to LTE<sub>4</sub>. To identify such a receptor two human mast cell lines, LAD2 and LUVA, were selected that differentially responded to LTE<sub>4</sub> when analysed by intracellular signalling and gene expression. Comparative transcriptome analysis and recombinant gene overexpression experiments revealed CysLT<sub>1</sub> as a receptor responsible for potent LTE<sub>4</sub>-induced response in LAD2 but not in LUVA cells, an observation confirmed further by gene knockdown and selective inhibitors. Lentiviral overexpression of CysLT<sub>1</sub> in LUVA cells augmented intracellular calcium signalling induced by LTE<sub>4</sub> but did not restore full agonist responses at the gene expression level. Our data support a model where both an increased expression of G $\alpha$ q-coupled CysLT<sub>1</sub>, and sustained intracellular calcium mobilisation and extracellular signal-regulated kinase (Erk) activation, are required for LTE<sub>4</sub>-mediated regulation of gene expression in human cells. Our study shows for the first time that CysLT<sub>1</sub> expression is critically important for responsiveness to LTE<sub>4</sub> within a human cell system.

## Introduction

Cysteinyl leukotrienes (cysLTs) (LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub>) play pivotal roles in cell proliferation, differentiation, migration and regulation of immune responses implicated in a wide variety of disorders, including asthma, allergy, atherosclerosis and cancer<sup>1</sup>. CysLTs are products of the 5-lipoxygenase (5-LO) pathway. 5-LO converts arachidonic acid to an unstable intermediate LTA<sub>4</sub>, which is then conjugated to reduced glutathione by leukotriene C<sub>4</sub> synthase to form LTC<sub>4</sub>. After transport to the extracellular space LTC<sub>4</sub> is converted to LTD<sub>4</sub> and then to the terminal product LTE<sub>4</sub>, the most abundant cysLT in biological fluids. The biological actions of cysLTs are mediated by 2 currently identified G-protein coupled receptors (GPCR): cysLT type 1 (CysLT<sub>1</sub>) and 2 (CysLT<sub>2</sub>) receptors. They differ in binding affinities for different cysLTs. CysLT<sub>1</sub> is recognized as a high-affinity receptor for LTD<sub>4</sub>, whereas CysLT<sub>2</sub> binds LTC<sub>4</sub> and LTD<sub>4</sub> with similar affinity. LTE<sub>4</sub>, the most stable of the cysLTs, binds poorly to the classical CysLT<sub>1</sub> and CysLT<sub>2</sub> and is also much less potent than LTC<sub>4</sub> and LTD<sub>4</sub> in inducing cellular responses *in vitro*, showing a partial agonistic activity<sup>2-5</sup>. However *in vivo*, it is LTE<sub>4</sub> that has shown to be the most potent cysLT in eliciting influx of eosinophils and basophils into bronchial mucosa of asthmatic subjects and in enhancing airway responsiveness to histamine and increasing vascular permeability, suggesting the existence of one or more leukotriene receptors that have not been identified to date<sup>6-10</sup>. The potential presence of such a receptor has been demonstrated in CysLT<sub>1</sub>/CysLT<sub>2</sub> double knock-out mice<sup>11</sup> but human data are lacking.

The observations that asthmatic airways respond with enhanced bronchoconstriction to inhaled cysLTs, especially to LTE<sub>4</sub> in comparison with normal subjects<sup>10</sup> and that

infiltration of airways by mast cells is associated with disordered airway function in asthma <sup>12</sup> suggest that mast cells could be a potential target cell type expressing a putative receptor preferentially responding to LTE<sub>4</sub>. In fact, the possible existence of such a novel, LTE<sub>4</sub>-activated receptor has been suggested in human mast cells <sup>13</sup>. In this study LTE<sub>4</sub> has been shown to be the most potent of cysLTs in inducing cell proliferation and activation of gene expression in human primary mast cells and LAD2 human mast cell line. LTE<sub>4</sub>-mediated activities were resistant to knockdown of CysLT<sub>1</sub> and CysLT<sub>2</sub> but were dependent on PPAR- $\gamma$  signalling. Another study has suggested that the P2Y<sub>12</sub> receptor is required for LTE<sub>4</sub>-mediated responses <sup>14</sup> but these observations have not been confirmed <sup>15</sup>.

In order to identify such a receptor responding to LTE<sub>4</sub> we studied human mast cells and used transcriptome profiling by microarrays, recombinant GPCR overexpression models and methods analysing GPCR signalling. We characterize LTE<sub>4</sub> as a fully functional agonist activating human CysLT<sub>1</sub> and show for the first time that CysLT<sub>1</sub> expression is critically important for responsiveness to LTE<sub>4</sub> within a human cell system.

## Results

### **LTE<sub>4</sub> signals differently in LAD2 and LUVA cells**

LTE<sub>4</sub> has been shown to induce potent responses in LAD2 cells<sup>13</sup> offering a model for identification of the elusive receptor responsible for LTE<sub>4</sub> signalling. In order to compare responses between LTD<sub>4</sub> and LTE<sub>4</sub> microarray analysis of LAD2 cells stimulated with either vehicle control, LTD<sub>4</sub> or LTE<sub>4</sub> was carried out in the presence of L-cysteine (3 mmol/L) to inhibit dipeptidase enzyme responsible for converting LTD<sub>4</sub> to LTE<sub>4</sub><sup>16</sup>. Both leukotrienes significantly regulated expression of 64 genes including many chemokines, growth and transcription factors (Figure 1A and supplementary Table 1). LTE<sub>4</sub> was more potent in up and down regulation of gene expression than LTD<sub>4</sub> for the majority of analysed genes, providing strong evidence for a robust LTE<sub>4</sub> response in LAD2 cells. CCL4 and CSF2 were among the most upregulated genes in LAD2 cells and were selected for further analysis. qRT-PCR and ELISA analysis of LAD2 cells showed induction of CCL4 and CSF2 with LTE<sub>4</sub> consistently matching or being the more potent of the 2 ligands (Figure 1B). To verify whether this responsiveness to LTE<sub>4</sub> is characteristic for other mast cells, another human mast cell line, LUVA, was analysed to compare responses to LTD<sub>4</sub> and LTE<sub>4</sub>. Although in LUVA cells LTD<sub>4</sub> regulated gene expression in a similarly potent way to LAD2 cells, LTE<sub>4</sub> induced only very weak responses (Figure 1C). As intracellular calcium mobilisation is a secondary messenger signalling cue for classical leukotriene receptors, cysLT induced calcium mobilisation was analysed in both cell lines. In LAD2 cells, all cysLTs induced a concentration-dependent calcium mobilisation (Figure 1D), with LTD<sub>4</sub> and LTC<sub>4</sub> showing similar potency (LTC<sub>4</sub> EC<sub>50</sub>-1.3x10<sup>-9</sup> M, LTD<sub>4</sub> EC<sub>50</sub>-0.58x10<sup>-9</sup> M) and LTE<sub>4</sub> being the weakest of all 3 ligands but still

inducing a robust response (LTE<sub>4</sub> EC<sub>50</sub>-1.67x10<sup>-9</sup> M). In contrast, LTD<sub>4</sub> was the most potent ligand in LUVA cells (EC<sub>50</sub>-2.8x10<sup>-9</sup> M) followed by LTC<sub>4</sub> (EC<sub>50</sub>-1.7x10<sup>-8</sup> M), while LTE<sub>4</sub> induced very weak response (EC<sub>50</sub>-not determined)(Figure 1E). Similar potencies of cysLTs as in LUVA cells were detected in HEK293T cells transfected with human CYSLTR1 (Figure 1F) (LTC<sub>4</sub> EC<sub>50</sub>-1.12x10<sup>-8</sup> M, LTD<sub>4</sub> EC<sub>50</sub>-0.9x10<sup>-9</sup> M; LTE<sub>4</sub> EC<sub>50</sub>-8.32x10<sup>-8</sup> M). Therefore LAD2 and LUVA cells represent two human mast cell lines that respond differently to LTE<sub>4</sub> stimulation.

### **Comparison of GPCR gene expression profiles between LAD2 and LUVA cells**

A previous study <sup>13</sup> has suggested that in LAD2 cells LTE<sub>4</sub> signals through a novel, CysLT<sub>3</sub> receptor, different from classical CysLT<sub>1</sub> and CysLT<sub>2</sub>. As our observations in LAD2 and LUVA cells indicated that a potential LTE<sub>4</sub> receptor should be differentially expressed in LAD2 and LUVA cells, gene expression was compared between LAD2 and LUVA cells using microarray in order to identify the putative gene. A list of significantly differentially expressed genes (ANOVA p<0.05; > 2 fold difference) was generated and GPCR genes were filtered using the IUPHAR GPCR database <sup>17</sup>. Among 27 GPCRs that differed significantly in expression between LAD2 and LUVA cells (Figure 2A, supplementary Table 2), 10 GPCRs were considered orphan receptors (without known ligands)(GPR12, GPR37, GPR65, GPR85, GPR114, GPR137B, GPR174, MAS1L, MRGPRX2 and P2RY8). GPR65, MAS1L and MRGPRX2 were the most differentially expressed orphan GPCRs (9.9, 32.4 and 70.2 fold difference between LAD2 and LUVA cells, respectively). To ascertain whether cysLTs, and LTE<sub>4</sub> in particular, could mediate signalling through any of these receptors, plasmids encoding GPR65, MAS1L and MRGPRX2 were

transiently transfected into HEK293T cells and calcium mobilisation was analysed upon stimulation with cysLTs (Figure 2B). CYSLTR1 gene was among differentially expressed GPCRs and was used as a positive control for all experiments. No specific calcium responses were observed in any of the transfectants apart from cells transfected with CYSLTR1, which showed the predicted pattern of response to cysLTs. As co-transfections of GPCRs and  $G\alpha_{16}$  have been reported previously to direct signal transduction to phospholipase C and calcium signalling<sup>18</sup>, target genes were co-expressed with human  $G\alpha_{16}$  and responses to cysLTs measured using calcium mobilisation in order to analyse potential alternative GPCR signalling pathway. Similarly, no response was observed in any of our overexpression models apart from CYSLTR1 transfected cells (Figure 2C). Thus CysLT<sub>1</sub> was the receptor that was differentially expressed in LAD2 and LUVA cells (4.3 fold difference) and responded to cysLTs.

### **CysLT<sub>1</sub> is required for LTE<sub>4</sub> induced signalling in LAD2 cells**

To determine whether CysLT<sub>1</sub> could be involved in LTE<sub>4</sub> signal transduction, LAD2 and LUVA cells were pretreated with selective CysLT<sub>1</sub> and CysLT<sub>2</sub> antagonists, Montelukast and HAMI3379, respectively. Antagonists' selectivity was previously verified in HEK293T cell transfection models (supplementary Figure 1). qRT-PCR analysis of CCL4 gene expression in LAD2 cells showed that both LTD<sub>4</sub> and LTE<sub>4</sub> induced responses were fully inhibited by Montelukast while HAMI3379 had no effect (Figure 2D). In LUVA cells, LTD<sub>4</sub> signalling was again fully inhibited by Montelukast but not by HAMI3379 (Figure 2E). Analysis of calcium mobilisation in these cells showed a very similar picture, with Montelukast fully inhibiting LTE<sub>4</sub> responses in LAD2 as well as LTD<sub>4</sub> responses in LAD2 and LUVA cells while

HAMI3379 had no effect (Figure 2D, E). To verify whether the potent LTE<sub>4</sub> induced, Montelukast sensitive, response in LAD2 cells was attributable specifically to CysLT<sub>1</sub> signalling and not via another Montelukast sensitive receptor, stable CYSLTR1 receptor knockdown was generated in LAD2 cells using shRNA. Four shRNA targeting different regions of CYSLTR1 were transduced into separate LAD2 cell populations using lentiviral particles. qRT-PCR analysis of CYSLTR1 revealed shRNA “475” to significantly knock down CYSLTR1, without affecting CYSLTR2 mRNA expression (supplementary Figure 2). Knocking down of CYSLTR1 substantially inhibited intracellular calcium responses to LTD<sub>4</sub> and LTE<sub>4</sub> (Figure 3A, B), confirming a functional decrease in CysLT<sub>1</sub> expression. CCL4 and CSF2 mRNA and protein expression upon LTD<sub>4</sub> and LTE<sub>4</sub> stimulation were almost completely abrogated in CysLT<sub>1</sub> knocked down LAD2 cells (Figure 3C, D and supplementary Figure 3) identifying CysLT<sub>1</sub> as a receptor responsible for LTE<sub>4</sub> induced signalling in LAD2 cells.

### **Overexpression of CysLT<sub>1</sub> in LUVA cells does not determine LTE<sub>4</sub> responses**

Our GPCR expression profiles identified CYSLTR1 as more highly expressed in LAD2 than in LUVA cells. To test the hypothesis that the expression level of CYSLTR1 is relevant for mast cell responsiveness to LTE<sub>4</sub>, CYSLTR1 was stably overexpressed in LUVA cells using lentiviral transduction and positive clones were selected using puromycin. qRT-PCR confirmed a 3-fold increase in CYSLTR1 expression in the transduced population, a level similar to LAD2 cells (Figure 3E). Functional CYSLTR1 overexpression was confirmed using calcium assay and showed potent concentration-dependent increase in LTE<sub>4</sub> induced calcium responses (Figure 3F), again similar to responses observed in LAD2 cells. Stimulation of LUVA cells



overexpressing CysLT<sub>1</sub> and control empty vector-transduced cells with either LTD<sub>4</sub> or LTE<sub>4</sub> revealed no significant differences in CCL4 mRNA or protein induction between both cell lines (Figure 3G, H), showing that the expression level of CysLT<sub>1</sub> does not solely determine LTE<sub>4</sub> induced gene regulation, even though it allows for enhanced calcium mobilisation in response to LTE<sub>4</sub>.

### **Comparison of CYSLTR1 gene sequence between LAD2 and LUVA cells**

As genetic variations in the CYSLTR1 gene between LAD2 and LUVA cells could account for such differential responses to LTE<sub>4</sub>, promoter and coding regions of CYSLTR1 in both cell types were sequenced. DNA was extracted and CYSLTR1 promoter fragment containing 4 single nucleotide polymorphisms (SNPs)(rs321029, rs2637204, rs2806489, rs7066737) as well as the entire coding region were PCR amplified and sequenced. BLAST analysis of DNA sequences from LAD2 and LUVA cells revealed no differences between cell lines and showed that both cell lines share the same promoter homozygous haplotype, “CAAC” for 4 SNPs studied, respectively and homozygous T allele for rs320995 coding synonymous SNP. Human CYSLTR1 gene is localized to chromosome X thus lack of heterozygosity at the locus was consistent with the fact that both cell lines were derived from male donors.

### **CysLT-activated CysLT<sub>1</sub> signals through Gα<sub>q</sub>, calcium and Erk for gene regulation**

In order to compare CysLT<sub>1</sub> mediated signalling in LAD2 and LUVA, both cell types were pre-incubated with several signalling pathway inhibitors and gene expression was measured in response to LTD<sub>4</sub> and LTE<sub>4</sub> (Figure 4A, B). LTD<sub>4</sub>- and LTE<sub>4</sub>-induced CCL4 mRNA expression was potently inhibited by U0126 (MEK/Erk

pathway inhibitor), intracellular (BAPTA-AM) and extracellular (EDTA) calcium chelators but was not modified by pertussis toxin or GW9662 and T0070907 (PPAR- $\gamma$  inhibitors), suggesting that in both cell lines CysLT<sub>1</sub> couples to G $\alpha$ q, requires intracellular and extracellular calcium and Erk activation for regulation of gene expression.

### **LTE<sub>4</sub> activates prolonged signalling in LAD2 cells**

To further analyse the agonistic activity of LTE<sub>4</sub>, time course experiments of Erk phosphorylation were conducted and analysed by Western blotting (Figure 4C). In LAD2 cells stimulated with LTD<sub>4</sub>, Erk phosphorylation peaked at 7 minutes with a gradual decrease until 60 minutes. LTE<sub>4</sub> induced a peak of Erk phosphorylation later but with a more sustained phosphorylation, still being detectable after 60 minutes. In LUVA cells the time point of highest Erk phosphorylation was similar to LAD2 cells but LTE<sub>4</sub>-induced Erk phosphorylation was shorter than in LAD2 cells. Thus sustained Erk phosphorylation induced by LTE<sub>4</sub> in LAD2 but not in LUVA cells underlies an important difference in CysLT<sub>1</sub>-mediated responses between the cell lines.

We next compared calcium mobilisation kinetics in LAD2 cells; although LTD<sub>4</sub> induced a higher peak response than LTE<sub>4</sub>, the intracellular calcium levels decreased at a higher rate after LTD<sub>4</sub> stimulation while LTE<sub>4</sub> induced a long lasting plateau phase (Figure 4D). The sustained calcium signalling in response to LTE<sub>4</sub> was not observed in LUVA cells or in LUVA cells overexpressing CysLT<sub>1</sub> (Figure 4D).

As GPCR signalling is regulated through receptor desensitization, cross desensitization experiments with cysLTs were performed (Figure 4E). Prior stimulation with either LTC<sub>4</sub> or LTD<sub>4</sub> completely abrogated calcium response to

LTD<sub>4</sub> in LAD2 and LUVA cells, suggesting that both LTC<sub>4</sub> and LTD<sub>4</sub> can fully desensitize CysLT<sub>1</sub> in both cell lines. However, prior stimulation with LTE<sub>4</sub> caused only partial inhibition of the calcium response to LTD<sub>4</sub> in LAD2 and LUVA cells, showing partial agonistic/desensitizing activity of LTE<sub>4</sub> but no difference between the cell lines in LTE<sub>4</sub> mediated signalling. The sustained increased level of calcium in LAD2 but not in LUVA cells after LTE<sub>4</sub> stimulation was again the main difference observed between the cell lines in these experiments.

To analyse whether prolonged calcium/Erk signalling induced by LTE<sub>4</sub> in LAD2 cells affects gene expression, CCL4 mRNA expression was analysed in LAD2 and LUVA cells after short (5 minutes) and long (2 hours) term exposure to LTD<sub>4</sub> and LTE<sub>4</sub>, respectively. In LAD2 cells, 2 hour exposure to LTE<sub>4</sub> and LTD<sub>4</sub>, caused similar upregulation of CCL4 mRNA expression (Figure 4F). Stimulation of LAD2 cells with LTE<sub>4</sub> for only 5 minutes failed to induce potent CCL4 expression with mRNA levels being significantly lower than that induced by LTD<sub>4</sub>. In LUVA cells, no difference could be observed between different exposure times.

## Discussion

This study identifies  $\text{LTE}_4$  as a fully functional agonist activating human  $\text{CysLT}_1$  for regulation of gene expression in LAD2 cells although only weak, partial agonism of  $\text{LTE}_4$  signalling could be detected in LUVA cells. Our data suggest that increased expression of  $\text{CysLT}_1$  and induction of prolonged intracellular signalling are required for  $\text{LTE}_4$  functional agonism. Ever since the elucidation and cloning of human  $\text{CysLT}_1$  and  $\text{CysLT}_2$ ,  $\text{LTE}_4$  has been considered as a final, non-active leukotriene metabolite due to its weak efficacy in recombinant systems and poor binding affinities compared to  $\text{LTC}_4$  and  $\text{LTD}_4$ <sup>6</sup>. However, it was  $\text{LTE}_4$  that was shown to be the most potent  $\text{cysLT}$  in inducing inflammatory and contractile responses in asthmatic subjects. Our observation that  $\text{LTE}_4$  can induce full agonistic activity through  $\text{CysLT}_1$  could be of relevance for explaining this discrepancy between potent *in vivo* activity of  $\text{LTE}_4$  observed in asthmatic patients and weak *in vitro* potency for classical  $\text{cysLT}$  receptors. Early studies analysing the effects of  $\text{cysLTs}$  *in vivo* revealed a disproportionate augmentation in relative responses to  $\text{LTE}_4$  inhalation in asthmatic patients when compared to healthy individuals<sup>10</sup>.  $\text{LTE}_4$  responsiveness was increased more than 200 fold in asthmatics while responses to  $\text{LTC}_4$  and  $\text{LTD}_4$  were increased 6 and 9 fold respectively. More recent clinical studies suggest that  $\text{CysLT}_1$  is more highly expressed in asthmatic airways compared to healthy individuals<sup>19</sup>, with further increase observed in asthma exacerbations and in a sub-phenotype of asthma, patients with aspirin-exacerbated respiratory disease (AERD)<sup>20,21</sup>. This increased  $\text{CysLT}_1$  expression observed in AERD patients was significantly decreased following successful aspirin desensitisation, a procedure associated also with a significant reduction in sensitivity to inhaled  $\text{LTE}_4$ <sup>20</sup>. We found similar disproportionate augmentation in  $\text{LTE}_4$ -induced responses when comparing LAD2 and LUVA cells, a

model of relatively high versus low CysLT<sub>1</sub> expression. LTC<sub>4</sub> and LTD<sub>4</sub> were 2-3 times more potent at inducing calcium mobilisation in LAD2 cells compared to LUVA while LTE<sub>4</sub> showed nearly 60-fold difference. Such potent responses to cysLTs, including LTE<sub>4</sub> have been recently described in other human primary cells expressing high levels of CysLT<sub>1</sub>, T helper type 2 (Th2) lymphocytes<sup>22,23</sup> and group 2 innate lymphoid cells (ILC2)<sup>24</sup> supporting further our observation.

Prolonged intracellular signalling was identified as another potential contributing factor for the potency of LTE<sub>4</sub> responses. The sustained increase in intracellular calcium and Erk phosphorylation upon LTE<sub>4</sub> stimulation were observed in LAD2 but not in LUVA cells, suggesting that prolonged signalling could be critical for transcriptional regulation. LTE<sub>4</sub>, in contrast to LTC<sub>4</sub> and LTD<sub>4</sub>, shows only partial activity and does not desensitise CysLT<sub>1</sub> responses, a feature that can contribute to prolonged signalling in response to LTE<sub>4</sub> in LAD2 cells. In fact, in experiments with short term exposure to agonists LTE<sub>4</sub> showed only weak, partial agonist activity in comparison to LTD<sub>4</sub>, confirming important role of prolonged signalling in LTE<sub>4</sub> induced responses. Overexpression of CysLT<sub>1</sub> in LUVA did not restore sustained intracellular calcium and full agonism even though it increased peak calcium response to LTE<sub>4</sub>, suggesting that additional unidentified signalling molecules expressed in LAD2 cells but not in LUVA, are also required for full functional agonism of LTE<sub>4</sub>.

Mouse models provide strong evidence that CysLT<sub>1</sub> and CysLT<sub>2</sub> are not the only cysLT receptors as germline deletion did not diminish leukotriene-mediated inflammation<sup>11,14,25</sup>. Our data presented here provide an explanation for potent LTE<sub>4</sub> activity observed in humans but do not rule out the possibility of another cysLT receptor. Our study shows for the first time that CysLT<sub>1</sub> expression is critically

important for responsiveness to  $\text{LTE}_4$  within a human cell system. This could potentially be relevant for human cell types other than mast cells and could thus have important implications for diagnostics and targeted treatment of specific phenotypes of asthma.

## **Materials and Methods**

### **Reagents**

Leukotrienes (LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub>), Montelukast, MK-571, HAMI3379, U-0126, GW9662, T0070907 (all Cayman Chemical), EDTA (Ambion), BAPTA-AM, Pertussis Toxin, Calcium ionophore (A23187), (all Sigma-Aldrich) were obtained from the manufacturers.

### **Cell Culture**

HEK293T cells were cultured in DMEM medium supplemented with 2mmol/L glutamine, 10% fetal bovine serum and Penicillin/Streptomycin (50 units/ml) (all Life Technologies) in a humidified 5% CO<sub>2</sub> 37°C incubator. LAD2 cells (a kind gift from Dr. Arnold Kirshenbaum, NIAID, NIH, USA <sup>26</sup>) and LUVA cells (a kind gift from Dr. John Steinke, University of Virginia, USA <sup>27</sup>) were cultured in StemPro-34 medium supplemented with with L-glutamine (2 mmol/L), Pen/Strep (50 IU/ml) and with or without stem cell factor (SCF) (100 ng/ml) (all Life Technologies), respectively. Cells were hemidepleted weekly with fresh medium.

### **Transient transfections**

HEK293T cells cultured to above 60% confluence were transiently transfected as described previously <sup>4,15</sup> with a mixture of Lipofectamine 2000 (Life Technologies) and the following plasmids as indicated: pcDNA3.1-empty, pcDNA3.1-human CYSLTR1, pcDNA3.1-human CYSLTR2, pcDNA3.1-human GPR65, pcDNA3.1-human GNA15 (Gα<sub>16</sub>) (all the Missouri S&T cDNA Resource Center, Rolla, Mo) and

pCMV6-Kan/Neo- human MAS1L and human MRGPRX2 (Origene Technologies) in serum-free medium (Opti-MEM, Life Technologies) according to manufacturer's protocol. After incubation the transfection medium was removed and HEK293T cells were cultured for 36 hours before calcium mobilisation was assayed in response to stimulation with calcium ionophore (1 $\mu$ mol/L), LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub> (all 100 nmol/L).

### **Short hairpin RNA (shRNA) knockdown**

For stable gene silencing shRNA constructs targeting different regions of human CYSLTR1 (clone ID: V3LHS\_305475, V3LHS\_305478, V2LHS\_90946 and V2LHS\_90947) were purchased from ThermoScientific and used to generate lentiviral particles with the lentiviral packaging system (psPAX2, pMD2.G and PEG-it™ precipitation)(System Biosciences) according to manufacturer's protocol. LAD2 cells were transduced with viral particles for 24 hours and positive cells selected using Puromycin (2 $\mu$ g/ml)(Life Technologies). Efficiency of transduction was assessed by analysing GFP expression using flow cytometry.

### **CYSLTR1 overexpression**

CYSLTR1 gene was amplified from the pcDNA3.1-CYSLTR1 construct (UMR cDNA Resource Center) with primers containing restriction enzyme sites for NheI and BamHI (5'-AGGTGCTAGCATGGATGAAACAGGAAATT and 5'-GCGGGGATCCCTATACTTTACATATTTTC) and cloned into lentiviral vector pCDH (System Biosciences) encoding GFP and puromycin resistance under the EF1 promoter and a multiple cloning site under the CMV promoter. Viral particles were generated using lentiviral packaging system (System Biosciences). LUVA cells were



transduced, selected with puromycin (2µg/ml) and transduction efficiency was evaluated by GFP expression using flow cytometry.

### **DNA sequencing**

Total DNA was extracted using DNeasy Tissue kit (Qiagen) and fragments of CYSLTR1 gene were amplified using Platinum Taq Polymerase High Fidelity (Invitrogen) following manufacturer's protocol and primers: CYSLTR1 promoter 5'-AACTGGAGACTTGCAGGTTGCG, 5'-AACATCAAAGTGCTGCCCCAGG; CYSLTR1 coding region 5'-TCAATGCCTCACTACTATTGCTTG, 5'-TTGGTTTGGACTGGAAATGGG and sequenced by Source Bioscience Sanger service using custom designed primers: CYSLTR1 promoter 5'-TAAGATGGGAAGCAGGGACG, 5'-GGCTTCAATCAGCACATACC; CYSLTR1 coding region 5'-ATACCAAGTGCTTTGAGCC, 5'-GCATTTGGCTCTTTGGTG and 5'-GTTTGATTGTCTTGTGGGG.

### **Calcium mobilisation assay**

Calcium mobilisation assays were conducted using FLIPR calcium 4 assay kit (Molecular Devices) as described previously<sup>4,28</sup>. Cells ( $1.5 \times 10^5$ /well) were plated into poly-L-lysine coated 96 well plates in RPMI 1640 supplemented with 10mmol/L HEPES, incubated for 1 hour with FLIPR loading buffer prior to addition of ligand and fluorescent intensity was measured at 37°C using a Flexstation 3 (Molecular Devices). Controls included medium control with ethanol for leukotriene stimulations. Results were analysed with SoftMax Pro Software (Molecular Devices).

### **Real time PCR**

LAD2 and LUVA cells were stimulated for 2 hours in the presence of L-cysteine (3 mmol/L) with LTD<sub>4</sub> and LTE<sub>4</sub> (both 100 nmol/L) and vehicle control. In some experiments as indicated cells were pretreated with U0126 (1 µmol/L; 30 min), BAPTA-AM (30 µmol/L; 30 min), EDTA (2.5 mmol/L; 5 min), pertussis toxin (PTX)(100ng/ml; overnight), GW9662 (10 µmol/L; 30 min) or T0070907 (1 µmol/L; 30 min). Total cellular RNA was isolated using the miRNeasy mini kit (Qiagen), DNAase treated (Ambion) and reverse transcribed using RevertAid M-MuLV (Fermentas). Expression of mRNA encoding selected genes was measured using real time PCR on an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Commercially available primer probe sets: 18S rRNA - 4319413E (Applied Biosystems) and individually designed assays using the Universal Probe Library (UPL) (Roche): CYSLTR1- probe 71, primers 5'-GGAGAGGGTCAAAGCAACAA, 5'-TGCAGAAGTCCGTGGTCATA; CYSLTR2- probe 21, primers 5'-TGATGTGACACTGCCGTTCT, 5'-TCATGGCTTCCTCAATAATGC; CCL4- probe 20, primers 5'-CAGCACAGACTTGCTTGCTT, 5'-CTTCCTCGCAACTTTGTGGT; CSF2- probe 1, primers 5'-GCCCTTGAGCTTGGTGAG, 5'-TCTCAGAAATGTTTGACCTCC were used. All primers/probes were tested for optimal efficiency of amplification. Relative gene expression was normalized to 18S rRNA. Data were analysed using SDS2.1 software (Applied Biosystems).

### **Microarray Analysis**

Total cellular RNA was isolated using the miRNeasy mini kit (Qiagen), DNase treated (Ambion), quality analysed on an Agilent 2100 Bioanalyzer (Agilent Technologies) and further processed with the Ambion WT Expression Kit (Applied

Biosystems) according to the manufacturers' instructions<sup>29</sup>. cRNA was fragmented, labelled, and hybridised to the Affymetrix Human Gene 1.0 ST Arrays using the GeneChip WT Terminal Labeling and Hybridization Kit (Affymetrix). GeneChip fluidics station 450 (Affymetrix) was used for processing of the arrays and fluorescent signals were detected with the GeneChip scanner 3000. Images were analysed with the GeneChip operating software (Affymetrix). Further analysis was performed with the Partek Genomics Suite (Partek). RMA processing and quantile normalization was applied, and after Median Polish and gene level probeset summarization, differentially expressed genes were identified using ANOVA. Data were submitted to Gene Expression Omnibus database (accession number GSE75603).

### **Western Blot Analysis**

Total protein lysates were prepared using lysis buffer containing 1mM protease inhibitor cocktail (Roche), 25µg proteins loaded onto a 10% Bis-Tris NuPage gel (Invitrogen) and transferred onto a nitrocellulose membrane (Invitrogen). The membrane was incubated with primary antibodies against phospho-p44/42 MAPK and p44/p42 MAPK (Extracellular-signal-regulated kinase (ERK))(Cell Signaling) overnight at 4°C, followed by secondary, horseradish peroxidase-conjugated antibody (goat anti rabbit IgG (Southern Biotech). Blots were developed using ECL plus Detection Reagent (GE Healthcare) and visualized on a Chemidoc MP System (BioRad). Data were analysed using Image Lab 4.1 software (BioRad).

### **ELISA**

LAD2 and LUVA cells were stimulated for 6 hours in the presence of L-cysteine (3 mmol/L) with LTD<sub>4</sub> and LTE<sub>4</sub> (both 100 nmol/L) and vehicle control. CCL4 and

CSF2 concentrations were measured in supernatants using human CCL4 (MIP-1 $\beta$ ) and CSF2 (GM-CSF) duo set kits (R&D Systems, UK) following manufacturer's protocol.

### **Statistical analysis**

Data were analysed by means of one- or two- way ANOVA using GraphPad Prism software (GraphPad). Differences were considered significant at a p-value of less than 0.05.

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### **Author Contributions**

HRF, EF, WB and GW performed the experiments and analysed data. GW and THL conceived and designed the experiments. DJC contributed reagents/materials/analysis tools and analysed data. HRF, EF, THL and GW wrote the paper. All authors reviewed and provided comments upon preparation of the manuscript.

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### **Competing Financial Interests statement**

The authors declare no conflict of interests.

## Figure legends

Figure 1.  $\text{LTE}_4$  is a potent agonist in LAD2 but not in LUVA cells. (A) LAD2 cells ( $n=3$ ) were stimulated with vehicle control,  $\text{LTD}_4$  and  $\text{LTE}_4$  and gene expression was analysed using microarrays. Hierarchical clustering of significantly (ANOVA,  $p<0.05$   $\text{LTD}_4$  and  $\text{LTE}_4$  compared to control, False Discovery Rate=0.1) regulated genes is presented as a heat map. (B) LAD2 and (C) LUVA cells were stimulated and CCL4 or CSF2 gene expression were measured at mRNA and protein levels. Data expressed as mean  $\pm$  SEM from 3 separate experiments. (D) LAD2, (E) LUVA and (F) HEK293T transfected with CYSLTR1 cells were stimulated with indicated concentrations of  $\text{LTC}_4$ ,  $\text{LTD}_4$  and  $\text{LTE}_4$  and calcium mobilisation was measured. Data from 3 experiments run in triplicate, presented as mean  $\pm$  SEM of baseline corrected peak intracellular calcium response. Relative fluorescence unit (RFU).

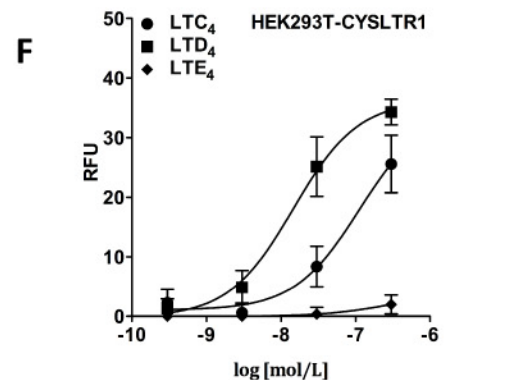
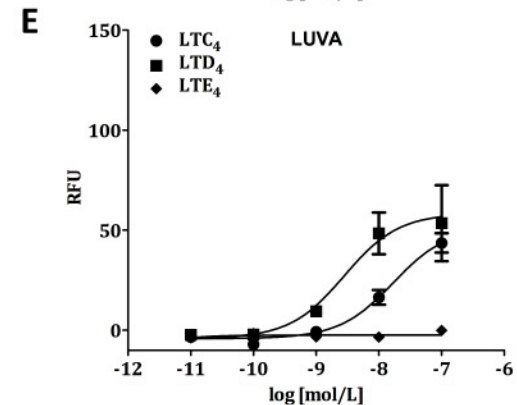
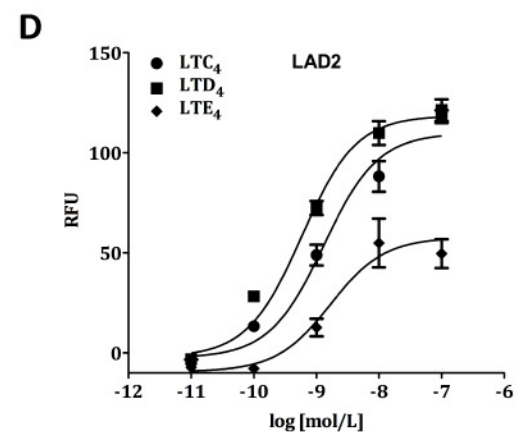
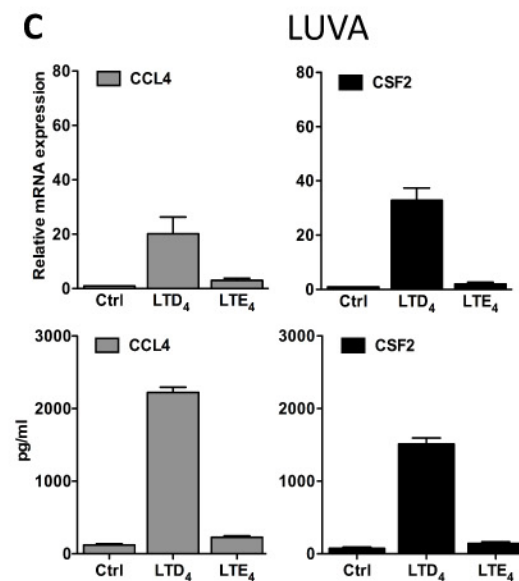
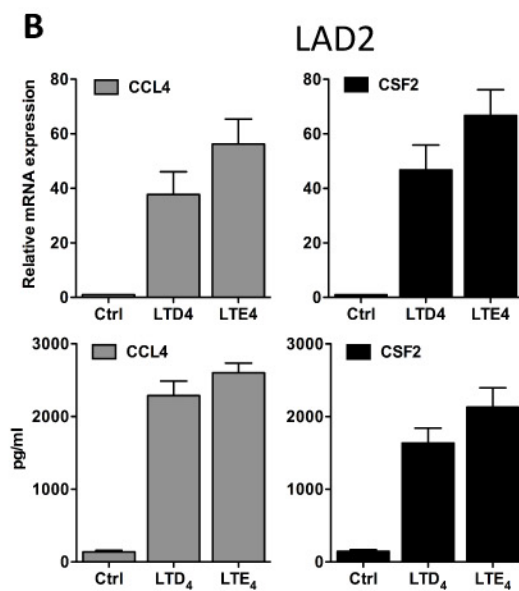
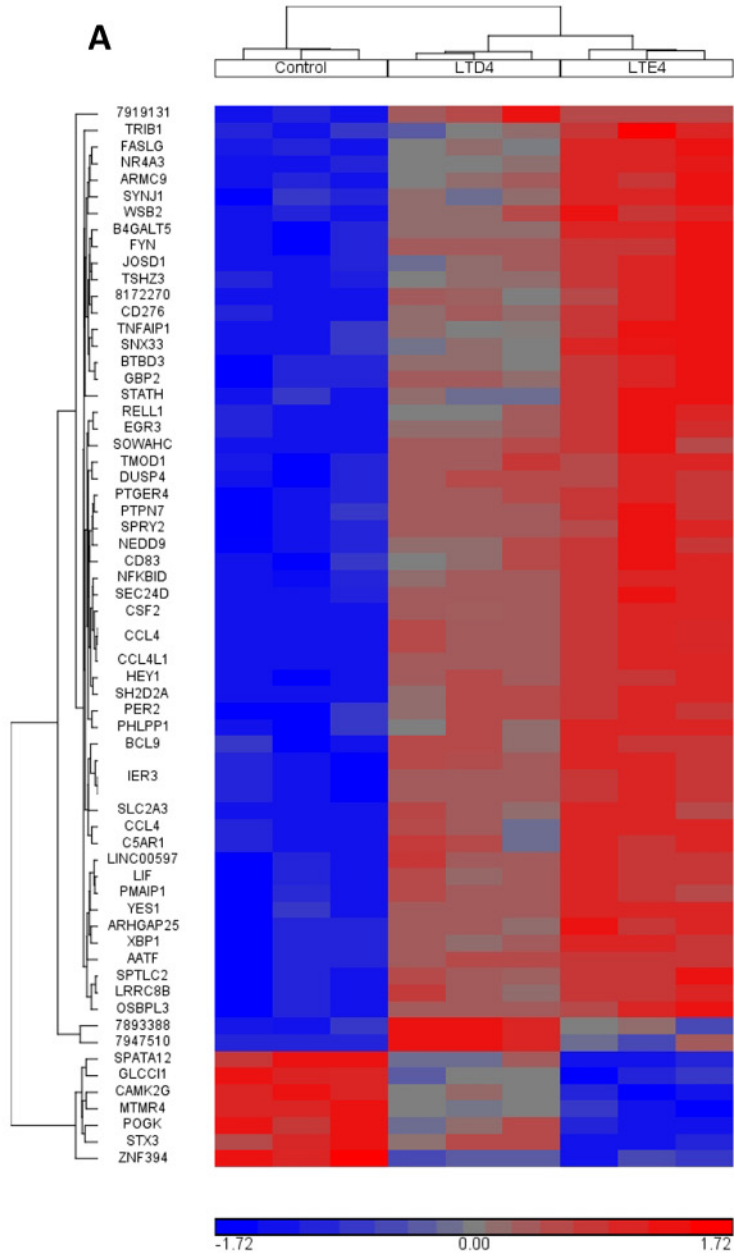
Figure 2. Comparison of GPCR gene expression profiles between LAD2 and LUVA cells. (A) Microarray gene expression was compared in LAD2 and LUVA cells ( $n=3$ ) and hierarchical clustering of differentially expressed GPCRs (ANOVA,  $p<0.05$ ,  $> 2$  fold difference) is presented as a heat map. Intracellular calcium mobilisation was analysed in HEK293T cells transiently transfected with the genes of interest (B) and co-transfected with  $\text{G}\alpha 16$  (C). Data expressed as percentage of peak calcium ionophore response, mean  $\pm$  SEM from 3 experiments run in triplicate. LAD2 (D) and LUVA (E) cells were pre-treated with Montelukast (100 nmol/L) and HAMI3379 (1  $\mu\text{mol/L}$ ) for 10 minutes, stimulated with  $\text{LTD}_4$  and  $\text{LTE}_4$  (both 100 nmol/L) and CCL4 mRNA expression or calcium mobilisation was measured. Data expressed as a fold difference in comparison to vehicle control or as baseline corrected peak calcium

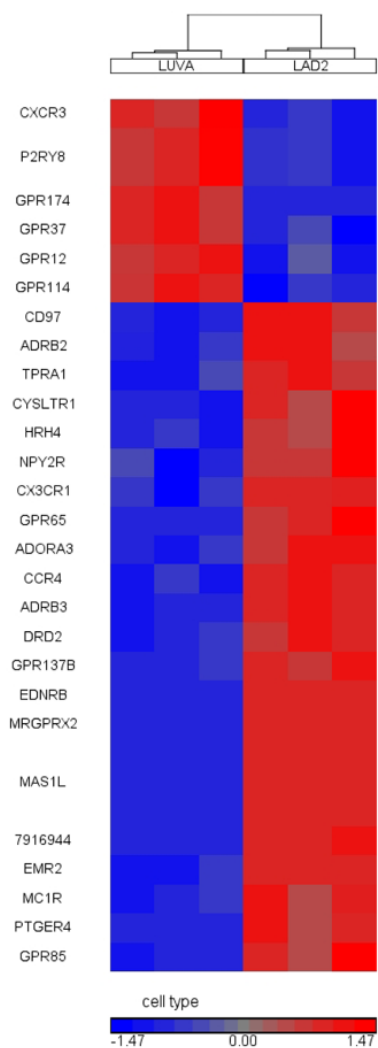
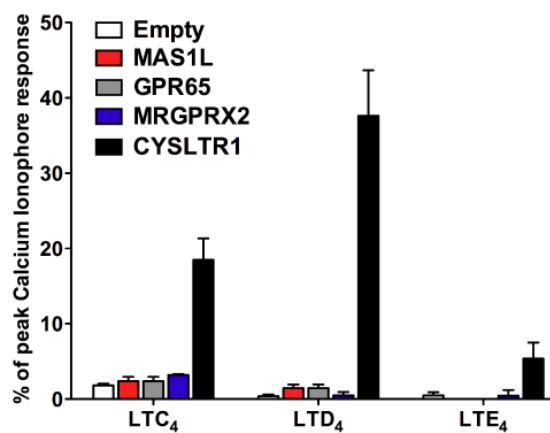
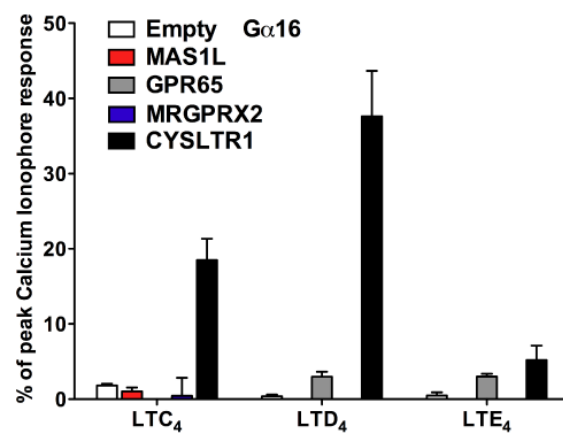
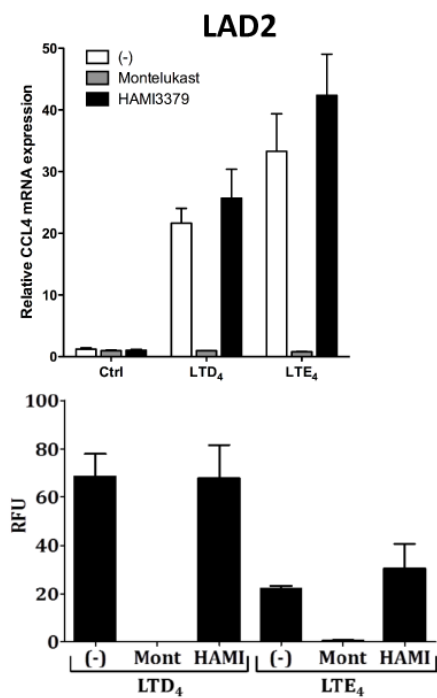
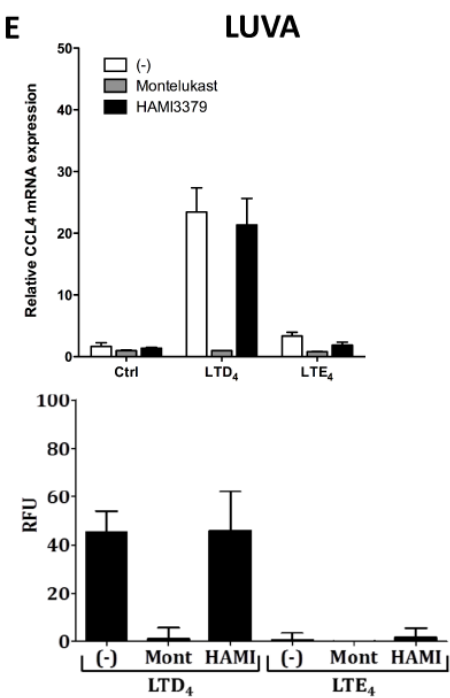
response. Mean  $\pm$  SEM from 3 separate experiments. Relative fluorescence unit (RFU).

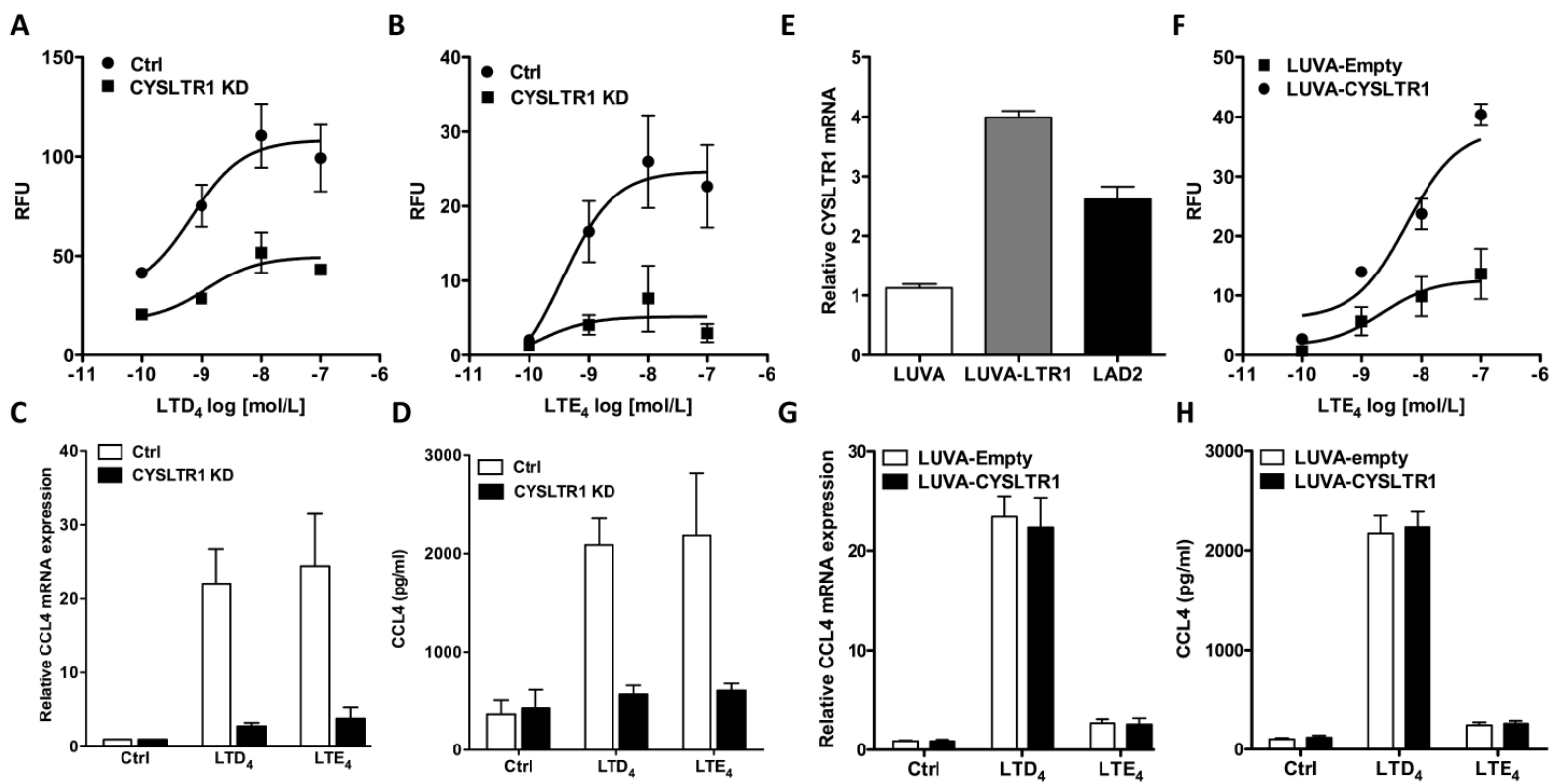
Figure 3. CysLT<sub>1</sub> is required for LTE<sub>4</sub> induced signalling in LAD2 cells. Calcium mobilisation responses to LTD<sub>4</sub> (A) and LTE<sub>4</sub> (B) in Empty control (Ctrl) and CYSLTR1 knocked down (CYSLTR1 KD) LAD2 cells. Baseline corrected peak calcium responses from 3 experiments run in triplicate presented as mean  $\pm$  SEM. (C) Control and CYSLTR1 knocked down LAD2 cells were stimulated with vehicle control, LTD<sub>4</sub> or LTE<sub>4</sub> for 2 (mRNA) (C) or 6 hours (protein) (D) before analysis. Data expressed as fold difference in comparison to vehicle control for CCL4 mRNA and as CCL4 supernatant concentrations. Mean  $\pm$  SEM from 3-5 experiments, relative fluorescence unit (RFU). (E) LUVA cells were stably transduced with empty (LUVA-empty) or CYSLTR1 overexpression (LUVA-CYSLTR1) vectors and relative CYSLTR1 mRNA expression was measured and compared to LAD2 cells. Mean  $\pm$  SEM, n=6. (F) Calcium mobilisation response to a range of LTE<sub>4</sub> concentrations was evaluated in empty control and CYSLTR1 transduced LUVA cells. Mean  $\pm$  SEM of baseline corrected peak calcium responses, n=9. Control empty vector and CYSLTR1 transduced LUVA cells were stimulated as indicated before CCL4 mRNA (G) or protein (H) expression was measured. Mean  $\pm$  SEM of 3 separate experiments.

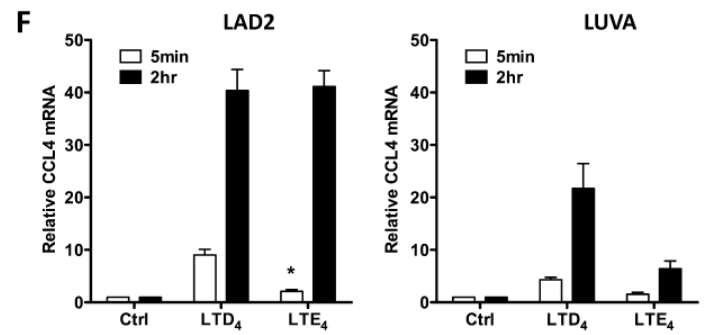
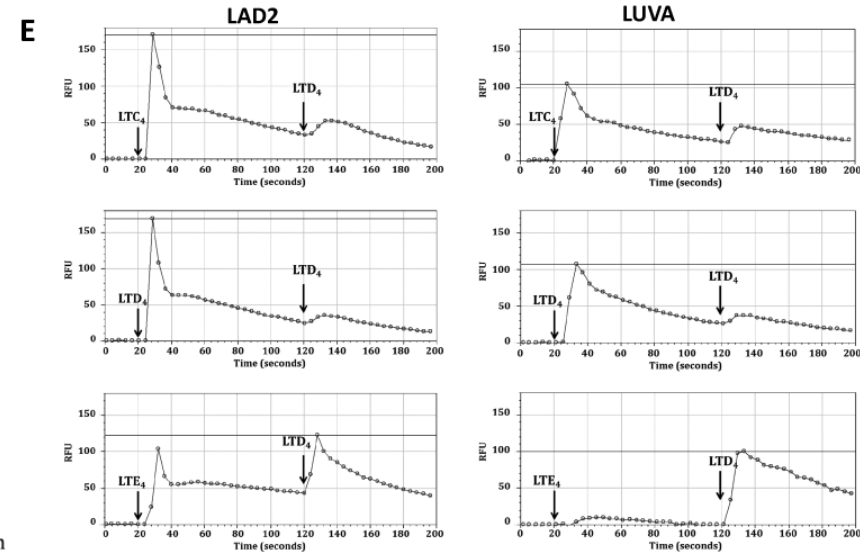
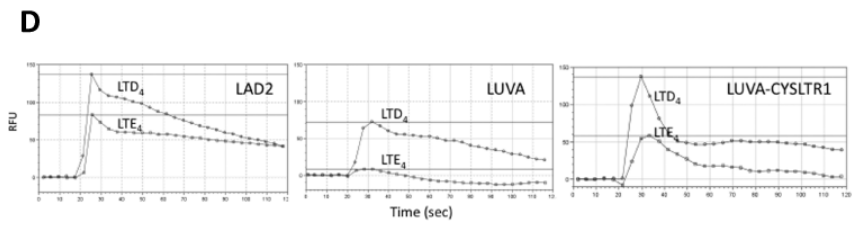
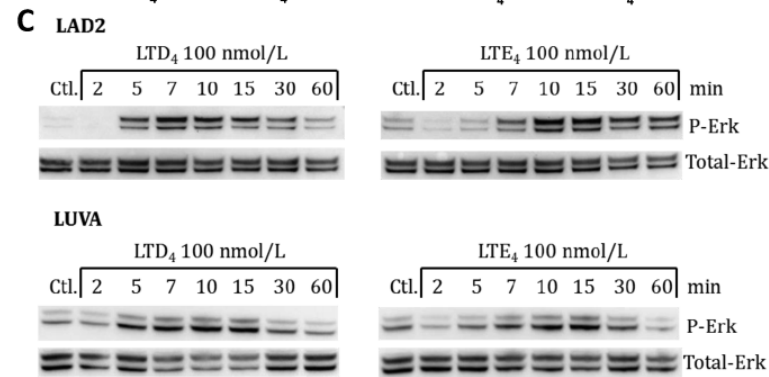
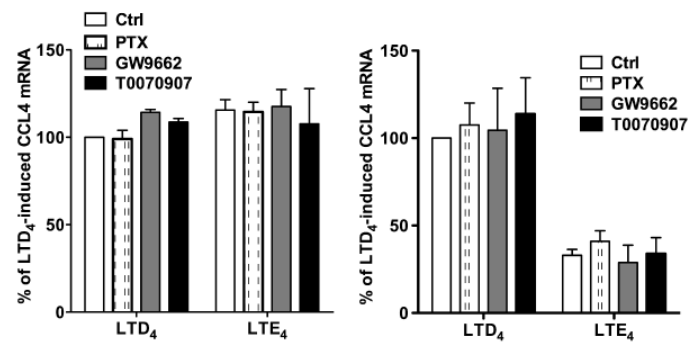
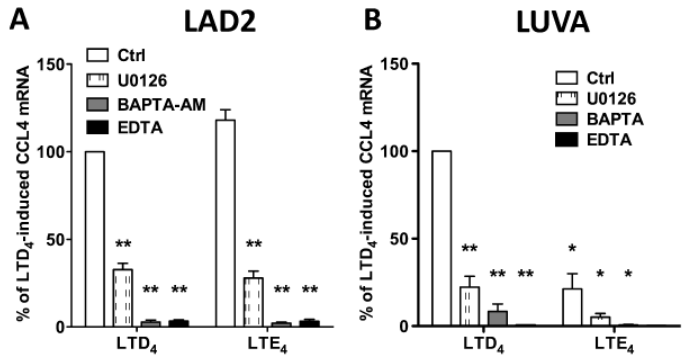
Figure 4. LTE<sub>4</sub> induces sustained signalling in LAD2 cells. LAD2 (A) and LUVA (B) cells were pre-treated with selected inhibitors and stimulated with LTD<sub>4</sub> or LTE<sub>4</sub>. Data from 3 separate experiments shown as % of LTD<sub>4</sub>-induced CCL4 mRNA expression (mean  $\pm$  SEM), \* p<0.05, \*\* p<0.001, ANOVA with Bonferroni post test compared to LTD<sub>4</sub> or LTE<sub>4</sub>. (C) LAD2 and LUVA cells were stimulated for time

indicated with vehicle control (Ctrl), LTD<sub>4</sub> or LTE<sub>4</sub> (both 100 nmol/L) and phosphorylated Erk and total Erk expression measured using specific antibodies. Results from a representative experiment of 3 performed. Calcium mobilisation traces of LAD2, LUVA or LUVA-CYSLTR1 cells stimulated as indicated with LTD<sub>4</sub> or LTE<sub>4</sub> (100 nmol/L) once (D) or twice (E). Representative of 3 separate experiments, relative fluorescence unit (RFU). Black arrows indicate start of stimulation. (F). LAD2 and LUVA cells were exposed for either 5 minutes or 2 hours to vehicle control, LTD<sub>4</sub> or LTE<sub>4</sub> (both 100 nmol/L) and CCL4 mRNA measured by qRT-PCR after 2 hours incubation. Mean  $\pm$  SEM data from 3 experiments shown as a fold change in comparison to controls. \*  $p < 0.05$ , 2-way ANOVA comparison between 5 min LTD<sub>4</sub> and LTE<sub>4</sub> stimulations.



**A****B****C****D****E**







## **Online supplementary material**

**Title: Leukotriene E<sub>4</sub> is a full functional agonist for human cysteinyl leukotriene type 1 receptor**

**Authors:** H. R. Foster<sup>1,2‡</sup>, E. Fuerst<sup>1,2‡</sup>, W. Branchett<sup>1,2</sup>, T. H. Lee<sup>1,2,†</sup>, D. J. Cousins<sup>1,2,3</sup>, G. Woszczek<sup>1,2\*</sup>

Table 1. List of genes (probes) significantly regulated by stimulation with LTD<sub>4</sub> or LTE<sub>4</sub> in comparison to vehicle control in LAD2 cells (ANOVA, p<0.05, False Discovery Rate = 0.1).

	ID	Gene assignment	Gene Symbol	Fold-Change (Control vs. LTD4)	Fold-Change (Control vs. LTE4)
1	15179	AY766446 // CCL4 // chemokine (C-C motif) ligand 4 // 17q12 // 6351 /// AY766447 // CCL	CCL4	-8.74218	-16.7445
2	16413	AY766446 // CCL4 // chemokine (C-C motif) ligand 4 // 17q12 // 6351 /// AY766447 // CCL	CCL4	-8.74218	-16.7445
3	15178	AY766447 // CCL4L1 // chemokine (C-C motif) ligand 4-like 1 // 17q12 // 9560 /// AY7664	CCL4L1	-7.27318	-14.2327
4	15176	AY766446 // CCL4 // chemokine (C-C motif) ligand 4 // 17q12 // 6351 /// ENST00000250151	CCL4	-6.27419	-16.7221
5	29743	AK292464 // EGR3 // early growth response 3 // 8p23-p21 // 1960 /// ENST00000317216 //	EGR3	-5.55947	-12.5264
6	13615	AF216224 // LINC00597 // long intergenic non-protein coding RNA 597 // 15q23-q24 // 816	LINC00597	-4.36168	-5.56335
7	18086	AF385434 // NFKBID // nuclear factor of kappa light polypeptide gene enhancer in B-cell	NFKBID	-3.15495	-5.53942
8	25305	BC108724 // CSF2 // colony stimulating factor 2 (granulocyte-macrophage) // 5q31.1 // 1	CSF2	-3.0202	-4.6602
9	4812	BC030607 // LRRC8B // leucine rich repeat containing 8 family, member B // 1p22.2 // 23	LRRC8B	-3.01226	-4.05937
10	29944	AF254637 // HEY1 // hairy/enhancer-of-split related with YRPW motif 1 // 8q21 // 23462	HEY1	-3.00429	-4.01398
11	6246	ENST00000464839 // GBP2 // guanylate binding protein 2, interferon-inducible // 1p22.2	GBP2	-2.81828	-4.74415

12	25033	L28175 // PTGER4 // prostaglandin E receptor 4 (subtype EP4) // 5p13.1 // 5734 /// ENST	PTGER4	-2.63092	-3.43541
13	27122	AF039067 // IER3 // immediate early response 3 // 6p21.3 // 8870 /// BC000844 // IER3 /	IER3	-2.61982	-3.43259
14	33032	AF039067 // IER3 // immediate early response 3 // 6p21.3 // 8870 /// BC000844 // IER3 /	IER3	-2.61982	-3.43259
15	29778	U21108 // DUSP4 // dual specificity phosphatase 4 // 8p12-p11 // 1846 /// ENST000002401	DUSP4	-2.61512	-3.12733
16	16610	D90070 // PMAIP1 // phorbol-12-myristate-13-acetate-induced protein 1 // 18q21.32 // 53	PMAIP1	-2.58693	-3.18639
17	32904	AF039067 // IER3 // immediate early response 3 // 6p21.3 // 8870 /// AF083421 // IER3 /	IER3	-2.51551	-3.31687
18	11755	BX648582 // SPRY2 // sprouty homolog 2 (Drosophila) // 13q31.1 // 10253 /// ENST0000037	SPRY2	-2.44659	-3.30198
19	23985	BC067219 // STATH // statherin // 4q13.3 // 6779 /// BX649104 // STATH // statherin //	STATH	-2.43164	-6.5496
20	30526	D78579 // NR4A3 // nuclear receptor subfamily 4, group A, member 3 // 9q22 // 8013 ///	NR4A3	-2.34479	-4.88248
21	18963	BC036652 // SOWAHC // sosondowah ankyrin repeat domain family member C // 2q13 // 65124	SOWAHC	-2.27147	-2.825
22	9302	---		-2.20735	-1.41984
23	6621	AF097744 // SH2D2A // SH2 domain containing 2A // 1q21 // 9047 /// ENST00000368199 // S	SH2D2A	-2.09425	-2.62985
24	26995	AK292682 // NEDD9 // neural precursor cell expressed, developmentally down-regulated 9	NEDD9	-2.01024	-2.61269
25	17456	BC008982 // C5AR1 // complement component 5a receptor 1 // 19q13.3-q13.4 // 728 /// ENS	C5AR1	-1.99449	-2.53677
26	29551	BC063292 // TRIB1 // tribbles homolog 1 (Drosophila) //	TRIB1	-1.95595	-4.18346

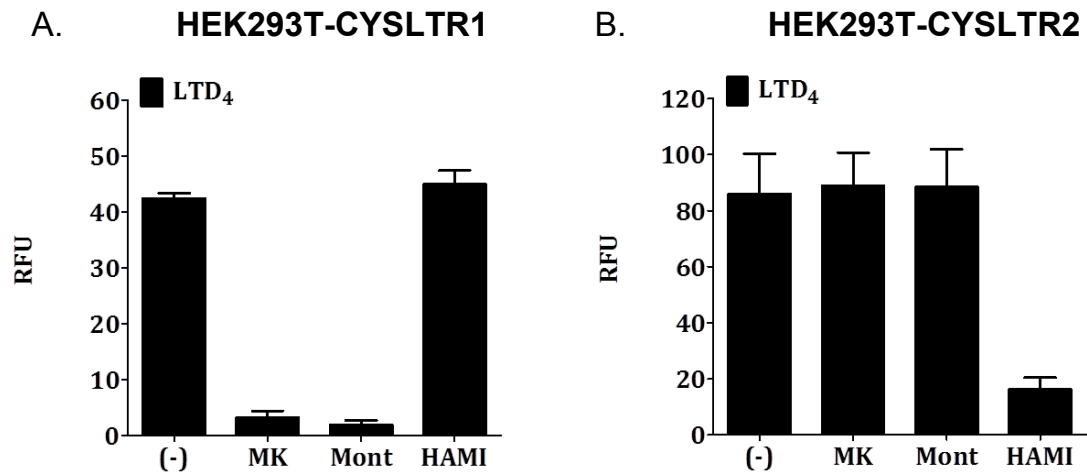
		8q24.13 // 10221 /// ENST000003			
27	22041	BC069540 // LIF // leukemia inhibitory factor // 22q12.2 // 3976 /// ENST00000249075 //	LIF	-1.89923	-2.26037
28	16663	ENST00000314574 // YES1 // v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1 // 18p11.	YES1	-1.80077	-2.27664
29	6842	BC001746 // PTPN7 // protein tyrosine phosphatase, non- receptor type 7 // 1q32.1 // 577	PTPN7	-1.79981	-2.23885
30	6402	---		-1.78997	-1.68385
31	26261	BC030830 // CD83 // CD83 molecule // 6p23 // 9308 /// ENST00000379153 // CD83 // CD83 m	CD83	-1.74841	-2.42664
32	891	---		-1.74642	-1.26037
33	20462	AK294876 // PER2 // period homolog 2 (Drosophila) // 2q37.3 // 8864 /// ENST00000254657	PER2	-1.70673	-1.98817
34	32175	---		-1.6855	-2.31655
35	22028	BC012841 // XBP1 // X-box binding protein 1 // 22q12.1 22q12 // 7494 /// ENST0000021603	XBP1	-1.68466	-2.0767
36	24733	AF130464 // SEC24D // SEC24 family, member D (S. cerevisiae) // 4q26 // 9871 /// AK2917	SEC24D	-1.57894	-1.96342
37	20554	AK299281 // BTBD3 // BTB (POZ) domain containing 3 // 20p12.2 // 22903 /// ENST00000254	BTBD3	-1.55008	-2.05834
38	30513	BC002660 // TMOD1 // tropomodulin 1 // 9q22.3 // 7111 /// ENST00000259365 // TMOD1 // t	TMOD1	-1.52537	-1.66137
39	28503	ENST00000313367 // OSBPL3 // oxysterol binding protein-like 3 // 7p15 // 26031 /// ENST	OSBPL3	-1.5228	-1.80363
40	22125	BC015026 // JOSD1 // Josephin domain containing 1 // 22q13.1 // 9929 /// ENST0000021603	JOSD1	-1.46992	-1.92819
41	13100	AK291721 // CD276 // CD276 molecule // 15q23-q24 // 80381 /// ENST00000318443 // CD276	CD276	-1.45467	-1.77488
42	27502	M14333 // FYN // FYN oncogene related to SRC, FGR, YES // 6q21 // 2534 ///	FYN	-1.44559	-1.68454

		ENST00000368			
43	21486	AF009039 // SYNJ1 // synaptojanin 1 // 21q22.2 // 8867 /// ENST00000382499 // SYNJ1 //	SYNJ1	-1.43387	-1.92248
44	13125	EF653821 // SNX33 // sorting nexin 33 // 15q24.2 // 257364 /// ENST00000308527 // SNX33	SNX33	-1.40268	-1.90385
45	24452	BC039540 // REL1 // RELT-like 1 // 4p14 // 768211 /// ENST00000314117 // REL1 // RELT	RELL1	-1.38026	-1.68682
46	15101	AK300584 // TNFAIP1 // tumor necrosis factor, alpha-induced protein 1 (endothelial) //	TNFAIP1	-1.3767	-1.78605
47	18746	AK290396 // ARHGAP25 // Rho GTPase activating protein 25 // 2p13.3 // 9938 /// AK297056	ARHGAP25	-1.34839	-1.54089
48	21157	AB004550 // B4GALT5 // UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide	B4GALT5	-1.3373	-1.59854
49	10655	AB209607 // SLC2A3 // solute carrier family 2 (facilitated glucose transporter), member	SLC2A3	-1.33586	-1.4728
50	18051	AK291466 // TSHZ3 // teashirt zinc finger homeobox 3 // 19q12 // 57616 /// ENST00000240	TSHZ3	-1.33225	-1.66953
51	5298	D38122 // FASLG // Fas ligand (TNF superfamily, member 6) // 1q23 // 356 /// ENST000003	FASLG	-1.3298	-1.73157
52	12611	BC005123 // SPTLC2 // serine palmitoyltransferase, long chain base subunit 2 // 14q24.3	SPTLC2	-1.31585	-1.41128
53	16621	BC014927 // PHLPP1 // PH domain and leucine rich repeat protein phosphatase 1 // 18q21.	PHLPP1	-1.29498	-1.45803
54	5010	BC116451 // BCL9 // B-cell CLL/lymphoma 9 // 1q21 // 607 /// ENST00000234739 // BCL9 //	BCL9	-1.28676	-1.36759
55	19447	AB058771 // ARMC9 // armadillo repeat containing 9 // 2q37.1 // 80210 /// AY219922 // A	ARMC9	-1.2482	-1.4451
56	11220	AF163324 // WSB2 // WD repeat and SOCS box containing 2 // 12q24.23 // 55884 /// AF2291	WSB2	-1.23277	-1.37082

57	15187	BC000591 // AATF // apoptosis antagonizing transcription factor // 17q12 // 26574 /// E	AATF	-1.13876	-1.16356
58	8552	AK290251 // STX3 // syntaxin 3 // 11q12.1 // 6809 /// AK297419 // STX3 // syntaxin 3 //	STX3	1.02997	1.14411
59	7942	BC034044 // CAMK2G // calcium/calmodulin-dependent protein kinase II gamma // 10q22 //	CAMK2G	1.0915	1.23204
60	5262	AB040946 // POGK // pogo transposable element with KRAB domain // 1q24.1 // 57645 /// E	POGK	1.12278	1.38229
61	16176	BC035609 // MTMR4 // myotubularin related protein 4 // 17q22-q23 // 9110 /// ENST000003	MTMR4	1.2065	1.43276
62	22512	AY221117 // SPATA12 // spermatogenesis associated 12 // 3p14.3 // 353324 /// ENST000003	SPATA12	1.23352	1.5721
63	27750	ENST00000223145 // GLCCI1 // glucocorticoid induced transcript 1 // 7p21.3 // 113263 //	GLCCI1	1.2636	1.50484
64	28820	AY642122 // ZNF394 // zinc finger protein 394 // 7q22.1 // 84124 /// BC017051 // ZNF394	ZNF394	1.3302	1.43708

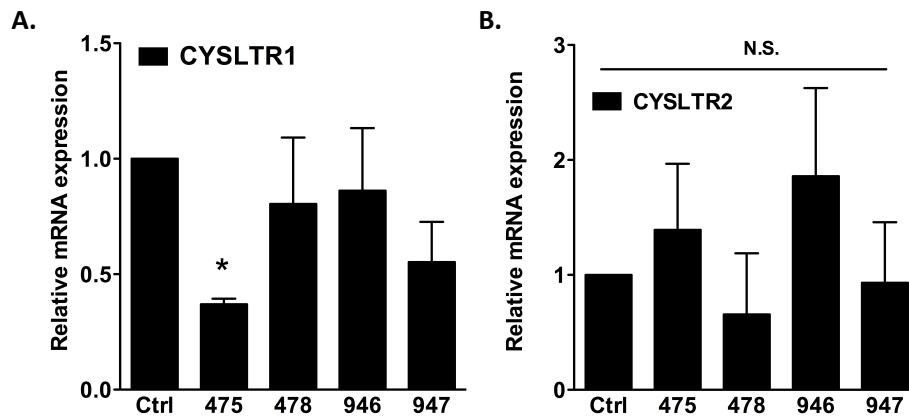
Table 2. Differentially expressed GPCRs in LAD2 cells compared to LUVA cells (ANOVA,  $p < 0.05$ , > than 2 fold difference). Orphan receptors selected for analysis are highlighted.

Gene	Description	Orphan?	Fold difference LAD2 vs. LUVA
ADORA3	Adenosine A3 receptor	No	2.27
ADRB2	Adrenergic $\beta 2$ receptor	No	2.39
ADRB3	Adrenergic $\beta 3$ receptor	No	6.52
CCR4	Chemokine receptor	No	4.16
CD97	Adhesion class receptor	No	2.87
CX3CR1	Chemokine receptor	No	4.76
CXCR3	Chemokine receptor	No	-3.60
CYSLTR1	Cysteinyl leukotriene receptor 1	No	4.32
DRD2	Dopamine receptor D2	No	4.90
EDNRB	Endothelin receptor type B	No	6.83
EMR2	Adhesion class receptor	No	4.50
GPR12	G-protein coupled receptor 12	Yes	-2.41
GPR37	G-protein coupled receptor 37	Yes	-2.05
<b>GPR65</b>	<b>G-protein coupled receptor 65</b>	<b>Yes</b>	<b>9.93</b>
GPR85	G-protein coupled receptor 85	Yes	2.94
GPR114	Adhesion class receptor	Yes	-2.14
GPR137B	G-protein coupled receptor 137B	Yes	4.12
GPR174	G-protein coupled receptor 174	Yes	-8.04
HRH4	Histamine H4 receptor	No	5.06
<b>MAS1L</b>	<b>MAS1 proto-oncogene like receptor</b>	<b>Yes</b>	<b>32.41</b>
MC1R	Melanocortin 1 receptor	No	2.02
<b>MRGPRX2</b>	<b>MAS-related GPR, member X2</b>	<b>Yes</b>	<b>70.23</b>
NPY2R	Neuropeptide Y2 receptor	No	2.30
P2RY8	Purinergic receptor P2Y, 8	Yes	-6.34
7916944	Prostaglandin E receptor 3	No	11.40
PTGER4	Prostaglandin E receptor 4	No	3.96
TPRA1	Transmembrane protein, adipocyte associated 1	No	2.52

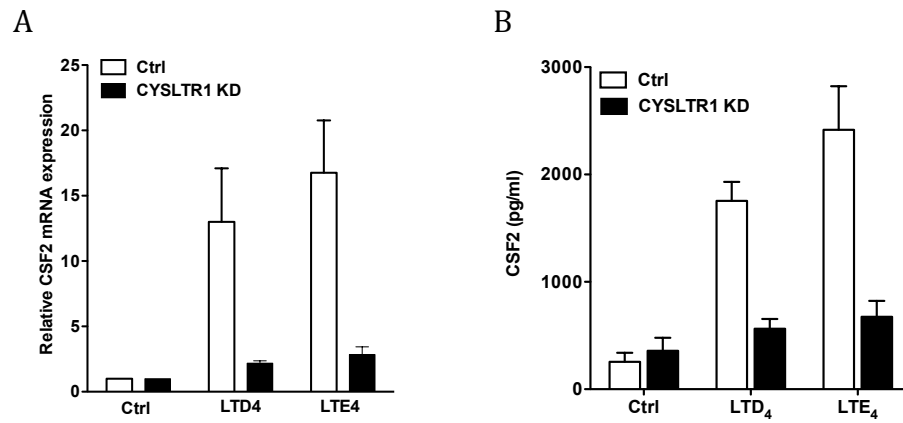


Supplementary Figure 1. The effect of inhibitors in HEK293T-CYSLTR1 and HEK293T-CYSLTR2 overexpression models. HEK293T cells were transiently transfected with human CYSLTR1 (A) or CYSLTR2 (B), preincubated with CYSLTR1 inhibitors (MK-571 (1  $\mu\text{mol/L}$ ), Montelukast (100 nmol/L)) or CYSLTR2 inhibitor (HAMI3379 (1  $\mu\text{mol/L}$ )) before stimulation with LTD<sub>4</sub> (100 nmol/L). Baseline corrected peak calcium mobilisation is presented as mean  $\pm$  SEM from 3 experiments run in triplicate. Relative fluorescence units (RFU).





Supplementary Figure 2. Four different shRNAs (475, 478, 946, 947) targeting CYSLTR1 were stably transduced into LAD2 cells and expression of CYSLTR1 (A) and CYSLTR2 (B) mRNA analysed using qRT-PCR for verification of successful gene knockdown. Mean  $\pm$  SEM of 3 experiments. \*  $p < 0.05$ , One way ANOVA with Bonferroni post test in comparison to control.



Supplementary Figure 3. CysLT<sub>1</sub> is required for LTE<sub>4</sub> induced CSF2 expression. Control and CYSLTR1 knocked down LAD2 cells were stimulated with vehicle control, LTD<sub>4</sub> or LTE<sub>4</sub> for 2 (mRNA) (A) or 6 hours (protein) (B) before analysis. Data expressed as fold difference in comparison to vehicle control for CSF2 mRNA and as CSF2 supernatant concentrations. Mean  $\pm$  SEM from 3 experiments.