Published in final edited form as:
Allergy. 2011 October ; 66(10): 1304–1311. doi:10.1111/j.1398-9995.2011.02647.x.

Cooperative and redundant signaling of leukotriene B_4 and leukotriene D_4 in human monocytes

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Abstract

Background—Leukotriene B_4 (LTB_4) and cysteinyl leukotrienes (cysLTs) are important immune mediators, often found concomitantly at sites of inflammation. Although, some of the leukotriene-mediated actions are distinctive (e.g. bronchial constriction for cysLTs), many activities such as leukocyte recruitment to tissues and amplification of inflammatory responses are shared by both classes of leukotrienes.

Objective—We used human monocytes to characterize leukotriene specific signaling, gene expression signatures and functions and to identify interactions between LTB_4 and cysLTs induced pathways.

Methods—Responsiveness to leukotrienes was assessed using oligonucleotide microarrays, real-time PCR, calcium mobilization, kinase activation and chemotaxis assays.

Results—Human monocytes were found to express mRNA for high- and low-affinity LTB_4 receptors, BLT_1 and BLT_2, but signal predominantly through BLT_1 in response to LTB_4 stimulation as shown using selective agonists, inhibitors and gene knock-down experiments. LTB_4 acting through BLT_1 coupled to G protein α inhibitory subunit activated calcium signaling, p44/42 mitogen-activated protein kinase, gene expression and chemotaxis. Twenty-seven genes, including immediate-early genes, transcription factors, cytokines and membrane receptors were significantly upregulated by LTB_4. LTB_4 and LTD_4 had similar effects on signaling, gene expression and chemotaxis indicating redundant cell activation pathways but co-stimulation with both lipid mediators was additive for many monocyte functions.

Conclusion—LTB_4 and LTD_4 display both redundant and cooperative effects on intracellular signaling, gene expression and chemotaxis in human monocytes. These findings suggest that therapies targeting either leukotriene alone may be less effective than approaches directed at both.

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None of the authors reports a conflict of interest related to material in this manuscript.

All authors participated either in the performance of experiments or in the interpretation of data presented in this manuscript.

Keywords

asthma; inflammation; monocytes; leukotrienes; receptors

Introduction

Leukotrienes are lipid mediators involved in the pathophysiology of many inflammatory diseases, such as bronchial asthma, allergic rhinitis, atherosclerosis and arthritis (1, 2). Two classes of leukotrienes are synthesized by stimulated leukocytes, LTB_4 and cysteinyl leukotrienes (cysLTs)(LTC_4, LTD_4, LTE_4). LTB_4 and CysLTs act by binding to distinct sets of specific G-protein coupled receptors (GPCRs) termed, BLT_1, BLT_2 and $CysLT_1$, $CysLT_2$, respectively (3). CysLTs have a clearly defined role in bronchial asthma and rhinitis, leading to airway constriction, increased vascular permeability and mucus secretion. They are also believed to play an important role in cell trafficking and innate immune responses (2). LTB_4 is characterized as a potent chemoattractant for myeloid cells (e.g. neutrophils) related to antibacterial responses and as the mediator associated with development of atherosclerosis and rheumatoid arthritis (4). However, there is evidence suggesting that LTB_4 may also play an important role in asthmatic inflammation, as increased levels of LTB_4 in sputum, plasma and bronchoalveolar lavage of asthmatic subjects and increased LTB_4 synthesis in macrophages and neutrophils from asthmatics have been reported (5–7). The LTB_4/BLT_1 pathway has also been shown to be essential for neutrophil and effector memory CD8+ cells recruitment into the lungs of allergen-induced airway inflammatory responses in mice (8, 9). In addition, production of leukotrienes is relatively resistant to corticosteroid treatment and in fact, corticosteroids were reported to increase BLT_1 expression on inflammatory cells, such as neutrophils and monocytes (10, 11).

Although some of the actions of cysLTs and LTB_4 are distinctive (e.g. bronchial contraction for cysLTs), many activities such as leukocyte chemotaxis and amplification of inflammatory responses are shared by both classes of leukotrienes. Many cells (monocytes/macrophages, mast cells, dendritic cells) when stimulated produce both, LTB_4 and cysLTs that activate specific receptors simultaneously in the same cell and may lead to parallel, independent receptor specific signaling or may activate similar intracellular signaling pathways. The inhibitory activities of leukotriene receptor antagonists have been studied in models analyzing receptor specific agonists (LTB_4/BLT_1; $LTD_4/CysLT_1$)(12, 13) but it is not known whether some common leukotriene-induced signaling pathways may be redundant when cells are exposed to both classes of leukotrienes in the presence of single receptor antagonists. The question whether co-stimulation of human cells with LTB_4 and cysLTs (as may often happen *in vivo*) may induce additive or synergistic signaling and functional effects in cells expressing LTB_4 and cysLT receptors has not been addressed.

We previously showed that $CysLT_1$ is the predominantly expressed cysLT receptor in human elutriated monocytes (14). As human monocytes express receptors for both cysLTs and LTB_4, in the current study we aimed to characterize the LTB_4 specific signaling, gene signature response and functions in human elutriated monocytes and to look for interactions between LTB_4 and LTD_4.

METHODS

Materials

LTB_4, LTD_4, 12 (S)-HETE, 12(S)-HHT, LY255283 (BLT_2 antagonist), montelukast and MK571($CysLT_1$ antagonists) (Cayman Chemical), pertussis toxin (Gαi/o inhibitor),

PD98059 (p44/42 MAPK inhibitor)(EMD Chemicals), DMSO (Sigma-Aldrich), anti-phospho-p44/42 and anti-phospho-p38 MAPK, anti- p44/42 and p38 antibodies (Cell Signaling) were obtained from the supplier. CP105696 (BLT_1 antagonist) was a generous gift from Pfizer (New York, NY).

Cell culture, calcium mobilization assay, chemotaxis assay and immunoblotting

Human elutriated monocytes from healthy donors were obtained through an institutional review board-approved protocol from the NIH Blood Bank (Bethesda, MD), cultured and calcium mobilisation, chemotaxis and immunoblotting were performed as previously described (14).

BLT_1 and BLT_2 knockdown

For siRNA knockdown experiments, Silencer Select pre-designed siRNA were used: 5'GGAAAAGGCUGUCUACAUUtt for BLT_1 and GCACAAUUGAAAACUUCAAtt for BLT_2. Silencer Select Negative Control siRNA (NC siRNA) (Ambion) were used as a negative control. Elutriated monocytes (5×10^6) were nucleofected with 4 μg of negative control and with either BLT_1 or BLT_2 specific siRNA using a Human Monocyte Nucleofector kit (Amaxa, Cologne, Germany) according to the manufacturer's protocol. After 24 hours, the media was replaced and the cells were used for calcium flux assay.

Microarray analysis

Total RNA was extracted from elutriated monocytes, processed and hybridized to Affymetrix HG U133 plus 2.0 arrays (Affymetrix) as previously reported (14). Data were normalized into S10 values using the MSCL Analyst's toolbox (P. J. Munson, GeneLogic Workshop of Low Level Analysis of Affymetrix GeneChip Data, 2001, software available at http://abs.cit.nih.gov/geneexpression.html. Genes with FDR values less than 10%, greater than 2 fold-change and greater than 50% present calls in one of the two treatment groups were termed significantly differentially expressed. Two-sided p-values for this test were reported as 1E-12 if their computed values was that or smaller. Raw data and CEL files were submitted to the National Center for Biotechnology Information Gene Expression Omnibus database (GSE24869).

Real-time PCR

mRNA expressions for selected genes were measured using real-time PCR performed on an ABI Prism 7900 sequence detection system (Applied Biosystems) using an RT kit and TaqMan Universal PCR master mix and commercially available probe and primer sets (Applied Biosystems): LTB4R- Hs00272624_s1, LTB4R2-Hs 00252658_s1, CYSLTR1-Hs00272624_s1, CYSLTR2-Hs00252658_s1, GPR17- Hs00171137_m1, FOSB-Hs00171851_m1, and EGR2- Hs00166165_m1. Relative gene expression was normalized to GAPDH transcripts and calculated as fold change compared with control transcripts.

RESULTS

LTB_4 induces gene expression in human monocytes

To determine whether LTB_4 can affect gene expression in human monocytes and globally identify LTB_4 target genes, elutriated monocytes from 5 healthy donors were stimulated with LTB_4 (50 nmol/L) or vehicle control (ethanol) for 30 minutes and analyzed with microarrays. LTB_4 stimulation significantly up-regulated 27 genes by 2 fold or more (Table 1).

LTB_4 signals through the BLT_1 in monocytes

$CysLT_1$, BLT_1, and BLT_2 were the three receptors predominantly expressed at the mRNA level in monocytes, while low levels of $CysLT_2$ mRNA and no mRNA for GPR17 (a putative cysLT receptor (15)) were found (Figure 1A). LTB_4-induced calcium flux was inhibited by the BLT_1 selective antagonist, CP105696 (Figure 1B). BLT_2-induced signaling by its specific, other than LTB_4, agonists: 12-HHT and 12-HETE (16, 17) was not detected (Figure 1C). Similarly, the BLT_2 antagonist, LY255283, did not inhibit LTB_4-induced calcium flux (Figure 1D). Further, siRNA was used to knock down either BLT_1 or BLT_2 expression. BLT_1 and BLT_2 siRNAs decreased their respective transcripts by 88±3.7% and 59±4.2% comparred to cells treated with NC siRNA (TaqMan; n=3, $p < 0.05$). BLT_1 down-regulation caused more than 50 % decrease in LTB_4 induced calcium flux whereas the BLT_2 knock-down cells showed no inhibition of response to LTB_4 (Figure 1E). These data suggest that the BLT_1 is the major functional LTB_4 receptor expressed in human monocytes.

LTD_4 augments the LTB_4-induced calcium flux

LTB_4 induced calcium mobilization with a half maximal effective concentration (EC_{50}) value of 1.17 nmol/L and the response reached plateau at 30 nM (Figure 2A). LTD_4 induced calcium flux with an EC_{50} value of 2.12 nmol/L and reached maximum effect at 100 nM. A significantly augmented calcium flux was observed in response to LTB_4/LTD_4 costimulation in comparison with LTB_4 and LTD_4 alone, with a lower EC_{50} value of 0.84 nmol/L.

As shown in Figure 2D, the LTD_4-induced calcium flux was completely inhibited by pretreatment with montelukast, but was not inhibited by CP105696. Similarly, the LTB_4-induced calcium flux was not inhibited by montelukast, but abolished by CP105696 (Figure 2E). Both inhibitors together completely inhibited the effect of both mediators together (Figure 2F). Stimulation with LTB_4 (Figure 2B) potently desensitized monocytes to subsequent stimulation with the same concentration of LTB_4 (as expected for homologous desensitization), but it did not have any effect on subsequent LTD_4 exposure. Similarly LTD_4 desensitized to subsequent stimulation with LTD_4 but not to LTB_4 (Figure 2C), confirming that LTB_4 and LTD_4 signal through separate receptors and independent pathways (BLT_1 and $CysLT_1$).

LTB_4 and LTD_4 signal in additive way through $G\alpha_{i/o}$-coupled BLT_1 and p44/42 MAPK in monocytes

LTB_4-induced calcium mobilization was fully inhibited by pertussis toxin (Ptx) pretreatment (Figure 3A) while residual calcium flux (less than 30 %) in response to LTD_4 was observed. We further tested the Ptx effect on combined LTB_4 and LTD_4 induced calcium flux and found more than 75 % inhibition of calcium flux by Ptx (Figure 3B), with the residual calcium mobilization contributed by LTD_4 signaling via its $CysLT_1$ receptor (Figure 3A). We then analyzed MAPK kinase activation. LTB_4 or LTD_4 induction of p44/42 phosphorylation was observed in 30 seconds and returned to baseline in 5 minutes (Figure 3C and 3D). The LTB_4 induced phosphorylation of p44/42 was abrogated by pretreatment with CP105696 (Figure 4E). An additive effect on p44/42 MAPK phosphorylation was observed when cells were stimulated with LTB_4 and LTD_4 together (Figure 4F) that was inhibited by Ptx pretreatment. While LTD_4 induction of p38 phosphorylation has been previously reported,(14) we detected no consistent p38 phosphorylation in response to LTB_4 stimulation (data not shown).

The additive effect of LTB_4 and LTD_4 co-stimulation on gene expression

We validated the array expression data for 2 immediate early genes (IEG) (*FOSB* and *EGR2*) using TaqMan real time PCR. *EGR2* and *FOSB* were induced by LTB_4 treatment and this effect was inhibited by CP105696 but not by the BLT_2 antagonist, LY255283 (Figure 4A). Figure 4B demonstrates the additive effect of LTB_4 (50 nmol/L) and LTD_4 (100 nmol/L) on *EGR2* and *FOSB* gene expression. The combined effect of LTD_4 and LTB_4 stimulation was partially inhibited by pretreatment with the BLT_1 and $CysLT_1$ antagonists, CP105696 and MK571, respectively (Figure 4B). The effect of LTD_4 and LTB_4 was substantially inhibited by PD98059 and completely inhibited by Ptx (Figure 4C), again suggesting similar but separate signaling pathways leading to immediate early gene expression.

LTD_4 augments LTB_4 induced chemotaxis of monocytes

A significant chemotactic activity in response to LTB_4, inhibited by the BLT_1 inhibitor, CP105696 (Figure 5A–B), was observed. LTD_4 also induced chemotactic response that was abrogated by MK571 (Figure 6A). Treatment of cells with LTB_4 and LTD_4 induced an additive effect (Figure 5A, C) that was only partially inhibited by MK571 and fully inhibited by Ptx pretreatment, showing that chemotaxis of monocytes in response to leukotrienes is mediated through $Ga_{i/o}$ coupled receptor activation.

Discussion

We report here a description of LTB_4 mediated signaling, target gene induction and chemotaxis in human monocytes, followed by an analysis of the cooperative and redundant responses induced by the co-administration of LTB_4 and LTD_4. In our model of human elutriated monocytes, LTB_4 stimulation induced calcium mobilization, p44/42 MAPK phosphorylation, gene expression and chemotaxis, showing that LTB_4 is a potent stimulus for monocyte activation. Potent inhibition of LTB_4 induced calcium flux, MAPK kinase activation, immediate-early gene expression as well as chemotaxis was observed with CP105696 treatment indicating that BLT_1 is responsible for all studied LTB_4-induced activities, further confirmed by BLT_1 and BLT_2 selective knock down experiments. This is in agreement with previous studies showing that LTB_4 induced MCP-1 production (12) and LTB_4 triggered adhesion of monocytes to endothelium (18) were inhibited by the BLT_1 antagonist, CP105696.

We provide here evidence in human monocytes that LTB_4 stimulates BLT_1 coupled to pertussis toxin (Ptx) sensitive $G\alpha_{i/o}$ causing intracellular calcium mobilization, p44/42 kinase activation, gene expression and chemotaxis. Our data in monocytes confirm early observations of predominant $G\alpha_{i/o}$ coupling of human BLT_1 observed in neutrophils (19) and differ compared to models of heterologously overexpressed BLT_1 where Ptx sensitive and insensitive G protein coupling was described, (20) underlining the importance of studying primary human cells.

We show for the first time in monocytes that LTB_4 induced the expression of 27 genes, belonging to families of immediate early genes, transcription activators, cytokines and membrane receptors. Several genes induced by LTB_4 in monocytes are associated with acute phase immune responses, e.g. IL-1β, TNFα and CCL3, consistent with the role of LTB_4 as an important mediator of innate immune responses. The role of identified transcription factors in regulation of gene expression in human monocytes has not been well studied. Egr genes are involved in monocyte activation and differentiation (21). Egr proteins have also been shown to interact with other transcription factors such as NF-κB and thereby modulate the transcription of genes encoding inflammatory cytokines (22). On the other hand, Egr2

may be involved in the negative regulation of T-cell proliferation and inflammation (23). Previous studies suggested that LTB_4 might induce MCP-1 (CCL2) and IL-6 in monocytes (12, 24). Our data confirm that CCL2 was also up-regulated (1.9 fold), but it did not cross the 2 fold threshold chosen for gene selection, while *IL-6* gene was not significantly changed. However, others studying changes in gene expression used 6–12 hours of LTB_4 stimulation. The induction of *EGR2* and *FOSB* mRNA by LTB_4 was only partially inhibited by the BLT_1 antagonist, CP105696, not affected by the BLT_2 antagonist, LY255283, but fully abolished by Ptx pretreatment (Figure 4), suggesting that LTB_4 might regulate gene expression in monocytes acting also through a different than BLT_1 or BLT_2, Ptx-sensitive receptor.

We compared our data with early gene signatures generated for LTD_4 in monocytes and for LTD_4 and thrombin in human umbilical vein endothelial cells (HUVECs) (14, 25). A similar set of genes was up-regulated in all these studies, including genes such as *FOSB, EGR2, EGR3, NR4A2* and *ATF3*. This is an interesting observation as in each case different GPCRs were involved in cell activation, namely BLT_1, $CysLT_1$, $CysLT_2$ and PAR_1 for LTB_4 and LTD_4 activated monocytes and LTD_4 and thrombin activated HUVECs, respectively. This suggests that signals from distinct GPCR subfamily members activate similar gene expression programs in response to ligands formed concomitantly *in vivo* at an inflammatory site. This implies the existence of an additional level of regulation that controls cell responsiveness and perhaps requires multiple different GPCRs to signal simultaneously in order to reach a combined threshold to fully activate the cell. Such a regulatory mechanism would be responsible for keeping cells non- or weakly responsive to single mediator(s) until a set of activating stimuli reach a threshold required for full cell activation. In support of this hypothesis, we observed an additive, enhanced gene expression in response to LTB_4 and LTD_4 costimulation and a similar enhancing effect on gene expression was reported by Uzonyi et al. in HUVECs stimulated with LTD_4 and thrombin (25). As most GPCRs become quickly desensitized to subsequent stimulation with the same agonist (as we showed here for LTB_4 and LTD_4), it is possible that only simultaneous activation of many different GPCRs by different mediators could lead to a functional activation of a particular cell *in vivo*. Such an additive effect of LTB_4 and LTD_4 costimulation was not only observed for gene expression, but was also seen at the level of signaling (calcium flux and p44/42 phosphorylation) and monocyte chemotaxis. The mechanism of this additive effect is not clear. Both agonists signal in parallel through specific receptors, as selective antagonists for BLT_1 and $CysLT_1$ affected only the receptor-specific part of the response. If these signaling pathways were completely nonredundant, one might expect a synergistic effect triggered by both agonists. A partial additive effect was observed in our study and no synergism, demonstrating redundancy in intracellular signaling induced by LTB_4 and LTD_4 in monocytes.

This observation may have important clinical implications. First, *in vivo* when multiple mediators are present at a site of inflammation, activation and functions of cells expressing BLT_1 and $CysLT_1$ might not be effectively inhibited by selective receptor antagonists as as these receptors have redundant functions. This finding could be relevant for choosing treatment strategies for diseases where enhanced synthesis of both leukotrienes have been identified, such as asthma, rhinitis and atherosclerosis. Blocking both receptors may be more effective than blocking either alone. Second, the observed additive effect of LTB_4/LTD_4 costimulation if clinically relevant, may favor leukotriene synthesis inhibitors (5-lipoxygenase or FLAP inhibitors) over antagonists of single leukotriene pathways as a therapeutic strategy for allergy and asthma, but properly designed clinical trials are needed to fully verify such a hypothesis.

Acknowledgments

This research was supported by the Intramural Research Program of the Clinical Center, NIH.

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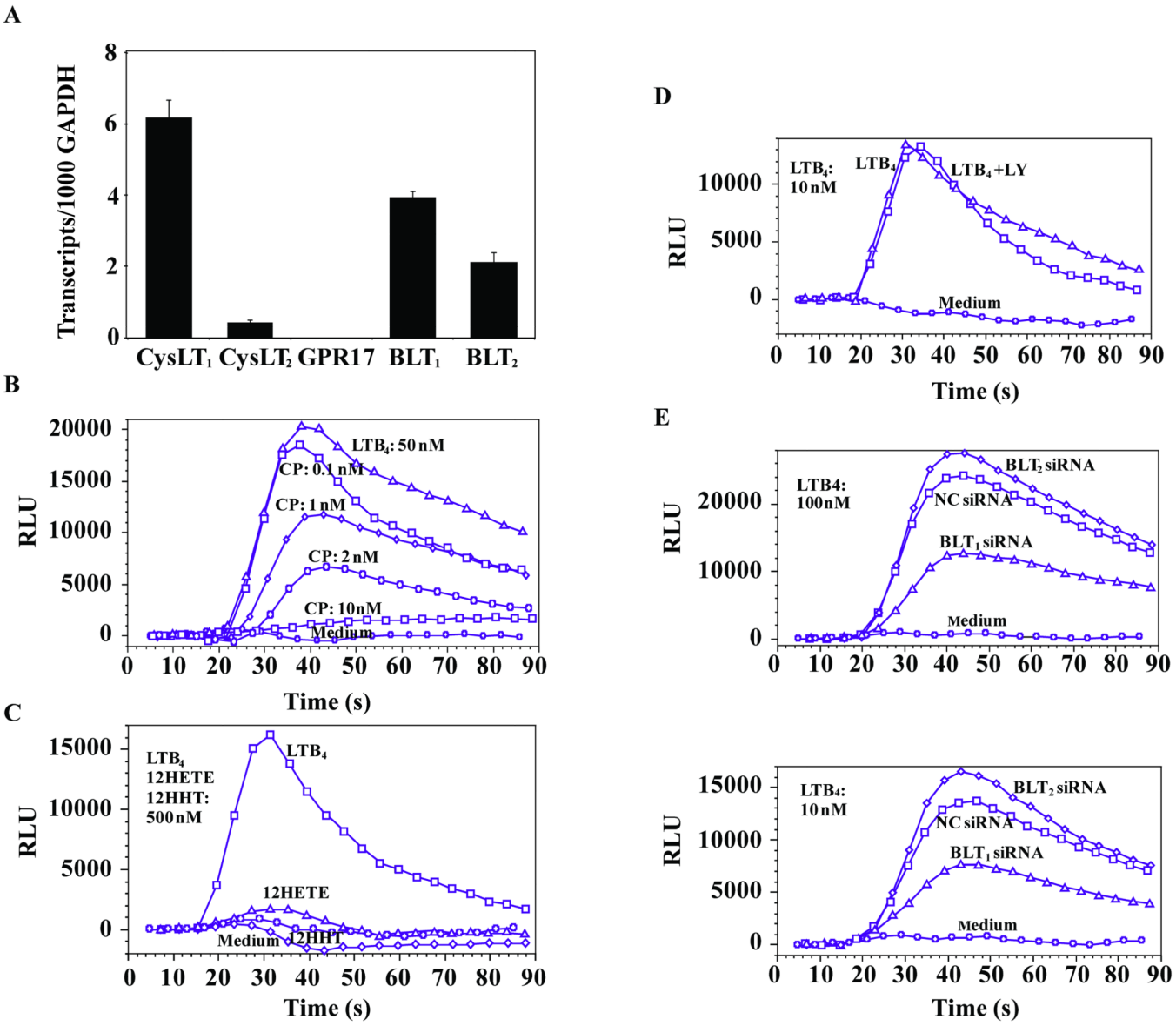

Figure 1.

BLT_1 is the major receptor mediating LTB_4-induced calcium mobilization in human monocytes. (A) The relative mRNA expression of $CysLT_1$, $CysLT_2$, GPR17, BLT_1 and BLT_2 measured by TaqMan is expressed per thousand copies of the control GAPDH gene and presented as mean values ± SD of 3 healthy donors. Monocytes were stimulated with LTB_4 (50 nmol/L) in the presence of increasing concentrations of CP105696 (B), or LTB_4, 12-HETE, 12-HHT (500 nmol/L) (C) or LTB_4 in presence of LY255283 (200 nmol/L) (D) or vehicle control (10 min pretreatment). (E) Monocytes were nucleofected with BLT_1 or BLT_2 siRNA or negative control oligonucleotides (NC siRNA) as described in the Methods section, cultured for 24 hours and calcium flux was measured in response to LTB_4 (100 or 10 nmol/L). Results of calcium flux experiments are shown as relative light units (RLU) from the FLEX 3 station. For the calcium flux experiments, the data are from one of three separate experiments each with similar results.

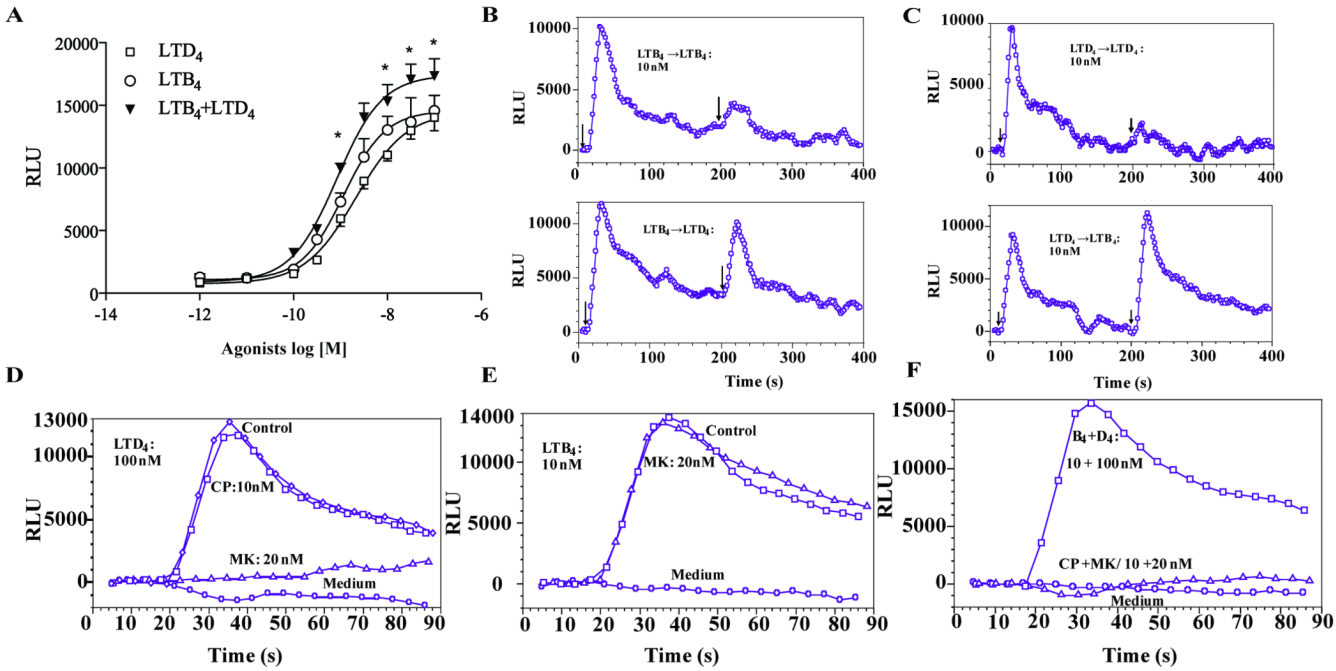

Figure 2.

LTB_4 and LTD_4 act via specific receptors to induce calcium in an additive manner. Monocytes were stimulated with different concentrations of LTB_4, LTD_4 or LTB_4+LTD_4 and calcium release was measured, as indicated in Methods (Figure 2A). Data presented as means ± SEM from 3–6 separate experiments each performed in triplicate. *$p<0.05$, analyzed by ANOVA. Representative traces of calcium flux induced by repeated exposure to LTD_4 (10 nmol/L) and LTB_4 (10 nmol/L) in monocytes (B and C). Monocytes were pretreated with MK (20 nmol/L) or CP105696 (10 nmol/L) for 20 min and stimulated with LTD_4 (100 nmol/L) (D), or LTB_4 (10 nmol/L) (E). The effect of treatment with both LTB_4 and LTD_4 was completely inhibited by pretreatment with both inhibitors (F). Data from one of three separate experiments, each with similar results, are shown.

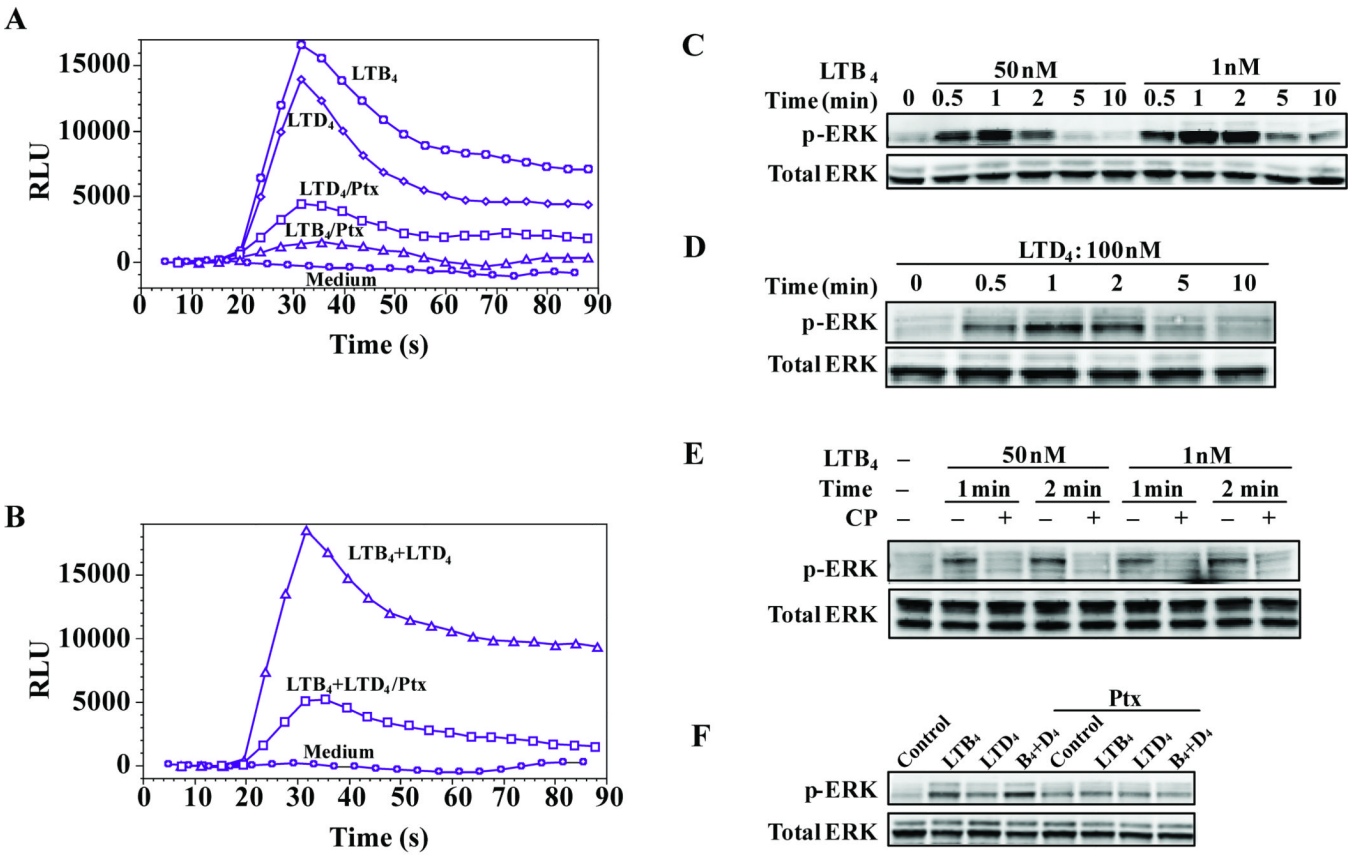

Figure 3.

LTB_4 and LTD_4 signal through $G\alpha_{i/o}$-coupled BLT_1 and p44/42 MAPK in monocytes. Monocytes were pretreated with pertussis toxin (100 ng/mL) overnight and then stimulated with LTB_4 (50 nmol/L) or LTD_4 (100 nmol/L) (A), or stimulated with LTB_4 and LTD_4 together (B). Monocytes were stimulated with LTB_4 (C) or LTD_4 (D) at different time points and phosphorylation of p44/42 was assayed by immunoblotting. (E) Cells were preincubated with CP105696 (10 nmol/L) for 20 min and stimulated with LTB_4 for 1 or 2 min. (F) Cells were preincubated with pertussis toxin (100 ng/mL) overnight and stimulated with LTB_4 (50 nmol/L), LTD_4 (100 nmol/L) or LTB_4 and LTD_4 for 2 min. Data are representative of three independent experiments.

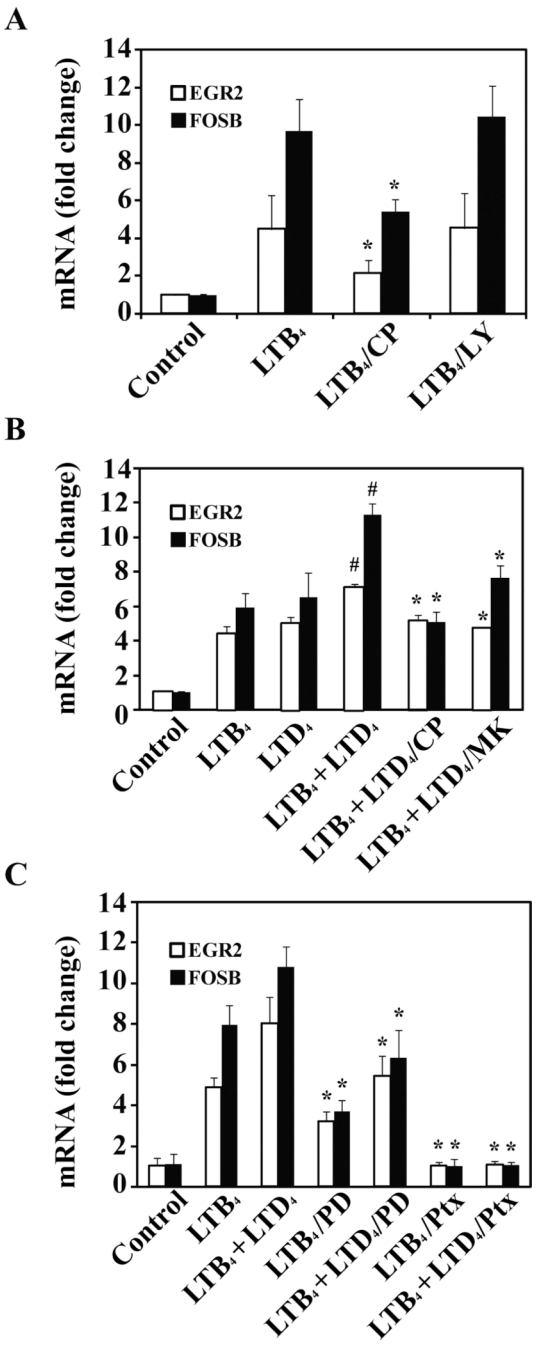

Figure 4.

The additive effect of LTB_4 and LTD_4 co-stimulation on gene expression. (A) Monocytes were preincubated with CP105696 (10 nmol/L), or LY255283 (200 nmol/L) or vehicle for 20 min and stimulated with LTB_4 (50 nmol/L) for 30 min. (B) Cells were pretreated with CP105696 (10 nmol/L), or MK571 (100 nmol/L) or vehicle for 20 min and then stimulated with LTB_4 (50 nmol/L), LTD4 or both for 30 minutes. (C) Cells were pretreated with PD98059 (10 μmol/L) for 1 hr or pertussis toxin (100 ng/mL) overnight and stimulated with LTB_4 (50 nmol/L) or LTB_4 and LTD_4 (50 nmol/L, 100 nmol/L) for 30 min. After the treatment, total RNA was extracted and subjected to TagMan analysis. Data are shown as a fold change in comparison with vehicle control treated cells. Mean ± SD from 3 experiments

done in triplicate. *$p < 0.05$ for agonist(s) plus inhibitor comparred to agonist(s) alone; #$p<0.01$ for LTB_4+LTD_4 comparred to LTB_4 or LTD_4 alone.

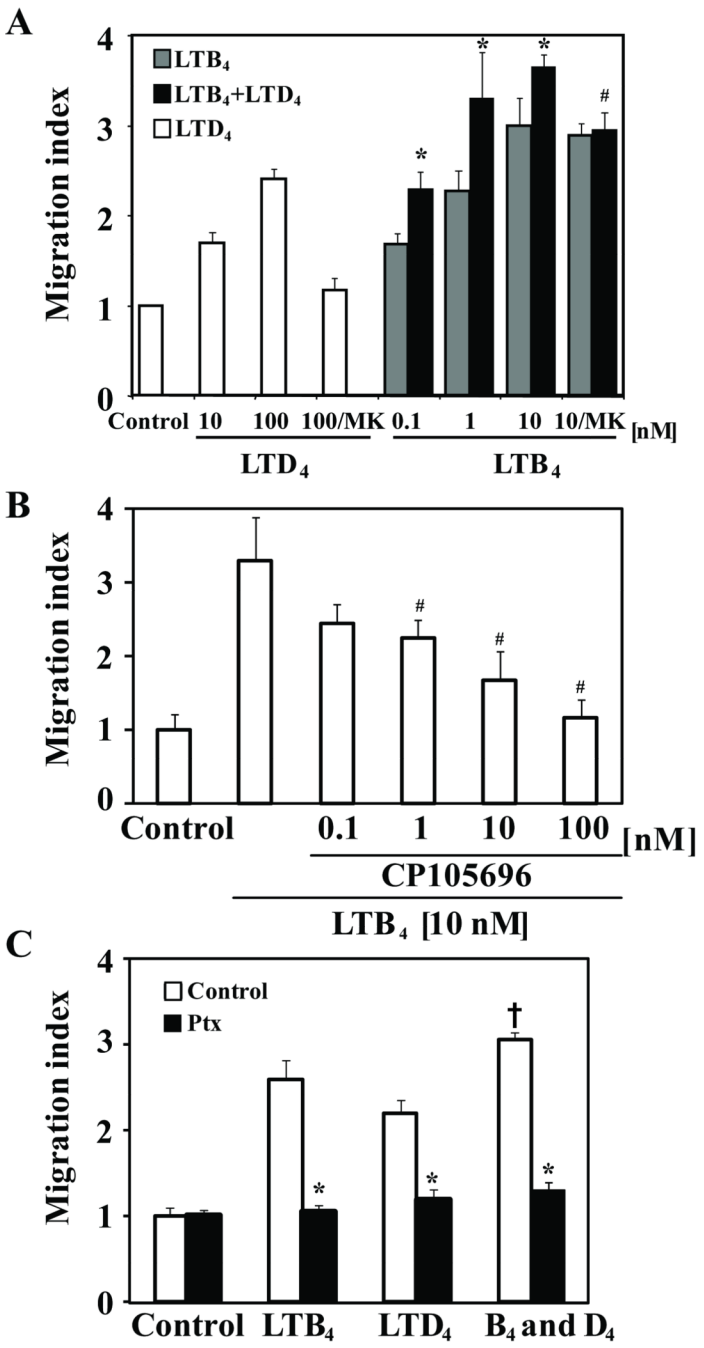

Figure 5.

LTD_4 augments LTB_4 induced chemotaxis of monocytes. (A) Chemotactic activity of monocytes to different concentrations of or LTB_4, LTD_4 or LTB_4 and LTD_4 (100 nmol/L) in the presence or absence of MK571 (MK; 100 nmol/L). *$p < 0.05$ for LTB_4+LTD_4 comparred to LTB_4 alone. #$p<0.05$ for LTB_4+LTD_4 comparred to LTB_4+LTD_4 plus MK571. (B) Cells were preincubated with different concentrations of CP105695 for 20 min and stimulated with LTB_4 (10 nmol/L). #$p<0.05$ for LTB_4 comparred to LTB_4 plus CP105696. (C) Cells were preincubated with pertussis toxin (100 ng/mL) overnight and stimulated with LTB_4 (10 nmol/L), LTD_4 (100 nml/L) or LTB_4 and LTD_4. *$p < 0.05$ for agonist(s) plus Ptx comparred to agonist(s) alone. +$p<0.05$ comparing LTB_4 and LTD_4 to

LTB_4 alone. Data from 3 different donors are presented as the migration index in comparison with vehicle control (mean ±SD).

TABLE I

Elutriated monocytes from 5 separate donors were stimulated with or without LTB_4 (50 nmol/L) for 30 minutes and analyzed by microarray as described in the Methods section. Values are mean +/− standard deviation (SD).

	Gene name	Fold Change (±SD)	P value for 2-sided consistency test
Cytokines			
IL1B	interlukin 1 β	2.6 (0.18)	8.14E-10
CCL3	chemokine (C-C motif) ligand 3	2.2 (0.08)	2.43E-10
TNF	tumor necrosis factor	2.0 (0.05)	3.36E-11
Membrane			
GPR109B	G protein-coupled receptor 109B	2.7 (0.09)	1.01E-12
CD83	CD83 molecule	2.1 (0.12)	8.20E-09
THBD	thrombomodulin	2.1 (0.08)	1.13E-09
CD44	CD44 molecule	2.0 (0.11)	1.57E-06
FCAR	Fc fragment of IgA receptor f	2.0 (0.03)	1.29E-11
Signal Transduction			
S100A10	S100 calcium binding protein A	2.4 (0.08)	1.68E-12
NEDD9	neural precursor cell express 9	2.2 (0.09)	8.43E-09
PSCDBP	pleckstrin homology Sec7	2.0 (0.05)	5.73E-10
Transcriptional Activation			
FOSB	FBJ murine osteosarcoma viral	12.2 (0.19)	1.00E-12
EGR3	early growth response 3	8.6 (0.11)	1.00E-12
EGR2	early growth response 2	5.4 (0.15)	1.00E-12
NR4A1	nuclear receptor subfamily 4	4.2 (0.22)	1.00E-12
NR4A2	nuclear receptor subfamily 4	3.7 (0.20)	1.00E-12
ATF3	activating transcription factor 3	2.8 (0.05)	1.00E-12
KLF10	Kruppel-like factor 10	2.6 (0.07)	1.00E-12
EGR1	early growth response 1	2.2 (0.24)	0.00055873
KLF4	Kruppel-like factor 4	2.2 (0.08)	3.23E-12
Metabolism			
AHR	aryl hydrocarbon receptor	2.0 (0.06)	2.76E-09
Cell Cycle and Apoptosis			
PHLDA1	pleckstrin homology-like domain A1	4.1 (0.10)	1.00E-12
RGC32	response gene to complement 32	2.3 (0.05)	1.24E-12
MCL1	myeloid cell leukemia sequence	2.9 (0.09)	1.00E-12
Other			
	transcribed locus	4.2 (0.16)	1.00E-12
LOC284454	hypothetical protein LOC284454	2.6 (0.14)	9.55E-10

	Gene name	Fold Change (±SD)	P value for 2-sided consistency test
TRIB1	tribbles homolog 1 (Drosophila) and LNRF1	2.3 (0.08)	4.13E-12
LONRF1	LON peptidase N-terminal domain	2.2 (0.04)	1.53E-12