

Cooperative and redundant signaling of leukotriene B₄ and leukotriene D₄ in human monocytes

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Abstract

Background: Leukotriene B₄ (LTB₄) 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 LTB₄- 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 LTB₄ receptors, BLT₁ and BLT₂, but signal predominantly through BLT₁ in response to LTB₄ stimulation as shown using selective agonists, inhibitors, and gene knock down experiments. LTB₄ acting through BLT₁ 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 (IEG), transcription factors, cytokines, and membrane receptors were significantly up-regulated by LTB₄. LTB₄ and LTD₄ had similar effects on signaling, gene expression, and chemotaxis indicating redundant cell activation pathways but costimulation with both lipid mediators was additive for many monocyte functions.

Conclusion: Leukotriene B₄ and LTD₄ 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.

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, Leukotriene B₄ (LTB₄), and cysteinyl leukotrienes (cysLTs) (LTC₄, LTD₄, LTE₄). LTB₄ and cysLTs act by

binding to distinct sets of specific G-protein-coupled receptors (GPCRs) termed, BLT₁, BLT₂ and CysLT₁, CysLT₂, respectively (3). Cysteinyl leukotrienes 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). LTB₄ 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 LTB₄ may also play an important role in asthmatic inflammation, as increased levels of LTB₄ in sputum, plasma, and bronchoalveolar lavage of asthmatic subjects and increased LTB₄ synthesis in macrophages and neutrophils from asthmatics have been reported (5–7). The LTB₄/BLT₁ 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 BLT₁ expression on inflammatory cells, such as neutrophils and monocytes (10, 11).

Although some of the actions of cysLTs and LTB₄ 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 LTB₄ 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 (LTB₄/BLT₁; LTD₄/CysLT₁) (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 costimulation of human cells with LTB₄ and cysLTs (as may often happen *in vivo*) may induce additive or synergistic signaling and functional effects in cells expressing LTB₄ and cysLT receptors has not been addressed.

We previously showed that CysLT₁ is the Predominantly expressed cysLT receptor in human elutriated monocytes (14). As human monocytes express receptors for both cysLTs and LTB₄, in the current study, we aimed to characterize the LTB₄-specific signaling, gene signature response, and functions in human elutriated monocytes and to look for interactions between LTB₄ and LTD₄.

Methods

Materials

Leukotriene B₄, LTD₄, 12 (S)-HETE, 12(S)-HHT, LY255283 (BLT₂ antagonist), montelukast and MK571 (CysLT₁ antagonists) (Cayman Chemical, Ann Arbor, MI, USA), pertussis toxin (Ptx) (Gai/o inhibitor), PD98059 (p44/42

MAPK inhibitor) (EMD Chemicals, Rockland, MA, USA), DMSO (Sigma-Aldrich, St. Louis, MO, USA), anti-phospho-p44/42 and anti-phospho-p38 MAPK, and anti-p44/42 and anti-p38 antibodies (Cell Signaling, Beverly, MA, USA) were obtained from the supplier. CP105696 (BLT₁ antagonist) was a generous gift from Pfizer (New York, NY, USA).

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, USA) and cultured, and calcium mobilization, chemotaxis, and immunoblotting were performed as previously described (14).

BLT₁ and BLT₂ knockdown

For siRNA knock down experiments, Silencer Select Pre-designed siRNA were used: 5'GGAAAAGGCUGUCUA CAUUt for BLT₁ and GCACAAUUGAAAACUUCAAtt for BLT₂. Silencer Select Negative Control siRNA (NC siRNA) (Ambion, Austin, TX, USA) were used as a negative control. Elutriated monocytes (5 × 10⁶) were nucleofected with 4 μg of negative control and with either BLT₁- or BLT₂-specific siRNA using a Human Monocyte Nucleofector kit (Amaxa, Cologne, Germany) according to the manufacturer's protocol. After 24 h, the media were 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, Santa Clara, CA, USA) 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 < 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, Foster City, CA, USA) using an RT kit and TaqMan Universal PCR master mix and commercially available probe and primer sets (Applied Biosystems): LTB4R-Hs00272624_s1, LTB4R2-Hs00252658_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

Leukotriene B₄ induces gene expression in human monocytes

To determine whether LTB₄ can affect gene expression in human monocytes and globally identify LTB₄ target genes, elutriated monocytes from five healthy donors were stimulated with LTB₄ (50 nM) or vehicle control (ethanol) for 30 min and analyzed with microarrays. LTB₄ stimulation significantly up-regulated 27 genes by 2-fold or more (Table 1).

Leukotriene B₄ signals through the BLT₁ in monocytes

CysLT₁, BLT₁, and BLT₂ were the three receptors predominantly expressed at the mRNA level in monocytes, while

low levels of CysLT₂ mRNA and no mRNA for GPR17 (a putative cysLT receptor (15)) were found (Fig. 1A). LTB₄-induced calcium flux was inhibited by the BLT₁ selective antagonist, CPI05696 (Fig. 1B). BLT₂-induced signaling by its specific, other than LTB₄, agonists: 12-HHT and 12-HETE (16, 17) was not detected (Fig. 1C). Similarly, the BLT₂ antagonist, LY255283, did not inhibit LTB₄-induced calcium flux (Fig. 1D). Further, siRNA was used to knock-down either BLT₁ or BLT₂ expression. BLT₁ and BLT₂ siRNAs decreased their respective transcripts by $88 \pm 3.7\%$ and $59 \pm 4.2\%$ compared to cells treated with NC siRNA (TaqMan; $n=3$, $P < 0.05$). BLT₁ down-regulation caused more than 50% decrease in LTB₄-induced calcium flux, whereas the BLT₂ knock down cells showed no inhibition of response to LTB₄ (Fig. 1E). These data suggest that the BLT₁ is the major functional LTB₄ receptor expressed in human monocytes.

Table 1 Genes up regulated by LTB₄ in human monocytes

	Gene name	Fold change ($\pm$ SD)	P value for two-sided consistency test
Cytokines			
	Interleukin 1 β	2.6 (0.18)	8.14E-10
	Chemokine (C-C motif) ligand 3	2.2 (0.08)	2.43E-10
	Tumor necrosis factor	2.0 (0.05)	3.36E-11
Membrane			
	G protein-coupled receptor 109B	2.7 (0.09)	1.01E-12
	CD83 molecule	2.1 (0.12)	8.20E-09
	Thrombomodulin	2.1 (0.08)	1.13E-09
	CD44 molecule	2.0 (0.11)	1.57E-06
	Fc fragment of IgA receptor f	2.0 (0.03)	1.29E-11
Signal transduction			
	S100 calcium binding protein A	2.4 (0.08)	1.68E-12
	Neural precursor cell express 9	2.2 (0.09)	8.43E-09
	Pleckstrin homology Sec7	2.0 (0.05)	5.73E-10
Transcriptional activation			
	FBJ murine osteosarcoma viral	12.2 (0.19)	1.00E-12
	Early growth response 3	8.6 (0.11)	1.00E-12
	Early growth response 2	5.4 (0.15)	1.00E-12
	Nuclear receptor subfamily 4	4.2 (0.22)	1.00E-12
	Nuclear receptor subfamily 4	3.7 (0.20)	1.00E-12
	Activating transcription factor 3	2.8 (0.05)	1.00E-12
	Kruppel-like factor 10	2.6 (0.07)	1.00E-12
	Early growth response 1	2.2 (0.24)	0.00055873
	Kruppel-like factor 4	2.2 (0.08)	3.23E-12
Metabolism			
	Aryl hydrocarbon receptor	2.0 (0.06)	2.76E-09
Cell cycle and apoptosis			
	Pleckstrin homology-like domain A1	4.1 (0.10)	1.00E-12
	Response gene to complement 32	2.3 (0.05)	1.24E-12
	Myeloid cell leukemia sequence	2.9 (0.09)	1.00E-12
Other			
	Transcribed locus	4.2 (0.16)	1.00E-12
	Hypothetical protein LOC284454	2.6 (0.14)	9.55E-10
	Tribbles homolog 1 (Drosophila) and LNRF1	2.3 (0.08)	4.13E-12
	LON peptidase N-terminal domain	2.2 (0.04)	1.53E-12

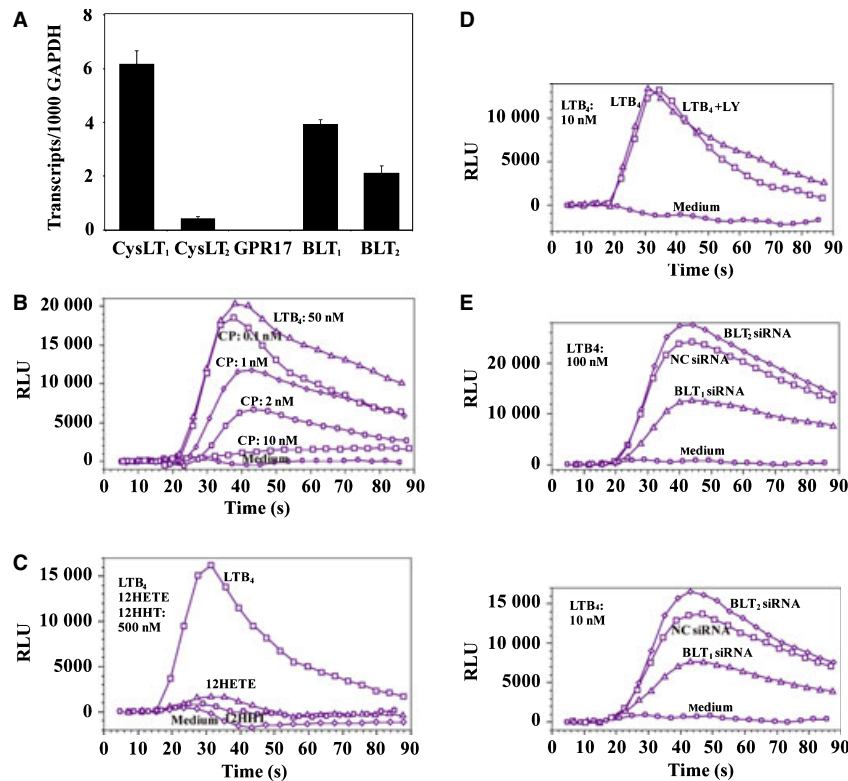


Figure 1 BLT₁ is the major receptor mediating LTB₄-induced calcium mobilization in human monocytes. (A) The relative mRNA expression of CysLT₁, CysLT₂, GPR17, BLT₁, and BLT₂ measured by TaqMan is expressed per thousand copies of the control *GAPDH* gene and presented as mean values $\pm$ SD of 3 healthy donors. Monocytes were stimulated with LTB₄ (50 nM) in the presence of increasing concentrations of CP105696 (B), or LTB₄, 12-HETE, 12-HHT (500 nM) (C) or LTB₄ in the presence of LY255283 (200 nM)

(D) or vehicle control (10 min pretreatment). (E) Monocytes were nucleofected with BLT₁ or BLT₂ siRNA or negative control oligonucleotides (NC siRNA) as described in the Methods section, cultured for 24 h and calcium flux was measured in response to LTB₄ (100 or 10 nM). 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.

Leukotriene D₄ augments the Leukotriene B₄-induced calcium flux

Leukotriene B₄-induced calcium mobilization with a half maximal effective concentration (EC₅₀) value of 1.17 nM/L and the response reached plateau at 30 nM (Fig. 2A). LTD₄-induced calcium flux with an EC₅₀ value of 2.12 nM/L and reached maximum effect at 100 nM. A significantly augmented calcium flux was observed in response to LTB₄/LTD₄ costimulation in comparison with LTB₄ and LTD₄ alone, with a lower EC₅₀ value of 0.84 nM.

As shown in Fig. 2D, the LTD₄-induced calcium flux was completely inhibited by pretreatment with montelukast, but was not inhibited by CP105696. Similarly, the LTB₄-induced calcium flux was not inhibited by montelukast, but abolished by CP105696 (Fig. 2E). Both inhibitors together completely inhibited the effect of both mediators together (Fig. 2F). Stimulation with LTB₄ (Fig. 2B) potently desensitized monocytes to subsequent stimulation with the same concentration of LTB₄ (as expected for homologous desensitization), but it did not have any effect on subsequent LTD₄ exposure. Simi-

larly, LTD₄ desensitized to subsequent stimulation with LTD₄ but not to LTB₄ (Fig. 2C), confirming that LTB₄ and LTD₄ signal through separate receptors and independent pathways (BLT₁ and CysLT₁).

Leukotriene B₄ and Leukotriene D₄ signal in additive way through G $\alpha_{i/o}$ -coupled BLT₁ and p44/42 MAPK in monocytes

Leukotriene B₄-induced calcium mobilization was fully inhibited by Ptx pretreatment (Fig. 3A), while residual calcium flux (<30%) in response to LTD₄ was observed. We further tested the Ptx effect on combined LTB₄- and LTD₄-induced calcium flux and found more than 75% inhibition of calcium flux by Ptx (Fig. 3B), with the residual calcium mobilization contributed by LTD₄ signaling via its CysLT₁ receptor (Fig. 3A). We then analyzed MAPK kinase activation. LTB₄ or LTD₄ induction of p44/42 phosphorylation was observed in 30 s and returned to baseline in 5 min (Fig. 3C,D). The LTB₄-induced phosphorylation of p44/42 was abrogated by pretreatment with CP105696 (Fig. 4E). An additive effect on p44/42 MAPK phosphorylation was observed when cells

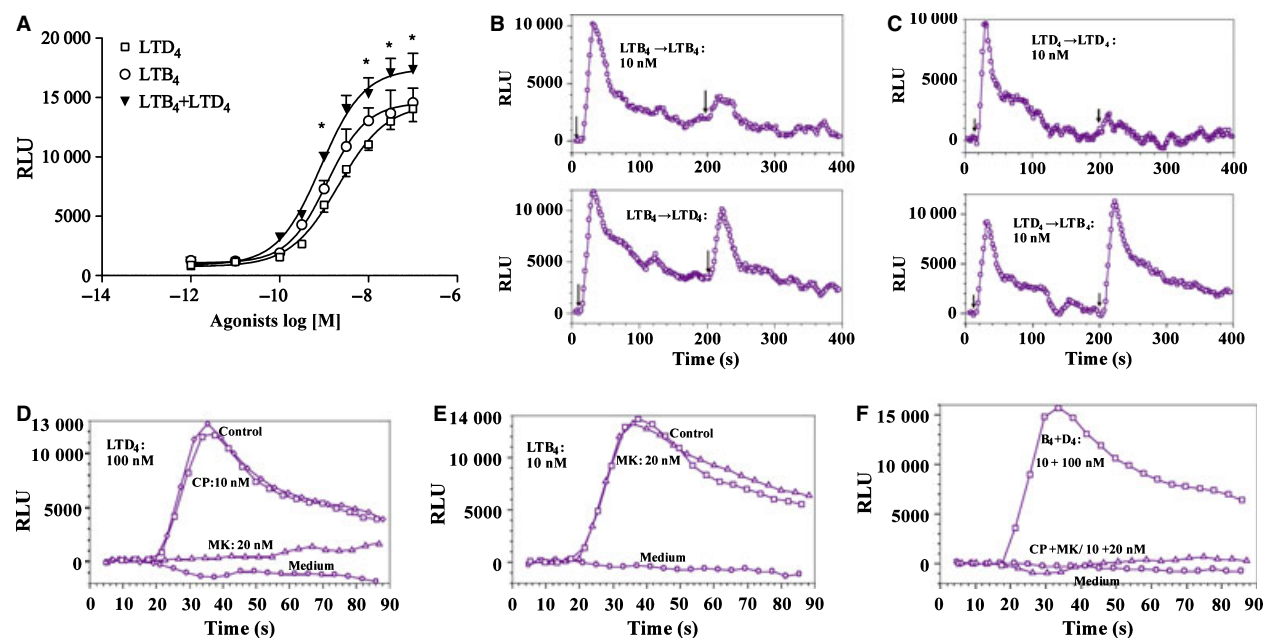


Figure 2 LTB₄ and LTD₄ act via specific receptors to induce calcium in an additive manner. Monocytes were stimulated with different concentrations of LTB₄, LTD₄ or LTB₄+LTD₄, and calcium release was measured, as indicated in Methods (Fig. 2A). Data presented as means ± SEM from 3-6 separate experiments each performed in triplicate. *P < 0.05 analyzed by ANOVA. Representative traces of calcium flux induced by repeated exposure to LTD₄

(10 nM) and LTB₄ (10 nM) in monocytes (B and C). Monocytes were pretreated with MK (20 nM/L) or CP105696 (10 nM) for 20 min and stimulated with LTD₄ (100 nM/L) (D), or LTB₄ (10 nM) (E). The effect of treatment with both LTB₄ and LTD₄ was completely inhibited by pretreatment with both inhibitors (F). Data from one of three separate experiments, each with similar results, are shown.

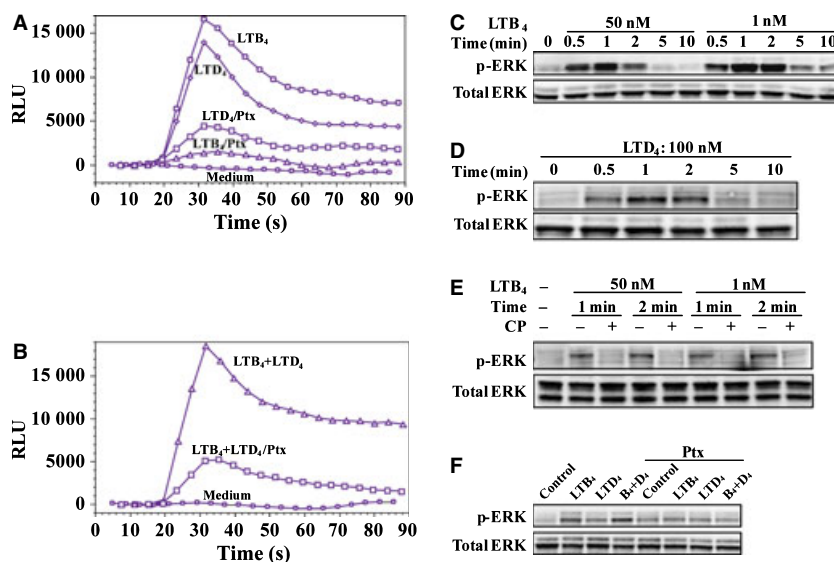


Figure 3 LTB₄ and LTD₄ signal through Gα_{i/o}-coupled BLT₁ and p44/42 MAPK in monocytes. Monocytes were pretreated with pertussis toxin (Ptx) (100 ng/mL) overnight and then stimulated with LTB₄ (50 nM) or LTD₄ (100 nM) (A), or stimulated with LTB₄ and LTD₄ together (B). Monocytes were stimulated with LTB₄ (C) or LTD₄ (D) at different time points, and phosphorylation of p44/42

was assayed by immunoblotting. (E) Cells were preincubated with CP105696 (10 nM) for 20 min and stimulated with LTB₄ for 1 or 2 min. (F) Cells were preincubated with Ptx (100 ng/mL) overnight and stimulated with LTB₄ (50 nM), LTD₄ (100 nM) or LTB₄ and LTD₄ for 2 min. Data are representative of three independent experiments.

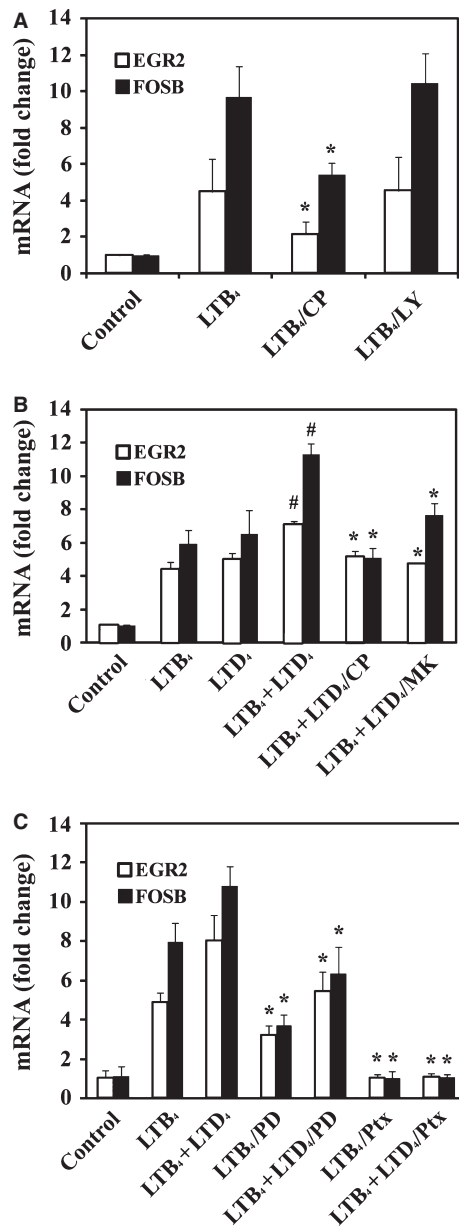


Figure 4 The additive effect of LTB₄ and LTD₄ costimulation on gene expression. (A) Monocytes were preincubated with CP105696 (10 nM), or LY255283 (200 nM) or vehicle for 20 min and stimulated with LTB₄ (50 nM) for 30 min. (B) Cells were pretreated with CP105696 (10 nM), or MK571 (100 nM) or vehicle for 20 min and then stimulated with LTB₄ (50 nM), LTD₄ or both for 30 min. (C) Cells were pretreated with PD98059 (10 μ M) for 1 h or pertussis toxin (100 ng/mL) overnight and stimulated with LTB₄ (50 nM) or LTB₄ and LTD₄ (50, 100 nM) 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 $\pm$ SD from three experiments performed in triplicate. * P < 0.05 for agonist(s) plus inhibitor compared with agonist(s) alone; # P < 0.01 for LTB₄ + LTD₄ compared with LTB₄ or LTD₄ alone.

were stimulated with LTB₄ and LTD₄ together (Fig. 4F) that was inhibited by Ptx pretreatment. While LTD₄ induction of p38 phosphorylation has been previously reported (14), we detected no consistent p38 phosphorylation in response to LTB₄ stimulation (data not shown).

The additive effect of Leukotriene B₄ and Leukotriene D₄ costimulation on gene expression

We validated the array expression data for two IEG (*FOSB* and *EGR2*) using TaqMan real-time PCR. *EGR2* and *FOSB* were induced by LTB₄ treatment, and this effect was inhibited by CP105696 but not by the BLT₂ antagonist, LY255283 (Fig. 4A). Figure 4B demonstrates the additive effect of LTB₄ (50 nM) and LTD₄ (100 nM) on *EGR2* and *FOSB* gene expression. The combined effect of LTD₄ and LTB₄ stimulation was partially inhibited by pretreatment with the BLT₁ and CysLT₁ antagonists, CP105696 and MK571, respectively (Fig. 4B). The effect of LTD₄ and LTB₄ was substantially inhibited by PD98059 and completely inhibited by Ptx (Fig. 4C), again suggesting similar but separate signaling pathways leading to immediate early gene expression.

Leukotriene D₄ augments Leukotriene B₄-induced chemotaxis of monocytes

A significant chemotactic activity in response to LTB₄, inhibited by the BLT₁ inhibitor, CP105696 (Fig. 5A,B), was observed. LTD₄ also induced chemotactic response that was abrogated by MK571 (Fig. 5A). Treatment of cells with LTB₄ and LTD₄ induced an additive effect (Fig. 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 G $\alpha_{i/o}$ -coupled receptor activation.

Discussion

We report here a description of LTB₄-mediated signaling, target gene induction, and chemotaxis in human monocytes, followed by an analysis of the cooperative and redundant responses induced by the coadministration of LTB₄ and LTD₄. In our model of human elutriated monocytes, LTB₄ stimulation induced calcium mobilization, p44/42 MAPK phosphorylation, gene expression, and chemotaxis, showing that LTB₄ is a potent stimulus for monocyte activation. Potent inhibition of LTB₄-induced calcium flux, MAPK kinase activation, immediate early gene expression as well as chemotaxis were observed with CP105696 treatment, indicating that BLT₁ is responsible for all studied LTB₄-induced activities, further confirmed by BLT₁ and BLT₂ selective knock down experiments. This is in agreement with previous studies showing that LTB₄-induced MCP-1 production (12) and LTB₄-triggered adhesion of monocytes to endothelium (18) were inhibited by the BLT₁ antagonist, CP105696.

We provide here evidence in human monocytes that LTB₄ stimulates BLT₁ coupled to Ptx sensitive G $\alpha_{i/o}$ causing intracellular calcium mobilization, p44/42 kinase activation, gene

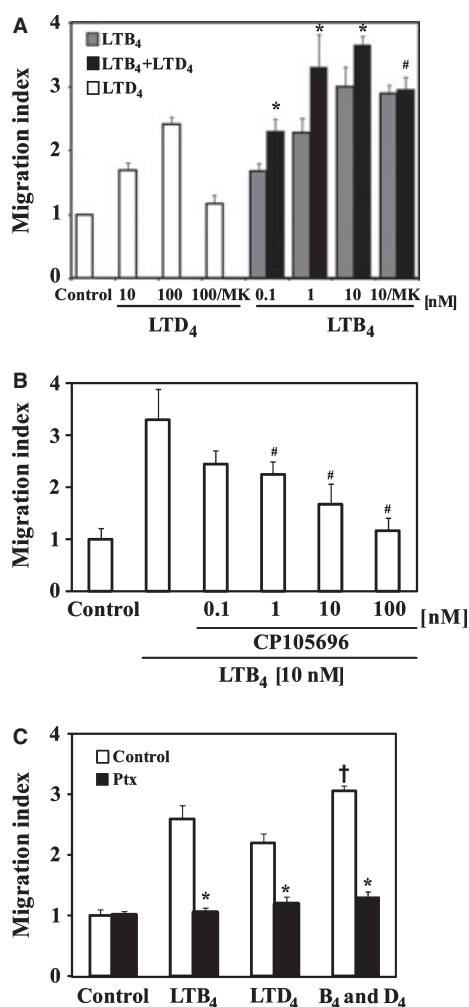


Figure 5 LTD₄ augments LTB₄-induced chemotaxis of monocytes. (A) Chemotactic activity of monocytes to different concentrations of or LTB₄, LTD₄ or LTB₄ and LTD₄ (100 nM) in the presence or absence of MK571 (MK; 100 nM). **P* < 0.05 for LTB₄ + LTD₄ compared with LTB₄ alone. #*P* < 0.05 for LTB₄ + LTD₄ compared with LTB₄ + LTD₄ plus MK571. (B) Cells were preincubated with different concentrations of CP105696 for 20 min and stimulated with LTB₄ (10 nM). #*P* < 0.05 for LTB₄ compared with LTB₄ plus CP105696. (C) Cells were preincubated with pertussis toxin (100 ng/mL) overnight and stimulated with LTB₄ (10 nM), LTD₄ (100 nM) or LTB₄ and LTD₄. **P* < 0.05 for agonist(s) plus Ptx compared with agonist(s) alone. †*P* < 0.05 comparing LTB₄ and LTD₄ with LTB₄ alone. Data from 3 different donors are presented as the migration index in comparison with vehicle control (mean ± SD).

expression, and chemotaxis. Our data in monocytes confirm early observations of predominant $G\alpha_{i/o}$ coupling of human BLT₁ observed in neutrophils (19) and differ compared with models of heterologously overexpressed BLT₁ where Ptx-sensitive and Ptx-insensitive G-protein coupling was described (20), underlining the importance of studying primary human cells.

We show for the first time in monocytes that LTB₄ induced the expression of 27 genes, belonging to families of immediate early genes, transcription activators, cytokines, and membrane receptors. Several genes induced by LTB₄ in monocytes are associated with acute-phase immune responses, e.g., IL-1 β , TNF α , and CCL3, consistent with the role of LTB₄ 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- κ B, thereby modulating 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 LTB₄ 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 h of LTB₄ stimulation. The induction of *EGR2* and *FOSB* mRNA by LTB₄ was only partially inhibited by the BLT₁ antagonist, CP105696, not affected by the BLT₂ antagonist, LY255283, but fully abolished by Ptx pretreatment (Fig. 4), suggesting that LTB₄ might regulate gene expression in monocytes acting also through a different than BLT₁ or BLT₂, Ptx-sensitive receptor.

We compared our data with early gene signatures generated for LTD₄ in monocytes and for LTD₄ 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 BLT₁, CysLT₁, CysLT₂, and PAR₁ for LTB₄- and LTD₄-activated monocytes and LTD₄- 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 to reach a combined threshold to fully activate the cell. Such a regulatory mechanism would be responsible for keeping cells nonresponsive 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 LTB₄ and LTD₄ costimulation, and a similar enhancing effect on gene expression was reported by Uzonyi et al. (25) in HUVECs stimulated with LTD₄ and thrombin. As most GPCRs become quickly desensitized to subsequent stimulation with the same agonist (as we showed here for LTB₄ and LTD₄), 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 LTB₄ and LTD₄ 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 BLT₁ and CysLT₁ 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 LTB₄ and LTD₄ 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 BLT₁ and CysLT₁ might not be effectively inhibited by selective receptor antagonists as these receptors have redundant functions. This finding could be relevant for choosing treat-

ment strategies for diseases where enhanced synthesis of both leukotrienes has 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 LTB₄/LTD₄ 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.

Conflict of interest

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

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