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Expression of functional leukotriene B₄ receptors on human airway smooth muscle cells

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Abstract

Background—Leukotriene B₄ (LTB₄) increases in induced sputum and exhaled breath condensate in people with asthma. Furthermore, the T_H2-type immune response and airway hyperresponsiveness induced by ovalbumin sensitization is markedly suppressed in LTB₄ receptor (BLT) 1 null mice. These studies suggest that LTB₄ may contribute to asthma pathophysiology. However, the direct effects of LTB₄ on human airway smooth muscle (ASM) have not been studied.

Objectives—We sought to determine the expression of LTB₄ receptors on human ASM and its functional role in mediating responses of human ASM cells, and the effect of LTB₄ on these cells.

Methods—Immunohistochemistry, RT-PCR, Western blotting, and flow cytometry were used to determine the expression of LTB₄ receptors. To determine the effect of LTB₄ on human ASM cells, cell proliferation was assessed by counting cells, and chemokinesis was assessed by gold particle phagokinesis assay.

Results—We confirmed expression of both BLT1 and BLT2 in human ASM cells in bronchial tissue and in cell culture. LTB₄ markedly induced cyclin D1 expression, proliferation, and chemokinesis of human ASM cells. LTB₄ also induced phosphorylation of both p42/p44 mitogen-

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activated protein kinase (MAPK) and downstream PI3 kinase effector, Akt1. However, we observed no induction of c-Jun N-terminal kinase or p38 MAPK. Notably, LTB₄-induced migration and proliferation of ASM cells were inhibited by the BLT1 specific antagonist, U75302, and by inhibitors of p42/p44 MAPK phosphorylation (U1026), and PI3 kinase (LY294002).

Conclusions—These observations are the first to suggest a role for a LTB₄-BLT1 signaling axis in ASM responses that may contribute to the pathogenesis of airway remodeling in asthma.

Keywords

Asthma; airway remodeling; airway smooth muscle cells; LTB₄; BLT

Asthma is a chronic respiratory disorder characterized by airway inflammation and hyperresponsiveness to various stimuli. That airway remodeling resulting from chronic inflammation contributes to the development of airway hyperresponsiveness has been suggested but not proven.^{1–3} In airway remodeling and hyperresponsiveness, airway smooth muscle (ASM) cells play an important role because of their multifunctional capacity for contraction, proliferation, migration, and synthesis of chemical mediators and cytokines. Among these, myocyte proliferation is a primary contributor to increased ASM mass.⁴ Growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor, and other inflammatory mediators such as histamine, acetylcholine, and leukotriene D₄, increase in asthmatic lungs and can induce ASM proliferation.^{5–8}

Thickening and fibrosis of subepithelial basement membrane resulting from the increase in number of myofibroblasts is an additional feature of airway remodeling. Geczy et al⁹ have shown that within 24 hours of acute airway allergen challenge, myofibroblast accumulation is induced in the submucosal compartment in patients with asthma. Because this response occurs more rapidly than the cell cycle duration for fibroblasts, migration of ASM cells to submucosal compartments of the airway wall has been suggested as a possible mechanism contributing to myofibroblast accumulation.¹

Leukotrienes are inflammatory mediators classified on the basis of their chemical structure. Both cysteinyl leukotrienes (cysLTs) and leukotriene B₄ (LTB₄) have been associated with airway inflammation in asthma. LTB₄ has been shown to increase in the airways of patients with severe asthma.^{10,11} However, its direct effects on human ASM cell responses that contribute to airway remodeling have not been studied, nor has the expression profile of LTB₄ receptors been described for human ASM cells.

We examined whether human ASM cells express LTB₄ receptors (BLTs) and whether these mediate effects of LTB₄ on proliferation and migration. Furthermore, we investigated the signaling pathways associated with LTB₄ effects on proliferation and migration, and assessed the functional role of BLTs in these processes.

METHODS

Human ASM cells and culture condition

ASM cell lines, immortalized by the stable expression of human telomerase reverse transcriptase (hTERT-ASM), and primary human ASM cells were prepared as we have previously described.^{12,13} The primary cultured human bronchial smooth muscle cells used to generate each hTERT-ASM cell line were prepared from macroscopically healthy segments of second-generation to fourth-generation main bronchus obtained after lung resection surgery from patients with a diagnosis of adenocarcinoma. All procedures were approved by the Human Research Ethics Board (University of Manitoba). As we have previously detailed,¹³ each cell line was thoroughly characterized to passage 10 and higher. Each hTERT cell line exhibits normal responsiveness to mitogens, and like primary human bronchial smooth muscle cultures, retains the capacity to express protein markers of the contractile phenotype, including smooth muscle–myosin heavy chain, smooth muscle- α -actin, and desmin, during prolonged serum withdrawal.^{13–15} Primary human ASM cells were used at passage 4 to 6, and hTERT-ASM cells were used at passage 12 to 20. All experiments were performed by using a minimum of 3 different primary or immortalized cell lines.

RNA extraction and RT-PCR

For details on the methods of RNA extraction and RT-PCR, please see this article's Methods section in the Online Repository at www.jacionline.org.

Preparation of cell lysates and Western blot analysis

For details on the methods of preparation of cell lysates and Western blot analysis, please see the Methods section in the Online Repository.

Flow cytometry analysis

Flow cytometry was performed to detect BLT on human ASM cells. Cells were grown to confluence and washed twice with warm PBS. Then cells were lifted by incubating in 0.5 mmol/L EDTA in PBS (37°C, 20 minutes). Collected cells were centrifuged (400g, 5 minutes, 4°C) and aliquoted into tubes containing 1.0×10^6 cells in 100 μ L washing buffer (PBS containing 1% BSA and 20 mmol/L glucose). To detect BLT1, cells were blocked for nonspecific binding with 5% BSA in PBS for 20 minutes at room temperature, and after dilution in washing buffer, the cells were incubated in the dark (overnight, 4°C) with 12 μ g/mL fluorescein isothiocyanate–conjugated mouse anti-BLT1 receptor (Serotec, Raleigh, NC), and with fluorescein isothiocyanate–mouse IgG_{2a} (BD Bioscience, Franklin Lakes, NJ) as a negative control. To detect BLT2, cells were incubated (overnight, 4°C) in washing buffer containing 12 μ g/mL rabbit anti-BLT2 (Cayman Chemical, Ann Arbor, Mich) or normal rabbit IgG (BD Bioscience) as a negative control. To finalize BLT2 staining, cells were centrifuged (400g, 5 minutes, 4°C), washed, and then incubated (30 minutes, room temperature) in the dark with 4 μ g/mL goat antirabbit phycoerythrin-conjugated antibody (BD Bioscience). Analysis of staining was performed with FACS Calibur flow cytometry (Becton Dickinson, Mountainview, Calif).

Proliferation assay

Cells were seeded in 12-well plates (40,000 cells per well) and grown to 50% to 70% confluence in Dulbecco's modified Eagle medium (DMEM) supplement with 5% FBS, streptomycin, and penicillin. Cells were maintained for 48 hours in DMEM and then were treated with and without 10^{-28} to 10^{-13} mol/L LTB₄ (Cayman Chemical) for 48 hours. When mitogen-activated protein kinase kinase (MEK) inhibitor, PI3 kinase, or BLT1 antagonist was used, it was added 1 hour before stimulation with LTB₄. To measure cell proliferation, cells were harvested and counted with a hemocytometer. EGF (Progen, Heidelberg, Germany) was used as a positive control in all experiments.¹⁶

Cell migration assay

Gold particle phagocytosis assay was performed to determine chemokinesis of human ASM cells as described elsewhere.^{17,18} Briefly, cells were seeded onto 6-well plates (4000 cells per well in DMEM) in which albumin-coated cover-glasses were overlaid with a uniform carpet of colloidal gold particles. Thereafter, cultures were incubated with and without 10^{-28} to 10^{-13} mol/L LTB₄ for 20 hours in DMEM. When MEK inhibitor, PI3 kinase inhibitor, or BLT1 antagonist was used, it was added 1 hour before stimulation with LTB₄. After 20 hours of incubation, cells were fixed with 10% formalin. The gold particle-free areas surrounding the cell were traced and analyzed with National Institute of Health (NIH) program (an NIH Image program); at least 100 individual cells were analyzed in each experiment, and 3 experiments were performed for each concentration of LTB₄, MEK inhibitor, PI3K inhibitor, and BLT1 antagonist tested. EGF was used as a positive control in all experiments.¹⁶

Immunohistochemistry

Normal bronchial tissue was obtained from patients undergoing lobectomy for lung cancer as approved by a local human ethics committee. Tissue specimens were fixed in 10% formalin and embedded in paraffin, and sections were prepared on slides. The sections were dewaxed in xylene, rehydrated through a graded series of ethanol solutions, and then immersed in methanol with 0.6% hydrogen peroxide for 30 minutes. The sections were then microwaved in 0.01 mol/L sodium citrate-buffered saline, pH 6.0, for 20 minutes at 92°C for antigen retrieval. The slides were blocked with 2% FBS at room temperature for 3 minutes and incubated at 4°C overnight in PBS containing rabbit polyclonal antibodies: anti-BLT1 receptor, anti-BLT2 receptor antibody (both 1:20; Cayman Chemical) or mouse mAb: anti- α smooth muscle actin antibody (1:400; Sigma, St Louis, Mo). Rabbit IgG was used as a negative control. Staining was performed by using the streptavidin-biotin complex method with 3,3'-diaminobenzidine substrate and 100 μ L hydrogen peroxidase in 50 mmol/L TRIS-HCl, pH 7.6.

Reagents

U0126 and LY294002 were obtained from Calbiochem (San Diego, Calif), and U75302 was obtained from Cayman Chemical.

Statistical analysis

Values were presented as the means $\pm$ SEMs. The statistical differences were determined by using the Bonferroni multiple comparisons test for paired comparisons. Differences were considered to be statistically significant at $P < .05$.

RESULTS

Immunohistochemical staining of human bronchus

To examine whether LTB₄ receptors are expressed *in vivo*, we performed immunohistochemical staining using human bronchus. Fig 1 shows staining for both BLT1 (A) and BLT2 (B) in human ASM cells, as confirmed by their overlapping distribution with smooth muscle α -actin staining in serial sections (C). In contrast, negative control specimens using rabbit IgG staining showed no immunoreactivity in any airway wall cells (Fig 1,D). BLT1 and BLT2 are also expressed by epithelial cells. These results clearly indicate that BLT1 and BLT2 are expressed by ASM and other cells of the airway wall *in vivo*.

Expression of LTB₄ receptors on human ASM cells in vitro

To examine the expression of receptors for LTB₄ in human ASM cells, mRNA and protein were evaluated with RT-PCR, Western blot analysis, and flow cytometry. As shown in Fig 2, mRNA for BLT1 and BLT2 was expressed in all 5 primary ASM cultures and hTERT-ASM cell lines. We next measured the abundance of BLT1 and BLT2 protein in 5 different primary and hTERT-immortalized human ASM cell cultures (Fig 3). Immunoblots for BLT1 revealed the presence of both the 80-kd nonglycosylated dimer form and the 60 kd glycosylated receptor, which is consistent with previous reports on the post translational modification of this receptor.¹⁹ The abundance of the latter varied somewhat among primary ASM cultures from different subjects. hTERT-ASM cell lines expressed both forms for BLT1 and also exhibited some variability between cell lines in the abundance of glycosylated receptor. We were also able to detect BLT2 in all primary ASM and hTERT-ASM cell cultures. However, there was considerable variability in the abundance between each cell line.

We next used flow cytometry to assess cell surface expression of BLT1 and BLT2 on individual hTERT-ASM cells (see this article's Fig E1 in the Online Repository at www.jacionline.org). The fluorescence histograms obtained clearly demonstrate significant labeling of each receptor subtype in approximately 25% myocytes from hTERT-ASM cell lines. Collectively our RT-PCR, Western blotting, and flow cytometry studies demonstrate that BLT1 and BLT2 are stably expressed on human ASM cells. Notably, because we observed no significant differences in BLT expression between primary and immortalized human ASM cells, subsequent functional studies were completed by using hTERT-ASM cell lines.

Effect of LTB₄ on ASM cell proliferation and migration

To assess proliferation, cells treated with LTB₄ for 48 hours were lifted from individual wells and counted with a hemocytometer (Fig 4, A). Proliferation was induced by 10^{-28} to

10^{-10} mol/L LTB₄ with a significant increase in cell number. However, no significant effect was observed at 10^{-11} to 10^{-13} mol/L LTB₄.

To evaluate ASM migration, we used a gold particle phagokinesis assay for chemokinesis. Cultures were treated with LTB₄, and cumulative migration responses were assessed 20 hours later (Fig 4, B). Concentration-dependent responses between 10^{-28} and 10^{-11} mol/L LTB₄ were observed. However, no significant effect was observed at 10^{-12} to 10^{-13} mol/L LTB₄.

Collectively, our data confirm LTB₄ has significant effects on important human ASM cell functions.

Intracellular signaling induced by LTB₄ in ASM cells

To examine further mechanisms associated with the proliferative effects of LTB₄, we performed Western blot analysis for cyclin D1 (see this article's Fig E2 in the Online Repository at www.jacionline.org). Cyclin D1 was clearly increased 6 hours after LTB₄ (10^{-28} mol/L) stimulation, and this effect was sustained through 12 hours. These data suggest that the primary effect of LTB₄-induced proliferation involved activation of ASM cell cycle progression.

To determine the functional significance of BLT in ASM cells, we next examined the ability of LTB₄ to induce signaling pathways associated with the regulation of cell division and migration. Signaling cascades involving p42/p44 mitogen-activated protein kinase (MAPK), p38 MAPK, c-Jun N-terminal kinase (JNK), and PI3 kinase are major signal transduction pathways for G-protein coupled receptor (GPCR)-stimulated proliferation of ASM.^{13,20} Thus, we examined LTB₄-induced phosphorylation of p42/p44 MAPK, p38 MAPK, JNK, and Akt1 (Fig 5). Phosphorylation of p42/p44 MAPK increased maximally at 20 to 30 minutes after LTB₄ stimulation, and returned to base levels after 120 minutes. Akt1 was also induced maximally 20 minutes after LTB₄ stimulation, returning to base levels 60 minutes after stimulation. Neither p38 MAPK nor JNK showed any evidence of increased levels of phosphorylation above base levels as long as 2 hours after LTB₄ stimulation. These data suggest that LTB₄ has a robust but transient inductive capacity for both the p42/p44 MAPK and PI3 kinase signal transduction pathways in human ASM. Thus, we next tested whether they play a role in the proliferative and cell migration responses of ASM cells in response to LTB₄.

Functional role of LTB₄ signaling in ASM proliferation and migration

To confirm a direct functional role of LTB₄ receptor-mediated signaling on cell proliferation, beginning 1 hour before and throughout the 48 hours of LTB₄ stimulation, we incubated human ASM cells with the selective BLT1 antagonist U75302 (1 μ mol/L), or with specific inhibitors for MEK (U0126, 1 μ mol/L) or PI3 kinase (LY294002, 25 μ mol/L) to prevent phosphorylation of p42/p44 MAPK and Akt1, respectively. Treatment with BLT1 antagonist virtually abolished pro-proliferative effects of LTB₄, strongly suggesting the BLT1 is chiefly responsible for mediating LTB₄ effects (Fig 6, A). A similar effect on LTB₄-induced proliferation was measured in cultures treated with MEK inhibitor, indicating p42/p44 MAPK is critical for LTB₄ effects via BLT1. Inhibition of PI3 kinase signaling also

significantly suppressed ASM proliferation, although this effect was less marked than that achieved by MEK inhibition. Collectively, these data strongly suggest a critical role for activation of p42/p44 MAPK and the PI3 kinase pathway in BLT1-mediated effects on ASM proliferation.

To examine whether ASM migration induced by LTB₄ was also mediated via BLT1 and is regulated by phosphorylation of p42/p44 MAPK and/or Akt1, we measured chemokinesis by using a gold particle phagokinesis assay (Fig 6, B). Similar to the effects we observed on proliferation, profound suppression of LTB₄-induced myocyte migration was achieved by using BLT1 antagonist (U75302, 1 μmol/L), inhibition of MEK (U0126, 1 μmol/L), and PI3 kinase (LY294002, 25 μmol/L). Collectively, these results confirm a central role for BLT1 in mediating effects of LTB₄ on ASM migration that involves signaling via p42/p44 MAPK and PI3 kinase pathways.

DISCUSSION

Our study is the first to describe the expression of LTB₄ receptors, the consequences of LTB₄ binding to BLT1 on proliferation, and migration of human ASM cells, and identifies the intracellular signaling pathways required for LTB₄-induced ASM responses. These observations are important because increased LTB₄ release is associated with airways inflammation and hyperresponsiveness in asthma. Moreover, the ASM cell responses we investigated, proliferation and migration, likely contribute to airway remodeling that is thought to be a principal determinant of fixed airway obstruction, particularly in patients with severe asthma. Thus, our data also provide some new directions for future studies focusing on the importance of LTB₄ in the pathogenesis of asthma.

Two receptor subtypes for LTB₄ have been cloned, including BLT1, a high-affinity receptor, and BLT2, which is a low-affinity receptor for LTB₄.^{21,22} This study clearly demonstrates that both BLT1 and BLT2 are expressed by and present on human ASM cells *in vivo* and *in vitro*. The presence of LTB₄ receptors in the human lung and cells such as leukocytes, neutrophils, eosinophils and T lymphocytes associated with the lung has been previously reported.²³ Back et al²⁴ have also shown that vascular smooth muscle cells express BLT1 receptor *in vitro* and *in vivo*. Our studies demonstrate for the first time the expression of both BLT1 and BLT2 on human bronchial smooth muscle cells.

The precise pathological impact of LTB₄ receptor expression on human ASM cells and how this may affect asthma pathogenesis and morbidity remain unresolved, but our data suggest this may be an important area for study in the near future. In human beings with asthma, LTB₄ is increased in induced sputum,¹⁰ exhaled breath condensate,¹¹ bronchoalveolar lavage fluid,²⁵ and blood.²⁶ To date, study of the expression of LTB₄ receptors cells from asthmatic lungs compared with nonasthmatic lungs has not been completed, and thus the impact of LTB₄ accumulation cannot be easily determined. Our data suggest LTB₄ promotes ASM proliferation and migration, which could contribute to airways remodeling.

Interestingly, BLT1 expression on human vascular smooth muscle cells can be markedly upregulated by IL-1β.²⁴ Similarly, IL-1β, TNF-α, and LTB₄ upregulate BLT1 expression by human umbilical vein endothelial cells.²⁷ A recent study using BLT^{-/-} mice showed that

BLT1 expression plays an important role in T-cell-derived IL-13 release and for the full development of allergen-induced airway hyperresponsiveness.²⁸ Thus BLT1 expression appears to both regulate expression of and be regulated by cytokines and proinflammatory biomolecules associated with the development of asthmalike symptoms. Our findings and those of other groups clearly suggest that rigorous assessment in ASM cells from subjects with asthma, or at least in animal models of allergic airways inflammation and airway remodeling, are warranted, although LTB₄ receptors expression and function may be quite different in asthma.

An increased mass of ASM is one of the key features that characterize airway remodeling in patients with asthma.^{29,30} Moreover, intrinsic differences in ASM between normal and asthmatic airway have been reported. For example, Johnson et al³¹ have reported that proliferation of ASM from subjects with asthma is increased compared with subjects without asthma. Our studies show that, in the absence of other mitogens, LTB₄ stimulates human ASM cell proliferation, and this response is blocked by the selective BLT1 antagonist U75302. Our data indicate the proliferative response of human ASM by LTB₄ involves increased cyclin D1. Because BLT1 is a GPCR,³² LTB₄ appears to be a mitogen of the same nature as other known GPCR mitogens such as thrombin and leukotriene D₄.⁵ Typically, stimulation of promitogenic GPCRs induces p21ras activation, leading to induction of both p42/p44 MAPK and PI3 kinase signaling cascades.³³ Indeed, a previous report using pancreas cancer cell lines has indicated that LTB₄ can stimulate both p42/p44 MAPK and PI3 kinase pathways.³⁴ Our results with human ASM cells mimic this effect because LTB₄ exposure resulted in marked phosphorylation of p42/p44 MAPK and Akt1. Importantly, proliferation in response to LTB₄ required p42/p44 MAPK phosphorylation and PI3 kinase signaling, because inhibition of these signaling cascades essentially abrogated LTB₄-induced cell growth. Collectively, our data confirm that BLT1 is essential for LTB₄-induced human ASM cell proliferation.

Growth factors such as platelet-derived growth factor,³⁵ EGF,¹⁶ cytokines such as IL-1 β ³⁵ and TGF- β / α ,³⁶ and inflammatory mediators such as leukotriene E₄³⁷ can induce ASM migration. Migration of human ASM may play a significant role in airway remodeling in asthma.³⁸ For example, it is possible that migration of ASM cells could contribute to ASM mass increase observed in airway remodeling, and the accumulation of myofibroblasts in the submucosa might result from migration of ASM cells.^{9,39} In our study, we clearly demonstrate that LTB₄ induces ASM migration and that this effect is dependent on BLT1, because blockade with U75302 abrogates the effect of LTB₄. Previous work with cultured human ASM cells has demonstrated that p42/p44 MAPK, p38 MAPK, PI3 kinase, and rho-kinase signaling pathways may regulate the migration response of human ASM.^{37,38} In the current study, we show that LTB₄ induces p42/p44 MAPK and Akt1 phosphorylation, but not p38 MAPK. Notably, Woo et al⁴⁰ have also reported that LTB₄ induces fibroblast migration via p42/p44 MAPK, but not p38 MAPK. Collectively, our data show that LTB₄-induced ASM cell migration via BLT1 requires both p42/p44 MAPK and PI3 kinase signal transduction.

Airway smooth muscle cells have many roles in the airway, including proliferation, migration, contraction, and synthesis of extracellular matrix, cytokines, and chemokinesis.

Because of their multifunctional behavior, these cells are emerging as therapeutic targets for asthma treatment. Several studies show that asthma drugs can regulate the function of human ASM, including migration and proliferation.^{41,42} To date, the clinical significance of these effects is not entirely clear.⁴³ Our data for LTB₄ parallel those for cysLTs: they both promote human ASM migration and proliferation in combination with other inflammatory mediators.^{20,37} Therefore, new therapeutic strategies that inhibit both LTB₄ and cysLT are likely needed to modulate human ASM function most effectively in asthma.

Conclusions

In conclusion, we demonstrate the functional expression of BLT1 on human ASM cells and its role in mediating LTB₄-dependent myocyte proliferation and migration. We also define key elements of the downstream signaling cascades induced by BLT1 in ASM cells activated by LTB₄ stimulation. These new findings provide evidence for future research direction focused on LTB₄ and BLT1 and their potential as therapeutic targets to regulate asthma pathogenesis and airway inflammation and remodeling.

METHODS

RNA extraction and RT-PCR

Total cellular RNA was isolated from cultured ASM cells with RNeasy mini kit extraction columns (Qiagen, Tokyo). Synthesis of cDNA was performed in a final reaction volume of 50 µL with 5 µg total RNA and 25 µL reverse transcriptase-mixtures consisting of 5× first-strand buffer, dithiothreitol, deoxy-nucleotide triphosphate, random primers, ribonuclease inhibitor, and Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen Life Technologies, Grand Island, NY). The reaction was carried out at 37°C for 60 minutes and 95°C for 5 minutes in a thermal cycler. The cDNA encoding genes of interest were amplified from 1 µL cDNA and 19 µL of the PCR mixture consisting of 10× PCR buffer, deoxynucleotide triphosphate, AmpliTaq gold polymerase (Roche, Branchburg, NJ), and specific primer. Primers for the glyceraldehyde-3-phosphate dehydrogenase, BLT1, and BLT2 receptors were purchased from Hokkaido System Science (Sapporo, Japan). Primer sequences were as follows: glyceraldehyde-3-phosphate dehydrogenase sense 5'-GTCATCCATGA CAACTTTGGTATCG-3' and antisense 5'-GCAGGTCAGGTCCAC CACTG-3' (product size, 255 bp); BLT1 receptor sense 5'-GAGTTCATCTCTCTGCTGGC-3' and antisense 5'-CCAGGTTCAGCAC CATCAGG-3' (139 bp); and BLT2 receptor sense 5'-CTTCTCATCGGGCAT CACAG-3' and antisense 5'-ATCCTTCTGGGCCTACAGGT-3' (88 bp). cDNA was denatured at 94°C for 9 minutes before the PCR reaction. The PCR products were separated and visualized on a 1% agarose gel consisting of 5 µg/mL ethidium bromide.

Preparation of cell lysates

Cells were washed twice with PBS, then lysed in radioimmunoprecipitation assay buffer (50 mmol/L TRIS-HCl, pH 7.4; 1% Triton-X 100; 0.25% sodium deoxycholate; 150 mmol/L NaCl; 1 mmol/L ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid; 0.1% SDS; 0.5 mmol/L Na₃VO₄; 1 mmol/L NaF; and protease inhibitors). Lysates were stored at -80°C until further use.

Western blot analysis

Equal amounts of total protein were subjected to electrophoresis, transferred to polyvinylidene fluoride membranes, then blocked with 5% nonfat dry milk in TRIS-buffered saline with 0.05% Tween 20 (TBST; 1 hour, room temperature) and subsequently incubated (overnight, 48C) in TBST containing primary rabbit antisera specific for BLT1 or BLT2 (dilution, 1:500; Cayman, Ann Arbor, Mich). For other immunoblots, polyclonal rabbit antibodies for phosphorylated and nonphosphorylated p42/p44 MAPK, a rabbit polyclonal antibody for p38, and a mouse mAb for phosphorylated p38 (1:200; Santa Cruz Biotechnology, Santa Cruz, Calif); a rabbit polyclonal antibody for JNK and a mouse mAb for phosphorylated JNK (1:200; Santa Cruz Biotechnology); rabbit antibodies for phosphorylated and nonphosphorylated Akt (1:1000; Cell Signaling Technology, Danvers, Mass); a mouse mAb for cyclin D1 (1:2000; Cell Signaling Technology); and mouse monoclonal anti- β -actin antibody (1:2000; Cell Signaling Technology) were used. After incubation with primary antibodies, the membranes were washed and incubated (1 hour, RT) with horseradish peroxidase-conjugated donkey antirabbit IgG or goat antimouse IgG (1:10,000 in TBST with 1% nonfat dry milk; GE Healthcare, Buckinghamshire, United Kingdom). Bands were subsequently visualized on film with enhanced chemiluminescence reagents (GE Healthcare).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations used

AHR	Airway hyperresponsiveness
ASM	Airway smooth muscle
BLT	Leukotriene B ₄ receptor
cysLT	Cysteinyl leukotriene
DMEM	Dulbecco's modified Eagle medium
EGF	Epidermal growth factor
GPCR	G-protein coupled receptor
hTERT-ASM	Human airway smooth muscle cell immortalized by stable ectopic expression of human telomerase reverse transcriptase
JNK	c-Jun N-terminal kinase
LTB₄	Leukotriene B ₄
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase

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Clinical implications

The LTB₄-BLT1 signaling axis in human airway smooth muscle may contribute to the pathogenesis of airway remodeling in asthma. Targeting this pathway may provide novel therapeutic strategies in the treatment of airway remodeling in asthma.

FIG 1.

Immunohistochemical staining of human bronchial tissue. Normal bronchial tissue was stained with anti-BLT1 antibody and BLT2 antibody. **A**, BLT1 staining. **B**, BLT2 staining. **C**, Smooth muscle α -actin staining. **D**, Rabbit IgG negative control. epi, epithelium; sm, smooth muscle.

FIG 2.

Expression of LTB₄ receptor mRNA in ASM cells *in vitro*. RT-PCR analysis was performed to detect BLT1 (*top*) and BLT2 (*middle*) mRNA in primary human ASM cells and hTERT-ASM cell lines each taken from 5 different donors. Each *lane* in the gels shown corresponds to samples from different cell cultures. Glyceraldehyde-3-phosphate dehydrogenase (*bottom*) was used as an internal loading control. Neutrophils obtained from a healthy donor were used as a positive control, and water was used as a negative control.

FIG 3.

Expression of BLT1 and BLT2 proteins in ASM cells. **A**, Expression of both the nonglycosylated dimer form receptor (~80 kD) and the glycosylated BLT1 receptor (BLT1R; ~60 kD). **B**, Expression of BLT2 receptor (*BLT2R*). Neutrophils obtained from a healthy donor were used as a positive control.

FIG 4.

Effects of LTB₄ on proliferation and migration of ASM cells. **A**, Results of proliferation assay for the effects of LTB₄. Measurements were taken from cells 48 hours after stimulation with 10⁻⁸ to 10⁻¹³ mol/L LTB₄. Stimulation with EGF (10 ng/mL) was used as a positive control. Results are presented as a percentage of negative control. Bars indicate the means ± SEMs from triplicate experiments using 3 cell lines. **P* < .001 vs control. **B**, Results of chemokinesis assay for the effects for LTB₄. Data are shown as percentage of gold particle-free areas compared with cells incubated in DMEM (control). Bars indicate the means ± SEMs from triplicate experiments using 3 cell lines. **P* < .001 vs control.

FIG 5.

LTB_4 phosphorylates p42/p44 MAPK and Akt1 in ASM cells. Equal amounts of cellular protein (20 μ g) were subjected to 10% SDS-PAGE and transferred to PVDF membranes. Blots shown are representative of the 3 different cell lines used in this study.

FIG 6.

BLT1 mediates effects of LTB₄ on ASM proliferation and migration via p42/p44 MAPK and PI3 kinase. **A**, Histogram showing results of proliferation assay 48 hours after LTB₄ addition. Results are presented as a percentage of negative control. Bars indicate the means $\pm$ SEMs from triplicate experiments using 3 cell lines. * P < .001 vs negative control; ** P < .001 vs stimulation with LTB₄ (10⁻²⁸ mol/L). **B**, Histogram showing results of cumulative cell migration assay after 20 hours of LTB₄ exposure. Data are shown as a percentage of gold particle-free areas compared with cells incubated in DMEM only (control). Bars indicate the means $\pm$ SEMs from at least 100 cells. * P < .001 vs negative control; ** P < .001 vs stimulation with LTB₄ (10⁻²⁸ mol/L).