LTD₄ induces HB-EGF-dependent CXCL8 release through EGFR activation in human bronchial epithelial cells

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McGovern T, Risse P-A, Tsuchiya K, Hassan M, Frigola G, Martin JG. LTD₄ induces HB-EGF-dependent CXCL8 release through EGFR activation in human bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 299: L808–L815, 2010. First published October 1, 2010; doi:10.1152/ajplung.00438.2009.—Airway epithelial cells release proinflammatory mediators that may contribute to airway remodeling and leukocyte recruitment. We explored the hypothesis that leukotriene D₄ (LTD₄) may trigger the release of proremodeling factors through activation of the EGF receptor (EGFR). We particularly focused on the effects of LTD₄ on release of heparin-binding EGF-like factor (HB-EGF) and IL-8 (CXCL8), a potent neutrophil chemoattractant that may be released downstream of EGFR activation. To address this hypothesis, both primary (NHBE) and transformed bronchial human epithelial cells (BEAS-2B) were grown on an air-liquid interface and stimulated with LTD₄. HB-EGF and CXCL8 were evaluated by ELISA in cell culture supernatants. To explore the EGFR signaling pathway, we used a broad-spectrum matrix metalloproteinase (MMP) inhibitor, GM-6001, two selective EGFR tyrosine kinase inhibitors, AG-1478 and PD-153035; an HB-EGF neutralizing antibody, and a specific small interfering RNA (siRNA) against the EGFR. Expression of the CysLTD₁ cysteinyl leukotriene receptor was demonstrated by RT-PCR and immunocytochemistry in both BEAS-2B and NHBE cells. Four hours after stimulation with LTD₄, HB-EGF and CXCL8 were significantly increased in cell culture supernatant. GM-6001 and montelukast, a specific CysLT₁ receptor antagonist, blocked the LTD₄-induced increase in HB-EGF. All inhibitors/antagonists decreased LTD₄-induced CXCL8 release. siRNA against EGFR abrogated CXCL8 release following stimulation with LTD₄ and exogenous HB-EGF. These findings suggest LTD₄ induced EGFR transactivation through the release of HB-EGF in human bronchial epithelial cells with downstream release of CXCL8. These effects may contribute to epithelial-mediated airway remodeling in asthma and other conditions associated with cysteinyl leukotriene release.

cysteinyl leukotrienes; heparin-binding-epidermal growth factor-like factor; interleukin-8; leukotriene D₄

CYSTEINYL LEUKOTRIENES (CysLTs) are potent lipid mediators derived from the arachidonic acid pathway that comprise leukotriene (LT) C₄, LTD₄, and LTE₄ (3). In the lung, CysLTs are mainly synthesized by mast cells, basophils, eosinophils, macrophages, neutrophils, and epithelial cells in response to a variety of stimuli (3). CysLTs are involved in the pathophysiology of asthma and contribute to causing bronchoconstriction, edema, mucus secretion, and increased inflammatory cell recruitment to the airways (14). High concentrations of CysLTs are present in the sputum of patients that have moderate to severe asthma in the course of acute exacerbations (1). CysLTs are also elevated in the sputum of patients with cystic fibrosis (22), a disease that is associated with a high prevalence of an asthmatic diathesis. CysLTs have been shown to be important agents in airway remodeling, and studies report that CysLT₁ receptor (CysLTr₁) antagonists reduce airway smooth muscle hyperplasia in experimental asthma in rats and, in addition, subepithelial fibrosis in mice (13, 23, 29). CysLTs exert their effects through G protein-coupled receptors, CysLTr₁ and CysLTr₂, although there may be additional receptors with complex interactions (4).

CysLT receptors are highly expressed in the lung, spleen, and peripheral blood leukocytes. In the lung, the expression of the CysLTr₁ predominates over the CysLTr₂ (19). Inhaled LTD₄ causes recruitment of inflammatory cells including neutrophils as well as increasing hyperresponsiveness to inhaled methacholine (9). CysLTs are weak chemoattractants for granulocytes, suggesting the possibility that their in vivo effects are mediated indirectly through the release of other chemoattractants.

IL-8 (CXCL8) is a member of the CXC chemokine family. It is a potent neutrophil chemoattractant (32), and its levels are elevated in the sputum of patients with asthma and correlated with neutrophilia (10). CXCL8 has properties that make it a potential contributor to airway remodeling by causing airway smooth muscle proliferation and migration (11). LTD₄ stimulation has been shown to induce gene transcription and protein release of CXCL8 in vitro (25). It has been reported that epithelial expression of CXCL8 is regulated by NF-κB, AP-1, and NF-IL-6, all of which are, in turn, regulated via the EGF receptor (EGFR) (25). However, the possible role of the EGFR in mediating the effects of LTD₄ on epithelial cells is uncertain, and whether LTD₄ release of CXCL8 occurs through this pathway has not been clarified.

The EGFR has been shown to play a key role in airway epithelial repair following mechanical wounding in a model system (2, 30). Innate immune responses of the airway epithelium mediated via the Toll-like receptor (TLR) 4 are modulated via activation of EGFR (16). In vivo studies in the Brown Norway rat have shown that inhaled LTD₄ induces an increase in inflammatory cells in bronchoalveolar lavage, goblet cell hyperplasia, and epithelial and airway smooth muscle cell proliferation, effects that are prevented by pretreatment with an EGFR tyrosine kinase inhibitor (25). LTD₄ also induces an increase in expression of heparin-binding EGF-like growth factor (HB-EGF), a ligand of EGFR, on airway epithelial cells in vivo (24), suggesting a possible role for this ligand in mediating the effects of LTD₄ on epithelial and smooth muscle remodeling. We reasoned therefore that LTD₄-induced CXCL8 release from human airway epithelial cells may be dependent on activation of EGFR via the proteolytic release of HB-EGF. To test this hypothesis, we examined the effects of LTD₄ on...
the release of three EGFR ligands [HB-EGF, amphiregulin, and transforming growth factor-α (TGF-α)] and CXCL8 by airway epithelial cells [transformed bronchial human epithelial cells (BEAS-2B) and normal human bronchial epithelial cells (NHBE)] and the effects of inhibitors of the mechanisms that lead to HB-EGF release. We also examined the effects of exogenous HB-EGF on CXCL8 release as well as an HB-EGF-specific neutralizing antibody on CXCL8 release triggered by LTD₄. Last, we used small interfering RNA (siRNA) to demonstrate that CXCL8 release via LTD₄ and HB-EGF is mediated through EGFR.

**METHODS**

*Reagents.* Tyrophostin AG-1478 (10 μM) and PD-153035 (3 μM), specific inhibitors of EGFR tyrosine kinase and LTD₄, were obtained from Cayman Chemical (Ann Arbor, MI). Human recombinant HB-EGF, a ligand of EGFR, and its function-blocking antibody (2.5 μg/ml) were obtained from R&D Systems (Minneapolis, MN). GM-6001 (25 μM), a broad-spectrum hydroxamic acid matrix metalloprotease (MMP) inhibitor [3-(N-hydroxycarbamoyl)-2(R)-isobutylpropiolyl-L-tryptophan methylamide], was obtained from Calbiochem (La Jolla, CA). Montelukast (10 μM), a specific CysLt1 antagonist, was provided by Merck Frost Canada.

*Cells.* BEAS-2B bronchial epithelial cell line (derived from adenovirus 12-SV40-transformed normal human bronchial epithelium) were obtained from the American Type Culture Collection and maintained in DMEM/F-12 with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) at 37°C under 5% CO₂. BEAS-2B cells were cultured in 75-cm² tissue culture flasks and grown to subconfluence. Medium was changed daily. Medium from the bottom chamber was collected for all experiments.

**Real-time PCR.** To determine basal expression level of the Cys-LTr1 in BEAS-2B and NHBE cells, real-time quantitative PCR (RT-qPCR) was performed using a LightCycler (Roche). Cells were serum-starved overnight before isolation of RNA with an RNaseasy Mini Kit (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer’s protocol. Each cDNA sample was synthesized using 0.5 μl of random hexamer primers, 0.5 μl of oligo(dT) primers, 1 μl of dNTP mix, 8 μl of RNA (1 μg), 10 μl of RNA-free H₂O, 2 μl of 10X cDNA synthesis buffer, 2 μl of 0.1 M DTT, 1 μl of RNaseOUT, and 1 μl of SuperScript III (Invitrogen). RT-qPCR was run using LightCycler software package version 3.5.3. Analysis was done using commercial software (LightCycler software package version 3.5.3). To verify the size of the amplification product, PCR reactions were analyzed on an ethidium bromide-stained 2.5% agarose gel.

**Immunocytochemistry for the Cys-LTr1.** Cells grown on culture inserts were fixed with 4% paraformaldehyde for 10 min. The membranes on which the cells were grown were removed from the inserts and embedded in paraffin. Sections (5 μm) of the membrane were cut by microtome to allow visualization of the cells adherent to the membrane. Sections were stained with hematoxylin and eosin for assessment of cellular morphology, and immunohistochemical staining for CysLt1 was performed with the Vectastain avidin-biotin-peroxidase complex (ABC) kit (Vector Laboratories, Burlingame, CA). Following deparaffination of sections, the slides were immersed in antigen-unmasking solution (Vector Laboratories) for 8 min. Sections were washed for 5 min twice with Tris-buffered saline (TBS; 0.5 M Tris-HCl, 1.5 M NaCl, pH 7.6). Cells were permeabilized using 0.2% Triton X-100 for 20 min, and universal blocking solution (Vector Laboratories) was applied for 20 min. Primary antibodies against human CysLt1 (Cayman Chemical) and control normal goat serum (Vector Laboratories) were used at a dilution of 1:250 were applied to tissue sections for incubation in a humidified chamber at room temperature for 1 h. The sections were then washed with TBS twice for 5 min. Biotinylated rabbit anti-goat IgG (Vector Laboratories) was applied to the tissue sections at a 1:50 dilution and incubated at room temperature for 45 min. Sections were washed twice with TBS. The slides were incubated with avidin-biotin complex alkaline phosphatase (Vector Laboratories) for 45 min. Last, Vector Red alkaline phosphatase (Vector Laboratories) was used to develop the sections for 15 min. For NHBE sections, nuclear staining was performed with Hoechst dye (Sigma-Aldrich) at a dilution of 1:2,000 in H₂O for 5 min. Following Hoechst staining, sections were washed in H₂O for 5 min. Sections were mounted with Vectamount mounting medium (Vector Laboratories). Immunofluorescence microscopy was used to visualize the Cys-LTr1.

**Measurement of EGFR ligands and CXCL8 by ELISA.** We assessed protein levels for HB-EGF, amphiregulin, TGF-α, and CXCL8 following stimulation with LTD₄ or HB-EGF at indicated time points and concentrations. Four hours following stimulation, cell supernatant was collected and frozen at −80°C. In some experiments, AG-1478 (10 μM), PD-153035 (50 μM), GM-6001 (25 μM), neutralizing antibody against HB-EGF (2.5 μg/ml), or montelukast (10 μM) were added at 30 min before the LTD₄ stimulation. After 4 h of incubation, supernatant was collected and run on an agarose gel to confirm the expected amplicon size of 85 bp. +, PCR products that were run with CysLt1 primers; −, negative control PCR products; Lad, ladder. n ≥ 3.

**Fig. 1. Expression of Cys-LT1 cysteinyl leukotriene receptor (Cys-LTr1) mRNA in primary and epithelial cell line.** RT-PCR was performed to determine presence of Cys-LTr1. RNA was isolated from confluent cells grown in an air-liquid interface. Cys-LTr1 was detected in both transformed bronchial human epithelial (BEAS-2B; A) and normal human bronchial epithelial (NHBE; B) cells. PCR products from both cell types were run on an agarose gel to confirm the expected amplicon size of 85 bp. +, PCR products that were run with Cys-LTr1 primers; −, negative control PCR products; Lad, ladder. n ≥ 3.
μM) were added to the cell cultures 30 min before (AG-1478, PD-15035, GM-6001, and montelukast) or simultaneously with (neutralizing antibody against HB-EGF) the addition of LTD₄. The concentrations of various EGFR ligands and CXCL8 in the cell culture medium were determined using sandwich ELISA assays (R&D Systems).

**EGFR knockdown using EGFR-specific siRNA transfection.** BEAS-2B cells were seeded into 6-well plates at 50% confluence (2 x 10⁵ cells per well) in antibiotic-free DMEM/F-12 supplemented with 10% FBS overnight. EGFR-specific siRNA (SC-29301; sense strand A, 5'-CUCUGAGGAAAAAGAAGU-3'; sense strand B, 5'-CGUAAAGGAAAUCACAGGG-3'; sense strand C, 5'-ACAGUG-GAGCGAAUUCU-3') and the negative control siRNA (SC-37007; siencer negative control) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A concentration of 80 pmol of siRNA targeting EGFR gene and nonspecific silencer region was transfected into cells according to manufacturer’s protocol. Cells were allowed to recover for 48 h and were put in serum-free medium and stimulated with 1 μM LTD₄ or 250 pg/ml HB-EGF for 4 h. Supernatant and cell lysates were collected for ELISA and Western blot analysis.

**Western analysis of EGFR.** BEAS-2B cells were cultured and transfected with EGFR siRNA or the appropriate control (scrambled siRNA) as described above. Cultured cells were lysed with the following lysis buffer: 50 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 2 mM DTT, and 1:100 dilution of protease inhibitor cocktail (Complete Mini Roche). Equal amounts of cell lysate (20 μg) were separated by an 8% SDS-PAGE gel and then transferred onto a nitrocellulose membrane, which was further incubated for 1.5 h with 5% skim milk in TBS containing Tween 20. Membranes were cut to separate high and low molecular mass proteins and incubated overnight at 4°C with a rabbit polyclonal antibody for total EGFR (cat. no. SC-03; 1:500 dilution; Santa Cruz Biotechnology) and β-actin mouse monoclonal antibody (dilution 1:10,000; Sigma-Aldrich) as a loading control. Proteins were visualized using secondary antibodies conjugated to horseradish peroxidase (Amersham, Oakville, Ontario, Canada) and enhanced chemiluminescence (ECL; Amershamb) on a FluorChem 8000 imaging system (Alpha Innotech, San Leandro, CA).

**Statistical analysis.** All data are expressed as means ± SE with at least n = 3 independent observations per experiment. In experiments that contained only two groups, statistical differences were determined by an unpaired Student’s t-test. When comparing more than
two means, two-way ANOVA was used, and post hoc analysis was performed using the Newman-Keuls test. GraphPad Prism (La Jolla, CA) was used to evaluate the data. Differences were considered significant for $P$ values of <0.05.

RESULTS

**CysLTr1 expression on epithelial cells.** To confirm the capacity of BEAS-2B and NHBE cells to express CysLTr1 mRNA, we performed semiquantitative RT-PCR. Following cell culture in an air-liquid interface system, both BEAS-2B and NHBE cells were tested. RT-qPCR confirmed that both cell types express CysLTr1. Similar crossing points were noted for both BEAS-2B and NHBE, indicating CysLTr1 mRNA expression to be comparable and supporting the relevance of findings on BEAS-2B for primary cells (NHBE) in our experiments. Amplicon size for CysLTr1 (85 bp) was verified on 2.5% agarose gels (Fig. 1). We demonstrated the presence of the CysLTr1 at the protein level by immunocytochemistry. Using a polyclonal CysLTr1 antibody and immunofluorescent secondary antibody, we were able to detect expression of CysLTr1 protein (Fig. 2, red) on the cell membrane in both BEAS-2B and NHBE cells.

**Effects of LTD$_4$ on release of EGFR ligands by epithelial cells.** Once we confirmed mRNA and protein expression of the CysLTr1 on BEAS-2B and NHBE cells, we examined the effects of LTD$_4$ on the chosen three EGFR ligands. We found significant increases in the release of HB-EGF by both BEAS-2B and NHBE cells (Fig. 3, A and B). However, under identical conditions, we did not detect increases in TGF-$\alpha$ (Fig. 3, C and D) or amphiregulin (Fig. 3, E and F). Next, we constructed a concentration-response curve to LTD$_4$ for the release of HB-EGF from the cells (Fig. 4A). A significant release of HB-EGF was observed at 1 $\mu$M LTD$_4$. Using this concentration, we examined the time course of release of HB-EGF (Fig. 3A).

![Graph of HB-EGF](image1.png)

**Fig. 3.** Leukotriene $D_4$ (LTD$_4$) induces release of heparin-binding EGF-like factor (HB-EGF) but not amphiregulin or transforming growth factor-$\alpha$ (TGF-$\alpha$). BEAS-2B and NHBE cells grown in air-liquid interface were stimulated with LTD$_4$ (1 $\mu$M) for 4 h. Following incubation, supernatant was collected and assayed for presence of EGF receptor (EGFR) ligands HB-EGF, amphiregulin, and TGF-$\alpha$. Both cell types released significant amounts of HB-EGF (A and B), but no changes were seen in the other ligands (C–F). $n \geq 3$; *$P < 0.05$. 

![Graph of TGF-$\alpha$](image2.png)

![Graph of amphiregulin](image3.png)
P LTD4, two selective tyrosine kinase inhibitors were used, AG-1478 and PD-153035 potently inhibited the release of CXCL8 from LTD4-exposed cells with 250 pg/ml HB-EGF caused a robust release of CXCL8 in both cell types (Fig. 6). Additionally, we confirmed that LTD4-mediated CXCL8 release was present in both cell types (Fig. 5, B and C).

CXCL8 release has been demonstrated to occur downstream of the activated EGFR (5). To confirm the involvement of EGFR in the release of CXCL8, we showed that treatment of the cells with 250 pg/ml HB-EGF caused a robust release of CXCL8 in both cell types (Fig. 6). Additionally, a selective tyrosine kinase inhibitor, AG-1478, was successful at blocking CXCL8 release from exogenously administered HB-EGF in both cell types (Fig. 6, A and B). To further implicate specific involvement of the EGFR in the release of CXCL8 from epithelium by LTD4, we used a neutralizing antibody to HB-EGF (2.5 ng/ml). The neutralizing antibody treatment attenuated the release of CXCL8 following LTD4 stimulation (Fig. 7, A and B). In each instance, the inhibitors reduced CXCL8 levels to values at or below basal levels (Fig. 7, A and B). The inhibitors on their own did not affect basal CXCL8 levels (Fig. 7, A and B).

**Effects of EGFR knockdown with siRNA on HB-EGF and LTD4 induced CXCL8 release.** We used a specific siRNA targeting the EGFR to knockdown this pathway in BEAS-2B cells. Following a 24-h transfection period and a further delay of 48 h, we examined the level of EGFR by Western analysis. There was a substantial reduction in EGFR induced by siRNA compared with the scrambled siRNA treatment (Fig. 8A). This result is representative of three experiments. The treatment of BEAS-2B cells with specific siRNA completely abrogated the release of CXCL8 by LTD4 (Fig. 8B). It also blocked the release of CXCL8 triggered by HB-EGF (Fig. 8B).

**DISCUSSION**

In the current study, we have shown that LTD4 induces the release of CXCL8 by bronchial epithelial cells through an EGFR-dependent mechanism. We demonstrated the transcript and protein for the CysLT1 and 2 receptors on the epithelial cells, and using specific pharmacological inhibitors, a neutralizing antibody against HB-EGF and EGFR-specific siRNA, we demonstrated that the CysLT1 mediated the effects of LTD4 by a cascade of reactions involving MMPs, release of HB-EGF, and downstream activation of the EGFR. Furthermore, we confirmed the role of HB-EGF as the dominant ligand driving this mechanism.

CysLTs have long been recognized as contributors to the pathogenesis of asthma. In our study, we used human bronchial epithelial cells that were donor (NHBE) and cell line-derived (BEAS-2B) to evaluate the mechanisms by which LTD4 may cause remodeling through EGFR-dependent pathways involving the airway epithelium. We first confirmed the presence of the CysLT1 by RT-PCR. Interestingly, both cell types demonstrated similar levels of mRNA expression for CysLT1.

**Graphs**

A) LTD4, HB-EGF, and supernatant was collected over a 24-h period. At 2, 4, 8, and 24 h, there was a significant increase in HB-EGF in the supernatant of LTD4-exposed cells compared with controls. n ≥ 3; *P < 0.05, **P < 0.01.

B) HB-EGF over a 24-h period (Fig. 4B). We chose the 4-h time point for subsequent experiments in this study.

**Role of the EGFR in LTD4-induced release of CXCL8.** We performed a time course over a 24-h period in BEAS-2B cells and found that LTD4 caused a time-dependent increase in the release of CXCL8 (Fig. 5A). The 4-h incubation time with LTD4 was chosen for the remainder of the experiments. Additionally, we confirmed that LTD4-induced CXCL8 release was present in both cell types (Fig. 5, B and C).

**Fig. 4.** LTD4 induces concentration- and time-dependent release of HB-EGF. Four hours after exposure to LTD4, there is a concentration-dependent increase in the release of HB-EGF in BEAS-2B cells. A final concentration of 1 μM was chosen as it caused a significant increase in HB-EGF release (A). n ≥ 3; *P < 0.05. B: cells were exposed to 1 μM LTD4 and supernatant was collected over a 24-h period. At 2, 4, 8, and 24 h, there was a significant increase in HB-EGF in the supernatant of LTD4-exposed cells compared with controls. n ≥ 3; *P < 0.05, **P < 0.01.

**Fig. 5.** LTD4 induces CXCL8 release. A) LTD4 caused a time-dependent increase in the release of CXCL8 in the BEAS-2B line. B) LTD4 caused an increase localized to the airway epithelium of LTD4-treated animals (24, 28). LTD4 causes airway epithelial and smooth muscle remodeling in the rat, an effect attenuated by the use of the selective EGFR tyrosine kinase inhibitor, AG-1478. Morphometric analysis of HB-EGF protein expression demonstrated an increase localized to the airway epithelium of LTD4-treated rats, suggesting this ligand as the potential cause of the EGFR

Previous work has implicated the EGFR in CysLT1-mediated airway remodeling in allergen-sensitized and -challenged animals (24, 28). LTD4 causes airway epithelial and smooth muscle remodeling in the rat, an effect attenuated by the use of the selective EGFR tyrosine kinase inhibitor, AG-1478. Morphometric analysis of HB-EGF protein expression demonstrated an increase localized to the airway epithelium of LTD4-treated rats, suggesting this ligand as the potential cause of the EGFR.
transactivation. Recently, studies have shown that CysLTs can induce transactivation of the EGFR in airway smooth muscle cells, but whether this was ligand-dependent was not established (21). LTD₄ synergizes with EGF in promoting growth of fibroblasts, but the effect does not seem to involve transactivation of the EGFR and is not mediated by either CysLTr1 or -2 (31). The current study provides a potential link between the remodeling effects of LTD₄ and activation of the EGFR via the release of HB-EGF. LTD₄ induced the release of HB-EGF protein from both primary and bronchial epithelial cell lines in a time- and concentration-dependent manner. Several other G protein-coupled receptor agonists (endothelin, thrombin, and lysophosphatidic acid) have been also implicated in the activation of the EGFR, although again the release of EGFR ligands that might have mediated these effects was not examined (8).

IL-8 (CXCL8) is a potent chemoattractant for neutrophils and a key mediator of neutrophilia in asthma (18). LTD₄ is able to induce release of CXCL8 in a time-dependent manner, and this release is dependent on EGFR activation. Furthermore, we demonstrate that LTD₄-induced CXCL8 release acts via an MMP-dependent mechanism as using the broad-spectrum MMP inhibitor GM-6001 blocks CXCL8 release. This result is consistent in both BEAS-2B and NHBE cells. Whether the same MMP is responsible for the cleavage of HB-EGF in response to other stimuli such epithelial compression (7) or wounding (2) has not been elucidated. There is added significance to the current findings in relationship to mechanisms of airway remodeling mediated by CysLTs since CXCL8 itself is a mitogen for airway smooth muscle and may also cause migration of airway smooth muscle cells (11).

CysLTs may not be the only agonists to lead to activation of the EGFR in the setting of airway disease. Previous studies have demonstrated that activation of the EGFR follows mechanical compression of the airway epithelium (7, 26, 27). This also involves HB-EGF, as a neutralizing antibody prevents the phosphorylation of the EGFR and downstream activation of mitogen-activated kinases. TLR agonists have also been shown to activate a cascade involving ADAM17 and the release of TGF-β1 (16). However, we were not able to detect an increase in TGF-β following LTD₄ stimulation, suggesting mechanisms of actions distinct from those that follow TLR activation involving ADAM17. Finally, we used siRNA specific against EGFR to test the direct involvement of EGFR in LTD₄- or HB-EGF-induced CXCL8 release. The treatment of BEAS-2B cells with specific siRNA for EGFR successfully reduced protein expression, and the release of CXCL8 induced by LTD₄ and

![](http://ajplung.physiology.org/)

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Fig. 5. LTD₄ induces the release of CXCL8 protein from bronchial epithelial cells. BEAS-2B cells were stimulated with 1 μM LTD₄ and allowed to incubate for given times over a 24-h period. Medium was collected at the indicated time points and assayed by ELISA for CXCL8 protein content. LTD₄ induced a significant release of CXCL8 at 4, 8, and 24 h (A). Both BEAS-2B (B) and NHBE (C) cells secreted a significant amount of CXCL8 following stimulation at 4 h. n ≥ 3; *P < 0.05, **P < 0.01, ***P < 0.001.

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Cleavage of EGFR ligands has been linked to various MMPs (6, 12). MMPs cleave the proform of HB-EGF, solubilizing it and allowing it to bind to EGFR. The precise MMP responsible for this action in our current experiments has not been elucidated. However, it appears that more than one MMP may be potentially responsible for the mechanism of cleavage. We demonstrate that LTD₄-induced CXCL8 release acts via an MMP-dependent mechanism as using the broad-spectrum MMP inhibitor GM-6001 blocks CXCL8 release. This result is consistent in both BEAS-2B and NHBE cells. Whether the same MMP is responsible for the cleavage of HB-EGF in response to other stimuli such epithelial compression (7) or wounding (2) has not been elucidated. There is added significance to the current findings in relationship to mechanisms of airway remodeling mediated by CysLTs since CXCL8 itself is a mitogen for airway smooth muscle and may also cause migration of airway smooth muscle cells (11).
HB-EGF was abrogated entirely. We noted an increase in baseline levels of IL-8, an effect most likely attributable to activation of TLR3. Previous studies have shown TLR3 activation to be induced by siRNA in epithelial cells, and IL-8 levels have been shown to increase following TLR3 activation (5, 15). Despite this, our data indicate that specific knockdown of EGFR inhibits LTD₄- and HB-EGF-induced IL-8 increase. Additionally, these findings support the conclusions derived from the use of the pharmacological antagonists of the EGFR.

We conclude that stimulation with LTD₄ sets off a cascade of signaling events that lead to the release of potent asthma mediators. These mediators have historically been implicated in the pathogenesis of asthma and remodeling. In this study, we show for the first time that the mechanism by which this process is taking place is hinged on EGFR activation through an HB-EGF-dependent system.

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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Fig. 6. CXCL8 release induced by exogenous HB-EGF is inhibited by AG-1478. A concentration curve using various concentrations of HB-EGF was done to establish comparable levels of CXCL8 at 4 h as to those following LTD₄ stimulation (data not shown). A concentration of 250 pg/ml HB-EGF was chosen as it induced comparable amounts of CXCL8 release from both BEAS-2B and NHBE cells 4 h following stimulation. Following incubation with HB-EGF, supernatant was collected, and protein concentration was determined by ELISA. Exogenous HB-EGF (250 pg/ml) induced significantly high levels of CXCL8 in both cell types (A, P < 0.01; B, P < 0.05). Pretreatment with AG-1478 (10 μM) 30 min before addition of HB-EGF attenuated this effect, limiting CXCL8 levels to that of non-HB-EGF-stimulated cells (A and B). AG-1478 alone had no effect on CXCL8 release on either cell type. n ≥ 3; P < 0.05, P < 0.01.

Fig. 7. LTD₄-induced CXCL8 release is inhibited by EGFR inhibitors, metalloproteinase inhibitors, a CysLT1 antagonist, and a neutralizing antibody. BEAS-2B and NHBE cells were stimulated with 1 μM LTD₄ for 4 h. Thirty minutes before stimulation, various inhibitors were added. AG-1478 (10 μM), PD-153035 (PD; 3 μM), GM-6001 (25 μM), and montelukast (MK; 10 μM) all attenuated the increase in CXCL8 release compared with cells that received LTD₄ only (A and B). AG-1478 alone had no effect on CXCL8 release on either cell type. n ≥ 3; *P < 0.05.
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Fig. 8. Small interfering (si) RNA knockdown of EGFR results in diminished release of CXCL8 following LTD4 and HB-EGF stimulation. siRNA targeted against EGFR was transfected into BEAS-2B cells, and cells were harvested for analysis by Western blotting 72 h later. Cells treated with siEGFR had less EGFR expression than those treated with scrambled siRNA (A). B: Following transfection, transfected cells were stimulated with LTD4 (1 μM) or HB-EGF (250 pg/ml) for 4 h. Supernatant was assayed for CXCL8 by ELISA and showed that knocking down EGFR limits the release of CXCL8 following stimulation. n = 3; *P < 0.05, **P < 0.01.


