

# Leukotriene E<sub>4</sub>-induced pulmonary inflammation is mediated by the P2Y<sub>12</sub> receptor

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Of the potent lipid inflammatory mediators comprising the cysteinyl leukotrienes (LTs; LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub>), only LTE<sub>4</sub> is stable and abundant in vivo. Although LTE<sub>4</sub> shows negligible activity at the type 1 and 2 receptors for cys-LTs (CysLT<sub>1</sub>R and CysLT<sub>2</sub>R), it is a powerful inducer of mucosal eosinophilia and airway hyperresponsiveness in humans with asthma. We show that the adenosine diphosphate (ADP)-reactive purinergic (P2Y<sub>12</sub>) receptor is required for LTE<sub>4</sub>-mediated pulmonary inflammation. P2Y<sub>12</sub> receptor expression permits LTE<sub>4</sub>-induced activation of extracellular signal-regulated kinase in Chinese hamster ovary cells and permits chemokine and prostaglandin D<sub>2</sub> production by LAD2 cells, a human mast cell line. P2Y<sub>12</sub> receptor expression by LAD2 cells is required for competition between radiolabeled ADP and unlabeled LTE<sub>4</sub> but not for direct binding of LTE<sub>4</sub>, suggesting that P2Y<sub>12</sub> complexes with another receptor to recognize LTE<sub>4</sub>. Administration of LTE<sub>4</sub> to the airways of sensitized mice potentiates eosinophilia, goblet cell metaplasia, and expression of interleukin-13 in response to low-dose aerosolized allergen. These responses persist in mice lacking both CysLT<sub>1</sub>R and CysLT<sub>2</sub>R but not in mice lacking P2Y<sub>12</sub> receptors. The effects of LTE<sub>4</sub> on P2Y<sub>12</sub> in the airway were abrogated by platelet depletion. Thus, the P2Y<sub>12</sub> receptor is required for proinflammatory actions of the stable abundant mediator LTE<sub>4</sub> and is a novel potential therapeutic target for asthma.

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Abbreviations used: 2-MesAMP, 2-methylthioadenosine monophosphate; 5-LO, 5-lipoxygenase; ADP, adenosine diphosphate; AERD, aspirin-exacerbated respiratory disease; BAL, bronchoalveolar lavage; cDNA, complementary DNA; CHO, Chinese hamster ovary; COX, cyclooxygenase; Cys-LT, cysteinyl LT; Der f, *Dermatophagoides farinae*; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; HIS, polyhistidine; LT, leukotriene; MC, mast cell; MIP-1β, macrophage inflammatory protein 1β; mRNA, messenger RNA; P2Y, purinergic; PAS, periodic acid-Schiff; PG, prostaglandin; PTX, pertussis toxin; shRNA, short hairpin RNA.

Cysteinyl leukotrienes (LTs [cys-LTs]) are lipid inflammatory mediators generated in vivo by mast cells (MCs), eosinophils, myeloid DCs, basophils, and macrophages (Kanaoka and Boyce, 2004). They abound in mucosal inflammation, play a validated role in human asthma (Wenzel et al., 1990; Israel et al., 1996; Liu et al., 1996), and are important mediators in mouse models of pulmonary inflammation, remodeling, and fibrosis (Beller et al., 2004; Henderson et al., 2006; Kim et al., 2006). LTC<sub>4</sub>, the parent cys-LT, is synthesized from arachidonic acid, which is liberated by calcium-dependent cPLA<sub>2</sub> (cytosolic phospholipase A<sub>2</sub>) from membrane phospholipids (Clark et al., 1991). Arachidonic acid is then converted to LTA<sub>4</sub> by 5-lipoxygenase (5-LO) in concert with 5-LO-activating protein (Dixon et al., 1990; Malaviya et al., 1993). LTA<sub>4</sub> is conjugated to reduced glutathione by LTC<sub>4</sub> synthase, a homotrimeric integral nuclear membrane protein (Ago et al., 2007), forming LTC<sub>4</sub>. LTC<sub>4</sub> is exported to

the extracellular space by a multidrug-resistant protein after synthesis (Robbiani et al., 2000), where it is converted to LTD<sub>4</sub> by γ-glutamyl leukotrienase-mediated removal of glutamic acid (Shi et al., 2001). LTD<sub>4</sub> is then converted to LTE<sub>4</sub> by dipeptidase-mediated removal of glycine (Lee et al., 1983). Thus, the cys-LTs comprise three ligands that form in a spatially and temporally distinct fashion. LTC<sub>4</sub> is the only intracellular cys-LT, and LTD<sub>4</sub> is the most powerful contractile agonist of the airway smooth muscle. The half-life of LTD<sub>4</sub> is short (minutes) because of its rapid conversion to LTE<sub>4</sub>, effectively limiting its duration of action in vivo. LTE<sub>4</sub> is stable and excreted in the urine (Sala et al., 1990). The stability of LTE<sub>4</sub> accounts for the fact that it is the dominant cys-LT detected

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in biological fluids. Consequently,  $\text{LTE}_4$  levels can be monitored in the urine (Drazen et al., 1992), sputum (Lam et al., 1988), and exhaled breath condensate (Csoma et al., 2002) as an index of the cys-LT synthetic pathway activity in human disease states such as asthma, where its concentrations can be markedly elevated.

To date, two G protein-coupled receptors (GPCRs) for cys-LTs, termed type 1 and type 2 cys-LT receptors ( $\text{CysLT}_1\text{R}$  and  $\text{CysLT}_2\text{R}$ ), have been cloned and characterized (Lynch et al., 1999; Heise et al., 2000). These receptors share 38% amino acid identity. Each is 24–32% identical to the purinergic (P2Y) class of GPCRs that regulate cellular responses to extracellular nucleotides (Mellor et al., 2001), suggesting a phylogenetic relationship between these two GPCR classes. The human  $\text{CysLT}_1\text{R}$ , encoded by a gene on chromosome Xq21.13, is a high-affinity receptor for  $\text{LTD}_4$  ( $K_d \sim 1$  nM; Lynch et al., 1999), whereas the human  $\text{CysLT}_2\text{R}$  is encoded by a gene on chromosome 13q14 and has equal affinity for  $\text{LTC}_4$  and  $\text{LTD}_4$  ( $K_d \sim 10$  nM; Heise et al., 2000). As neither receptor has significant affinity for  $\text{LTE}_4$ , the existence of an additional cys-LT receptor with a preference for  $\text{LTE}_4$  has long been suspected. Early studies demonstrated that purified  $\text{LTE}_4$  is more potent than  $\text{LTC}_4$  or  $\text{LTD}_4$  for inducing contraction of guinea pig tracheal rings (Lee et al., 1984), whereas  $\text{LTC}_4$  and  $\text{LTD}_4$  were more potent on peripheral lung. Of the three cys-LTs, only  $\text{LTE}_4$  potentiates the contractile response of guinea pig trachea to histamine, a response which can be blocked by the administration of indomethacin, a nonselective inhibitor of the cyclooxygenase (COX) enzymes (Lee et al., 1984).  $\text{LTE}_4$  inhalation by asthmatic individuals potentiates airway hyperresponsiveness to subsequent challenges with histamine. This potentiation is also blocked by oral administration of indomethacin (Christie et al., 1992a). Inhalation of  $\text{LTE}_4$ , but not of  $\text{LTD}_4$ , causes eosinophils, basophils, and MCs to accumulate in the bronchial mucosa of asthmatic individuals (Laitinen et al., 1993; Gauvreau et al., 2001). Patients with aspirin-exacerbated respiratory disease (AERD), a syndrome characterized by asthma, nasal polyposis, and marked cys-LT overproduction, exhibit selectively enhanced bronchoconstriction in response to  $\text{LTE}_4$  relative to  $\text{LTC}_4$  or to histamine when compared with aspirin-tolerant asthmatic individuals (Christie et al., 1993). Mice lacking both  $\text{CysLT}_1\text{R}$  and  $\text{CysLT}_2\text{R}$  ( $\text{Cysltr1/Cysltr2}^{-/-}$  mice) exhibit enhanced skin swelling in response to intracutaneous  $\text{LTE}_4$  relative to WT controls, indicating that a putative  $\text{LTE}_4$ -reactive GPCR (termed  $\text{CysLTE}_4\text{R}$ ) exists in the skin (Maekawa et al. 2008). Thus, the potency of  $\text{LTE}_4$  as an inducer of inflammatory and physiological effects in vivo is not explained by the pharmacology of the classical GPCRs for cys-LTs, which preferentially bind the metabolic precursors of  $\text{LTE}_4$ . Thus, the three cys-LTs are all potent mediators, and show considerable tissue specificity for their respective actions. Both a 5-LO inhibitor (zileuton) and the drugs that block  $\text{CysLT}_1\text{R}$  (Knorr et al., 1998) show clinical efficacy in asthma, despite the negligible activity of  $\text{LTE}_4$  at  $\text{CysLT}_1\text{R}$  and the fact that zileuton blocks only  $\sim 50\%$  of cys-LT

generation in vivo (Israel et al., 1996; Liu et al., 1996). Identification of receptors and pathways through which  $\text{LTE}_4$  exerts its effects may be highly significant in terms of the pathobiology of mucosal inflammation, as well as the treatment of asthma, AERD, and related diseases in which local concentrations of  $\text{LTE}_4$  are elevated and/or end-organ reactivity to  $\text{LTE}_4$  is high.

MCs respond strongly to cys-LTs and are a useful cell type for modeling cys-LT-induced signaling events and receptor functions. We previously demonstrated that human and mouse MCs express both  $\text{CysLT}_1\text{R}$  (Mellor et al., 2001) and  $\text{CysLT}_2\text{R}$  (Mellor et al. 2003) and that these receptors constitutively form heterodimers on this cell type (Jiang et al., 2007). Stimulation of MCs with  $\text{LTD}_4$ , the most potent agonist of the  $\text{CysLT}_1\text{R}$ , transactivates the kit tyrosine kinase (Jiang et al., 2006), induces calcium flux (Mellor et al., 2001), and phosphorylates extracellular signal-regulated kinase (ERK; Jiang et al., 2006). These signaling events amplify MC proliferation and induce their generation of cytokines and chemokines (Mellor et al., 2002).  $\text{CysLT}_1\text{R}$  is required for all of these  $\text{LTD}_4$ -induced responses, whereas  $\text{CysLT}_2\text{R}$  inhibits them (Jiang et al., 2007). MCs also express several P2Y receptors (Feng et al., 2004). We recently reported that  $\text{LTE}_4$  induces ERK activation and COX-2 expression, and causes prostaglandin (PG)  $\text{D}_2$  and macrophage inflammatory protein 1 $\beta$  (MIP-1 $\beta$ ) generation by LAD2 cells, a well-differentiated human MC line (Kirshenbaum et al. 2003; Paruchuri et al., 2008), and to a lesser extent by primary cord blood-derived human MCs (hMCs).  $\text{LTE}_4$ -mediated production of  $\text{PGD}_2$  by LAD2 cells was unaffected by short hairpin RNA (shRNA)-mediated knockdown of either  $\text{CysLT}_1\text{R}$  or  $\text{CysLT}_2\text{R}$  (Paruchuri et al., 2008), supporting the presence of a previously unrecognized  $\text{LTE}_4$ -reactive receptor on this cell type. A computer model (Nonaka et al., 2005) had predicted that the  $\text{P2Y}_{12}$  receptor, an adenosine diphosphate (ADP)-reactive GPCR which is the target of the thienopyridine anti-thrombotic drugs (Foster et al., 2001), could be an  $\text{LTE}_4$  receptor. In this study, we demonstrate a potent unique proinflammatory function for  $\text{LTE}_4$  in the lung, and we demonstrate that  $\text{P2Y}_{12}$  receptors are required for the functions of  $\text{LTE}_4$  in vitro and in the lung in vivo. Our results suggest that  $\text{P2Y}_{12}$  receptors may be a novel target for the treatment of asthma.

## RESULTS

### Recombinant human $\text{P2Y}_{12}$ receptors convey cellular responses to $\text{LTE}_4$

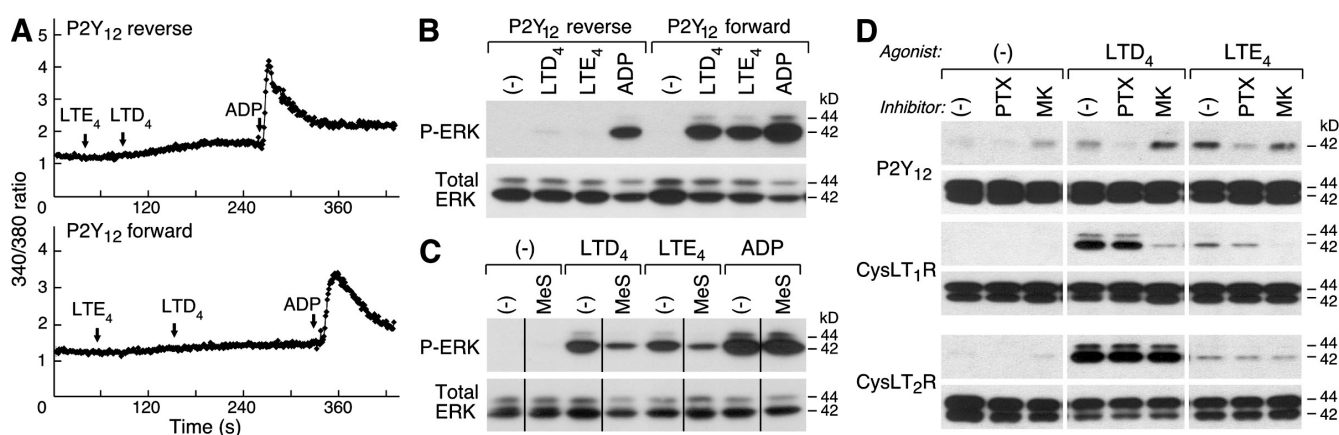
To determine if  $\text{P2Y}_{12}$  receptors could mediate responses to  $\text{LTE}_4$ , we stably transfected Chinese hamster ovary (CHO) cells (which do not natively express classical  $\text{CysLTR}$ s [Maekawa et al., 2001] or  $\text{P2Y}_{12}$  receptors) with constructs encoding the human  $\text{P2Y}_{12}$  receptor protein in forward and reverse orientation. The transfectants were stimulated with exogenous  $\text{LTC}_4$ ,  $\text{LTD}_4$ , or  $\text{LTE}_4$ . Fura-2 AM-loaded CHO cells expressing  $\text{P2Y}_{12}$  receptors failed to flux calcium in response to  $\text{LTE}_4$  but did exhibit a response to ADP, reflecting

endogenous P2Y<sub>1</sub> receptors (Fig. 1 A). However, CHO cells transfected with the P2Y<sub>12</sub> receptor construct in forward (but not reverse) orientation responded to LTE<sub>4</sub> (and to LTD<sub>4</sub>) with ERK activation (Fig. 1 B), which occurred at doses as low as 1 nM and reached a plateau at 100 nM (Fig. S1). The cys-LT-induced ERK activation was partially blocked by pretreatment of the cells with 2-methylthioadenosine monophosphate (2-MesAMP), a selective antagonist of the P2Y<sub>12</sub> receptor (Fig. 1 C), and was also sensitive to pertussis toxin (PTX; Fig. 1 D) but was resistant to MK571 (Fig. 1 D), an inhibitor which blocks CysLT<sub>1</sub>R and some P2Y receptors (Mamedova et al., 2005). Although ERK activation in the P2Y<sub>12</sub> receptor transfectants was at least as robust in response to LTE<sub>4</sub> as to LTD<sub>4</sub> (Fig. 1 D), CysLT<sub>1</sub>R and CysLT<sub>2</sub>R transfectants reacted to LTD<sub>4</sub> in marked preference to LTE<sub>4</sub>, even at high (500 nM) ligand concentrations. These responses were resistant to PTX and 2-MesAMP and, in the case of the CysLT<sub>1</sub>R transfectants, susceptible to MK571.

### P2Y<sub>12</sub> receptor requirement for LTE<sub>4</sub>-mediated activation and binding to LAD2 cells

We had previously reported that LAD2 cells (a well-differentiated human MC sarcoma line; Kirshenbaum et al., 2003) exhibited activation responses to LTE<sub>4</sub> > LTD<sub>4</sub>. To determine whether P2Y<sub>12</sub> receptors were responsible for these responses, Fura-2 AM-loaded LAD2 cells were stimulated with LTD<sub>4</sub> or LTE<sub>4</sub> in the presence or absence of 2-MesAMP or MK571. As reported previously (Paruchuri et al., 2008), MK571 blocked calcium responses of LAD2 cells to both ligands (unpublished data). In contrast, 2-MesAMP treatment

failed to attenuate LTD<sub>4</sub>- or LTE<sub>4</sub>-mediated calcium flux in LAD2 cells (Fig. 2 A). We determined the effect of MK571 and 2-MesAMP on MIP-1 $\beta$  generation by LAD2 cells in response to stimulation for 6 h with LTD<sub>4</sub> and LTE<sub>4</sub>, using IgE plus anti-IgE as a positive control. LTE<sub>4</sub> at doses of 100 and 500 nM induced the generation of large amounts of MIP-1 $\beta$ , exceeding the amounts generated in response to LTD<sub>4</sub> and to IgE plus anti-IgE (Fig. 2 B). Pretreatment of the LAD2 cells with 2-MesAMP blocked the LTE<sub>4</sub>-mediated increment in MIP-1 $\beta$  production by >50% (Fig. 2 B). 2-MesAMP also reduced the response to the higher concentrations of LTD<sub>4</sub>. MK571 suppressed the response to both ligands and was additive with 2-MesAMP for the suppression of the response to LTD<sub>4</sub>. Neither antagonist altered the production of MIP-1 $\beta$  in response to IgE plus anti-IgE. To exclude potential off-target effects of the inhibitors, samples of LAD2 cells were transfected with lentiviruses encoding a P2Y<sub>12</sub> sequence-specific shRNA, a CysLT<sub>1</sub>R-specific shRNA, or an empty vector control before stimulation. Knockdown of P2Y<sub>12</sub> receptors decreased the receptor messenger RNA (mRNA) expression by ~90% (Fig. 2 D) and did not alter expression of CysLT<sub>1</sub>R or CysLT<sub>2</sub>R proteins (not depicted). P2Y<sub>12</sub> receptor knockdown nearly abrogated MIP-1 $\beta$  production, completely eliminated PGD<sub>2</sub> production in response to LTE<sub>4</sub>, and minimally affected the response to LTD<sub>4</sub>. The knockdown of CysLT<sub>1</sub>R completely blocked MIP-1 $\beta$  and PGD<sub>2</sub> production in response to LTD<sub>4</sub> and slightly (but not significantly) decreased the responses to LTE<sub>4</sub> (Fig. 2 C, bottom). Neither knockdown altered MIP-1 $\beta$  generation or PGD<sub>2</sub> production in response to IgE-anti-IgE (Fig. 2 C). LTE<sub>4</sub>-mediated



**Figure 1. Activation by recombinant human P2Y<sub>12</sub> receptors in response to cys-LTs.** The human P2Y<sub>12</sub> receptor complementary DNA (cDNA) was cloned in forward and reverse orientation into the expression vector pEF1/HIS B and transfected into CHO cells using Fugene HD reagent. Stably expressing clones were selected using 1,000  $\mu$ g/ml G418, and expression of the construct was confirmed by cytofluorographic detection of the polyhistidine (HIS) tag. (A) Calcium fluxes in CHO transfectants in response to 500 nM LTD<sub>4</sub>, 500 nM LTE<sub>4</sub>, and 100  $\mu$ M ADP. Results in a second experiment were identical. (B) SDS-PAGE immunoblots showing phosphorylation of ERK2 by CHO cells stably expressing human P2Y<sub>12</sub> receptors in reverse (negative control) or forward orientations. Cells were stimulated with 100  $\mu$ M ADP (positive control ligand), 500 nM LTD<sub>4</sub>, or 500 nM LTE<sub>4</sub> for 15 min. The blots were stripped and reprobed with an antibody recognizing total ERK1 and ERK2. Dose responses are displayed in Fig. S1. (C) Effect of the selective P2Y<sub>12</sub> receptor antagonist 2-MesAMP (MeS) on ligand-induced ERK phosphorylation. P2Y<sub>12</sub> receptor-expressing CHO cells were stimulated with the same doses of agonists used in A, in the absence or presence of 100  $\mu$ M 2-MesAMP. (D) Comparison of P2Y<sub>12</sub> receptor-mediated responses to cys-LTs with those of recombinant human CysLT<sub>1</sub>R and CysLT<sub>2</sub>R expressed in CHO cells, and the effect of 1  $\mu$ M of the CysLT<sub>1</sub>R antagonist MK571. Data in B–D are from individual experiments that were repeated at least three times with similar results.

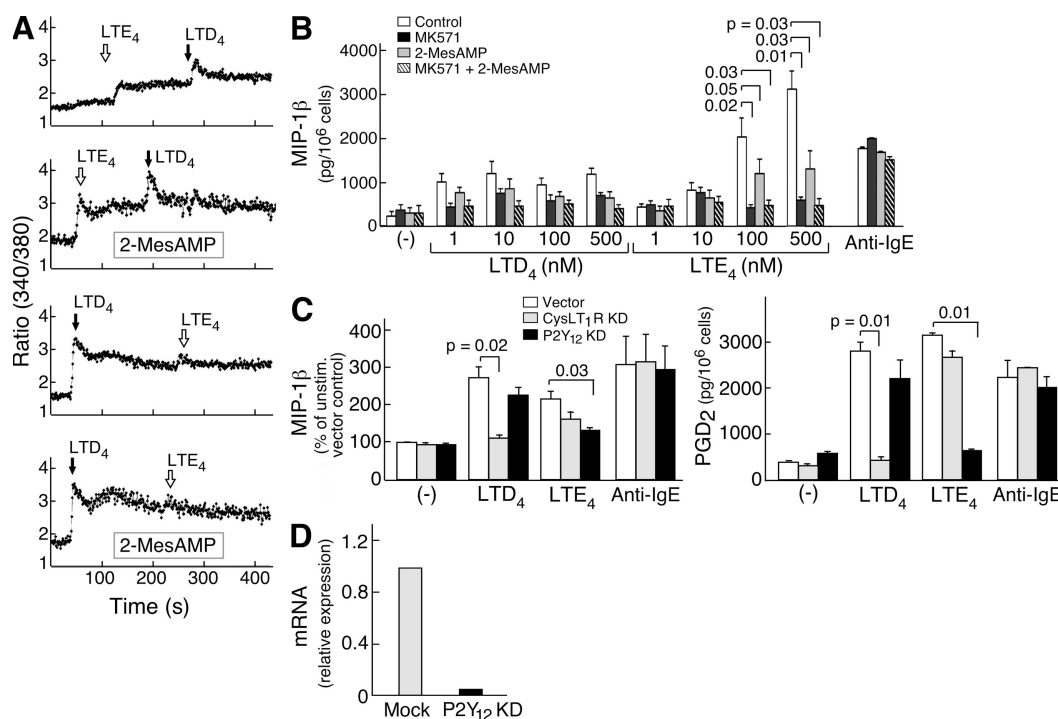
MIP-1 $\beta$  generation (Fig. S2 A) and ERK activation (Fig. S2 B) were not altered by treatment of LAD2 cells with apyrase to degrade extracellular ADP.

To determine whether P2Y<sub>12</sub> receptors directly mediated LTE<sub>4</sub> binding, membranes were prepared from LAD2 cells with and without P2Y<sub>12</sub> knockdown. We first performed competitive radioligand binding assays using [<sup>3</sup>H]ADP (the known natural ligand of P2Y<sub>12</sub> receptors) and unlabeled LTs as competitors. Unlabeled LTE<sub>4</sub> competed with labeled ADP, blocking 39  $\pm$  9 and 50  $\pm$  9% of specific ADP binding at doses of 0.1 and 1 nM, respectively, and reaching a plateau (60  $\pm$  7%) at 1  $\mu$ M (mean  $\pm$  SEM for four separate experiments, as shown for one experiment [Fig. S3 A]). LTE<sub>4</sub> was more efficacious than LTD<sub>4</sub> (Fig. S3 B). Knockdown of P2Y<sub>12</sub> receptors reduced binding of [<sup>3</sup>H]ADP by 40–60% and completely eliminated competition by LTE<sub>4</sub> (Fig. S3, A and B, right). LAD2 cell membranes weakly bound [<sup>3</sup>H]LTE<sub>4</sub> (converted from commercially prepared [<sup>3</sup>H]LTD<sub>4</sub>; Fig. S3 C), but specific binding of [<sup>3</sup>H]LTE<sub>4</sub> was not altered by the knockdown of P2Y<sub>12</sub> receptor (Fig. S3 C). To determine whether LTE<sub>4</sub> could block the ADP binding of P2Y<sub>12</sub> receptors expressed in isolation, the human forward and reverse P2Y<sub>12</sub> constructs were transiently expressed in COS-7 cells. The membranes

from the transfectants expressing the forward construct bound [<sup>3</sup>P]2-MesADP, a selective P2Y<sub>12</sub> receptor agonist. In contrast to ADP binding to LAD2 membranes, this binding was not competed by unlabeled LTE<sub>4</sub> or LTD<sub>4</sub> (Fig. S3 D). Additionally, these membranes failed to bind [<sup>3</sup>H]LTE<sub>4</sub> (unpublished data).

### LTE<sub>4</sub> potentiates allergen-induced bronchial inflammation

To determine whether LTE<sub>4</sub> could induce or potentiate histological signatures of pulmonary inflammation in mice, we determined the effect of 2.2 nmol of intranasal LTE<sub>4</sub> administered on three successive days on the lung histology of naive BALB/c mice. We compared the effect to that of LTD<sub>4</sub>. The lungs of naive BALB/c mice showed no evidence of cellular influx or goblet cell metaplasia after three doses of either cys-LT alone (unpublished data). Thus, we sought to determine whether either cys-LT amplified pulmonary inflammation induced by the inhalation of low-dose allergen in sensitized mice. 2 wk after sensitization with chicken egg OVA by i.p. injection, BALB/c mice received inhalation challenges on three consecutive days with low-dose OVA (0.1%) for 30 min. 30 min before each challenge, the mice received 2.2 nmol of intranasal LTD<sub>4</sub>, LTE<sub>4</sub>, or a buffer control. A cohort of mice treated



**Figure 2. Requirement for P2Y<sub>12</sub> receptors in LTE<sub>4</sub>-mediated MC activation.** (A) Calcium flux of LAD2 cells stimulated with 500 nM of the LTs in the presence or absence of 100  $\mu$ M 2-MesAMP. MK571 completely blocked both LTD<sub>4</sub>- and LTE<sub>4</sub>-mediated calcium fluxes (not depicted). Results in two additional experiments were similar. (B) Effect of 100  $\mu$ M 2-MesAMP, 1  $\mu$ M MK571, or both on MIP-1 $\beta$  generation by LAD2 cells in response to the indicated doses of LTD<sub>4</sub> and LTE<sub>4</sub>. Cells sensitized overnight with IgE and challenged with anti-IgE were used as the positive control. (C) Effect of shRNA-mediated knockdowns of CysLT<sub>1</sub>R or P2Y<sub>12</sub> receptors on MIP-1 $\beta$  generation (left) and PGD<sub>2</sub> generation (right) by LAD2 cells stimulated with 100 nM LTD<sub>4</sub> or 100 nM LTE<sub>4</sub>. Results in B and C are mean  $\pm$  SEM from three independent experiments each. Statistical differences were assessed by analysis of variance. (D) Effect of shRNA-mediated knockdown of P2Y<sub>12</sub> receptor mRNA expression by LAD2 cells. Real-time PCR was performed on an ABI PRISM 7700 Sequence detection system. FACS analysis was used to confirm the knockdowns of CysLT<sub>1</sub>R (not depicted). Mock, cells transfected with empty lentivirus. Results in two additional experiments were similar.



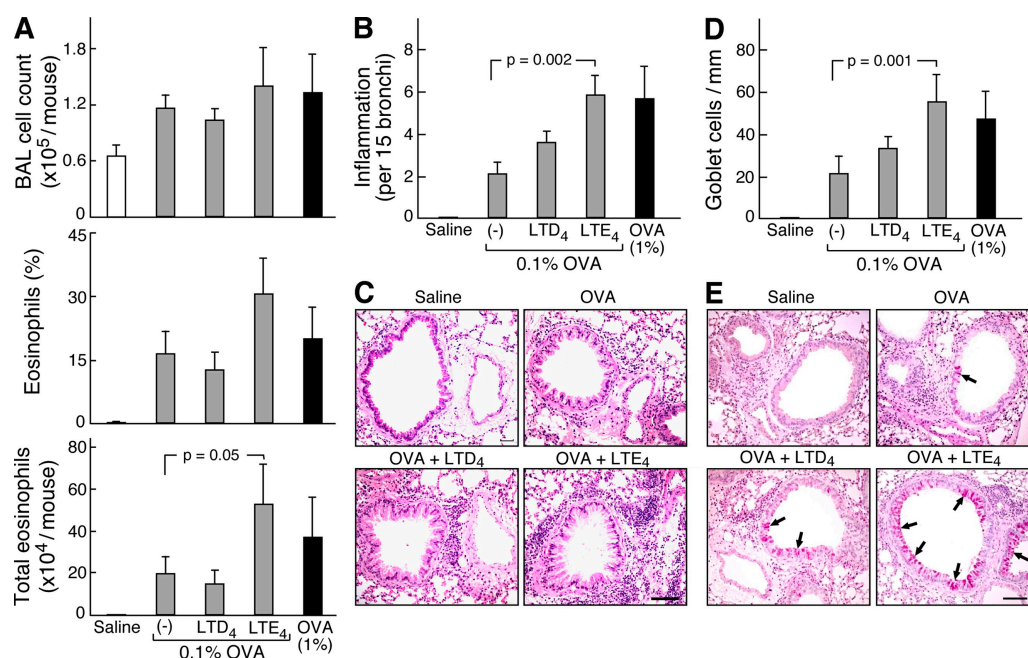
with 1% OVA were maintained as a positive control. The mice were then euthanized, their bronchoalveolar lavage (BAL) fluid was collected, and their lungs were examined histologically for evidence of inflammation and goblet cell metaplasia. Compared with saline-treated sensitized mice, mice challenged with low-dose OVA demonstrated low-grade BAL fluid eosinophilia (Fig. 3 A). These mice showed very mild pulmonary inflammation, as indicated by the accumulation of lymphocytes, plasma cells, and eosinophils around the bronchovascular bundles (Fig. 3, B and C). The administration of LTD<sub>4</sub> did not increase BAL fluid eosinophilia (Fig. 3 A) and slightly potentiated both bronchovascular inflammation (Fig. 3, B and C) and goblet cell metaplasia (Fig. 3, D and E). In contrast, LTE<sub>4</sub> significantly enhanced BAL fluid eosinophilia (Fig. 3 A), inflammation (Fig. 3, B and C), and goblet cell metaplasia (Fig. 3, D and E). The extent of the cellularity and goblet cell responses of the LTE<sub>4</sub>-treated animals approached the levels of these parameters in the mice treated with 1% OVA.

### LTE<sub>4</sub>-mediated pulmonary inflammation depends on P2Y<sub>12</sub> receptors and is independent of classical CysLTRs

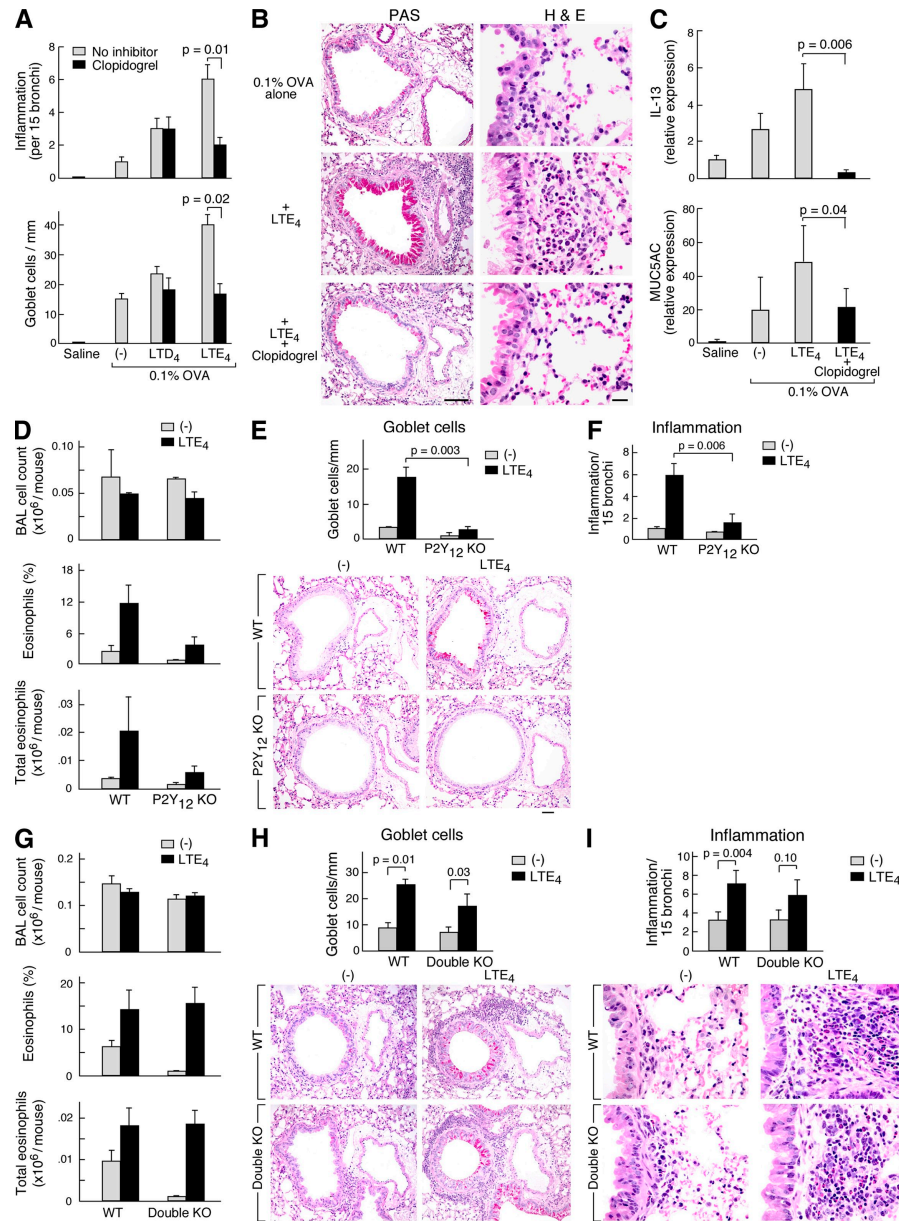
To determine whether P2Y<sub>12</sub> receptors accounted for the LTE<sub>4</sub>-mediated augmentation of pulmonary inflammation, sensitized mice were treated with clopidogrel, an antithrombotic agent that is converted in vivo to an active metabolite

that covalently binds to and irreversibly inactivates P2Y<sub>12</sub> receptors (Savi et al., 2006). Drug treatment began 2 d before the first administration of LTs to allow conversion of the prodrug. A separate cohort of sensitized mice was maintained without clopidogrel treatment as a control group. Both groups were challenged with low-dose OVA with or without additional LTE<sub>4</sub>. Treatment with clopidogrel eliminated LTE<sub>4</sub>-induced potentiation of both inflammation and goblet cell metaplasia (Fig. 4, A and B). To determine the effect of P2Y<sub>12</sub> receptor blockade on the induced expression of mRNAs encoding proteins involved in goblet cell metaplasia, real-time PCR was used to analyze the lungs of the mice for the expression of IL-13 and the goblet cell-associated glycoprotein MUC5AC. LTE<sub>4</sub> modestly increased the expression of both transcripts, whereas clopidogrel treatment substantially suppressed the expression of both (Fig. 4 C). There was no detectable induction of IL-4 or IL-5 transcripts (unpublished data).

Because pharmacologic antagonists can act in an off-target manner, we sought to determine the receptors required to mediate the effect of LTE<sub>4</sub> in the lungs of allergen-sensitized and -challenged mice using a molecular approach. First, we studied the ability of LTE<sub>4</sub> to amplify pulmonary inflammation in OVA-sensitized and -challenged C57BL/6 mice lacking P2Y<sub>12</sub> receptors (*p2ry12*<sup>-/-</sup> mice; Andre et al., 2003)



**Figure 3. LTE<sub>4</sub>-mediated amplification of allergen-induced pulmonary inflammation.** Male BALB/c mice (6–8 wk old) were sensitized on days 0 and 7 with i.p. injections of 10  $\mu$ g Alum-precipitated chicken egg OVA. Mice were challenged with LTs on days 13–15 and OVA on days 14–16 as described in the text. Mice were euthanized 24 h after their third and final aerosol challenge. (A) BAL fluid total cell counts (top) with percentages (middle) and total numbers per mouse (bottom) of eosinophils. (B) Quantitative analysis of pulmonary inflammation performed on 0.5- $\mu$ m-thick glycol methacrylate sections of lungs. The extent of cellular infiltration in 15 bronchovascular bundles of each mouse in hematoxylin and eosin (H&E)-stained sections was evaluated without knowledge of the treatment groups. (C) Representative fields of H&E-stained lungs from mice from the indicated experimental groups. (D) Morphometric analysis of goblet cell metaplasia, measured as the numbers of periodic acid–Schiff (PAS)-positive goblet cells per millimeter of bronchial basal lamina (determined by ImageJ image analysis software). (E) Representative PAS stains showing goblet cells (arrows). Results in A, B, and D are mean  $\pm$  SEM from eight to nine mice per group. The experiments were repeated three times. Statistical differences were determined by analysis of variance. Bars, 100  $\mu$ m.



**Figure 4. Role of P2Y12 receptors on potentiation of pulmonary inflammation by LTE<sub>4</sub>.** (A) Bronchovascular inflammation (top) and goblet cell metaplasia in sensitized mice challenged with low-dose OVA with or without the prior administration of 2.2 nmol LTD<sub>4</sub> or LTE<sub>4</sub> 30 min before each challenge. 500 µg/ml clopidogrel was added to the drinking water of the indicated groups of mice for 72 h before the first intranasal dose of LTs and was maintained throughout the treatment. Results are mean ± SEM from at least nine mice in each group. The experiments were repeated three times with similar results. (B) PAS stains (left) from representative mice in the indicated groups showing the effect of clopidogrel on goblet cell metaplasia. Higher magnification images of H&E stains (right) from the same animals showing cellular characteristics of the bronchovascular infiltrates. (C) Effect of clopidogrel administration on the steady-state expression of IL-13 and MUC5AC mRNA as determined by real-time PCR of whole lung RNA extracted 24 h after the last OVA challenge of the indicated groups. Data are mean ± SEM from four to five mice per group from a single experiment. Results in a second experiment were similar. (D–F) Male and female C57BL/6 *p2ry12*<sup>-/-</sup> mice and age- and sex-matched controls were sensitized and challenged with 0.1% aerosolized OVA on three consecutive days with or without intranasal LTE<sub>4</sub> 30 min before each challenge. (D) Total cell numbers (top), percentages of eosinophils (middle), and total numbers of eosinophils (bottom) in BAL fluid recovered 24 h after the last challenge with OVA. (E) Goblet cell metaplasia (top) and representative PAS stains (bottom) from WT and *p2ry12*<sup>-/-</sup> mice subjected to the same protocol. (F) Inflammation scores from the same mice. Data in D–F are from four mice per group. Results in a second experiment were similar. (G) BALB/c *Cysltr1/Cysltr2*<sup>-/-</sup> mice and age-matched WT controls were subjected to the same protocol as the *p2ry12*<sup>-/-</sup> mice. Total cell numbers (top), percentages of eosinophils (middle), and total numbers of eosinophils (bottom) in BAL fluid recovered 24 h after the last challenge with OVA. Results are from six mice per group. Three experiments were performed with similar results. (H) Goblet cell numbers (top) and representative PAS stains (bottom). (I) Quantitative assessment of bronchovascular inflammation as determined by H&E stain (top). Results are from six mice per group. Representative stains are shown (bottom). Error bars represent ± SEM. Bars, 100 µm.

along with age- and sex-matched C57BL/6 controls. Although cellular infiltration into the lung and BAL fluid was less pronounced in the C57BL/6 mice than in the BALB/c mice, LTE<sub>4</sub> potentiated BAL fluid eosinophilia (Fig. 4 D), goblet cell metaplasia (Fig. 4 E), and bronchovascular infiltration (Fig. 4 F) in the WT controls, all of which were severely blunted in the *p2ry12*<sup>-/-</sup> mice (Fig. 4, D–F). To determine whether classical GPCRs for cys-LTs were also required for the LTE<sub>4</sub> effects, BALB/c mice lacking both CysLT<sub>1</sub>R and CysLT<sub>2</sub>R (*Cysltr1/Cysltr2*<sup>-/-</sup> mice; Maekawa et al., 2008) were also studied. Because of limited numbers of available mice, all animals were sensitized and challenged with low-dose OVA, and half were treated with exogenous LTE<sub>4</sub>. Strikingly, the potentiation of OVA-induced BAL fluid eosinophilia (Fig. 4 G), goblet cell metaplasia (Fig. 4 H), and inflammation (Fig. 4 I) by LTE<sub>4</sub> were completely intact in the *Cysltr1/Cysltr2*<sup>-/-</sup> mice, indicating that LTE<sub>4</sub> was working independently of the known GPCRs for cys-LTs. Thus, the intrapulmonary actions of LTE<sub>4</sub> in vivo require P2Y<sub>12</sub> receptors but not classical cys-LT-reactive GPCRs.

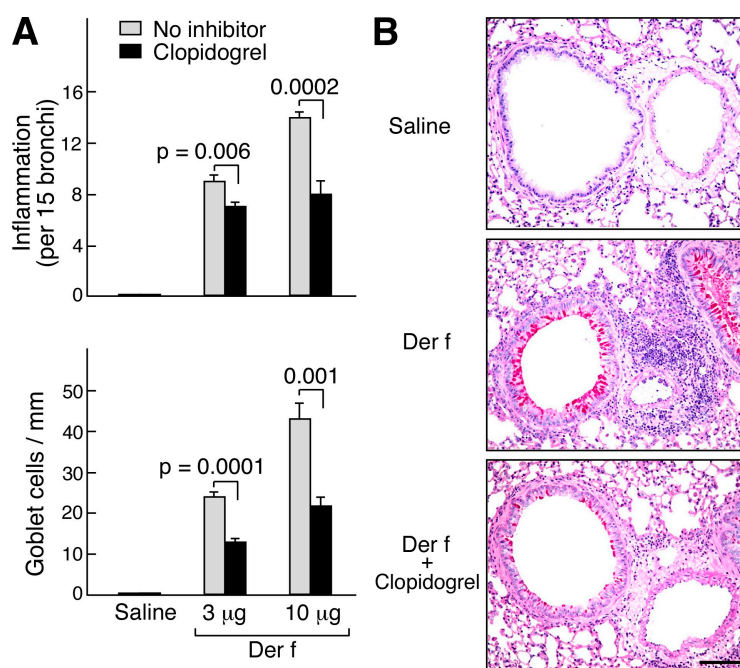
### Blockade of P2Y<sub>12</sub> receptors blunts pulmonary inflammation induced by house dust mite antigen

To determine whether pharmacologic blockade of P2Y<sub>12</sub> receptors altered pulmonary inflammatory responses in a more physiological model of pulmonary inflammation, C57BL/6 mice were administered an extract of house dust mite *Derma-tophagoides farinae* (Der f) intranasally twice weekly for 3 wk,

with or without clopidogrel treatment. Two different doses of Der f were used to elicit moderate (3 µg) and severe (10 µg) inflammation, respectively. At both antigen doses, the mice treated with clopidogrel showed ~75% attenuation of BAL fluid eosinophilia (unpublished data), as well as significant reductions in pulmonary inflammation, and goblet cell metaplasia compared with the cohort that did not receive clopidogrel (Fig. 5, A and B).

### LTE<sub>4</sub>/P2Y<sub>12</sub> receptor-mediated amplification of pulmonary inflammation requires platelets

Because P2Y<sub>12</sub> receptors are essential for normal platelet activation in vivo (Andre et al., 2003), we sought to determine whether platelets were required for the response of sensitized challenged mice to exogenous LTE<sub>4</sub>. Platelets were depleted in sensitized BALB/c mice by the i.v. injection of a rat mAb against mouse CD42b (GPIIbα; Nieswandt et al., 2000) or an isotype-matched control IgG 48 h before to the first administration of LTD<sub>4</sub> or LTE<sub>4</sub>. Treatment with the anti-CD42b antibody depleted platelets almost completely (99% depletion [unpublished data]) at the time of the first dose of LT. Platelet depletion resulted in a complete loss of the LTE<sub>4</sub>-mediated potentiation of airway eosinophilia, inflammation, and goblet cell metaplasia (Fig. 6, A and B). The effect of platelet depletion was identical to the effect of treatment of the mice with clopidogrel, and the two treatments were not additive (Fig. 6 B). Platelet depletion, like clopidogrel treatment, sharply reduced the LTE<sub>4</sub>-mediated expression of mRNA encoding IL-13



**Figure 5. Role of P2Y<sub>12</sub> receptors in inflammation mediated by dust mite allergen.** C57BL/6 mice were treated intranasally with the indicated dose of Der f extract twice weekly for 3 wk. Animals were euthanized 24 h after the last dose. (A) Effect of clopidogrel treatment on bronchovascular inflammation and goblet cell metaplasia in C57BL/6 mice subjected to intranasal challenge with the indicated doses of an extract from the house dust mite. Results are the mean ± SEM from five mice per group. The experiment was repeated three times with similar results. (B) Representative PAS stains of the lungs from mice in the indicated groups. Bar, 100 µm.



and MUC5AC (unpublished data). To determine whether LTE<sub>4</sub> alone induced platelet activation (leading to ADP release with potential resultant autocrine stimulation of P2Y<sub>12</sub> receptors), human blood platelets were stimulated with 1  $\mu$ M LTE<sub>4</sub> or with 100  $\mu$ M ADP, and degranulation was assessed by cytofluorographic detection of P-selectin (CD62P). As expected, ADP elicited CD62P expression, but no CD62P expression was detected in response to stimulation with LTE<sub>4</sub> (Fig. 6 C).

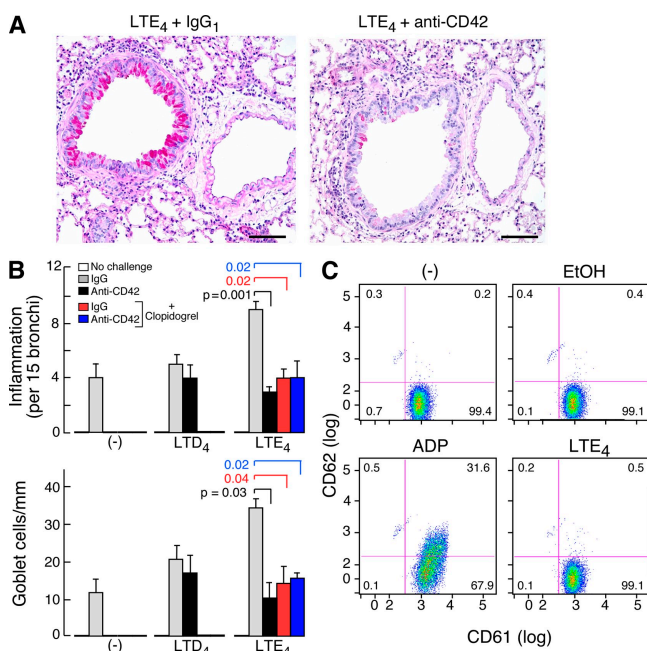
## DISCUSSION

This study establishes that P2Y<sub>12</sub> receptors are essential for the actions of LTE<sub>4</sub>, the only long-lived stable abundant member of the cys-LTs, in inflamed lung. Our findings help to explain long-recognized but unexplained properties of LTE<sub>4</sub> in airway biology. The involvement of cys-LTs in the pathobiology

of asthma is established by the fact that 5-LO inhibitors (Israel et al., 1996; Liu et al., 1996) and CysLT<sub>1</sub>R antagonists (Knorr et al., 1998) have clinical efficacy. The cloning and functional characterization of the CysLT<sub>1</sub>R (Lynch et al., 1999) and CysLT<sub>2</sub>R (Heise et al., 2000) explained the pharmacology of LTC<sub>4</sub> and LTD<sub>4</sub> predicted from studies of contractile tissues (Lee et al., 1984). The finding that neither GPCR showed significant binding or reactivity to LTE<sub>4</sub> was surprising given the plethora of data in human and animal studies that indicate the unique characteristics of this stable ligand relative to its short-lived precursors (Christie et al., 1992a, 1993; Laitinen et al., 1993; Gauvreau et al., 2001). Because we had previously demonstrated that LTE<sub>4</sub> could activate LAD2 cells by a mechanism independent of CysLT<sub>1</sub>R and CysLT<sub>2</sub>R (Paruchuri et al., 2008), and because LTE<sub>4</sub> was previously identified by an *in silico* model as a potential surrogate ligand for the P2Y<sub>12</sub> receptor (Nonaka et al., 2005), we undertook this study to determine whether the P2Y<sub>12</sub> receptor was a bona fide LTE<sub>4</sub>-reactive receptor and to determine its contribution to LTE<sub>4</sub>-induced proinflammatory events.

We first established that recombinant human P2Y<sub>12</sub> receptor protein conveyed activation responses by CHO cells to LTE<sub>4</sub>. Unlike most members of the P2Y receptor class, native P2Y<sub>12</sub> receptors do not couple to G $\alpha$ q proteins or activate calcium flux; instead, they induce signaling through PTX-sensitive G $\alpha$ i2 proteins when stimulated with ADP (Foster et al. 2001; Lova et al., 2002; Woulfe et al., 2002). It was, thus, not surprising that CHO cells expressing P2Y<sub>12</sub> receptors failed to flux calcium in response to cys-LTs (Fig. 1 A) or that blockade of P2Y<sub>12</sub> receptors on LAD2 cells with 2-MesAMP failed to alter cys-LT-mediated calcium flux (Fig. 2 A), which was totally abrogated by MK571. However, heterologously expressed P2Y<sub>12</sub> receptors responded to LTE<sub>4</sub> with PTX-sensitive ERK activation (Fig. 1). The dose range for this response (Fig. S1) is similar to the LTE<sub>4</sub> dose range required to compete with radiolabeled ADP for binding to LAD2 cell membranes (Fig. S3) and is consistent with that reported by Nonaka et al. (2005) using a P2Y<sub>12</sub>-G $\alpha$ 16 fusion protein to demonstrate calcium flux, also in CHO cells. The finding that cys-LT-induced ERK activation in the transfectants was resistant to MK571 (Fig. 1 D) implies that P2Y<sub>12</sub> receptors could contribute to an element of cys-LT-driven pathobiology that is relatively selective for LTE<sub>4</sub> and may resist conventional CysLT<sub>1</sub>R antagonists. This element may be especially relevant to pathological situations where LTE<sub>4</sub> is abundant as a result of its relative stability.

In our previous study, LTE<sub>4</sub> had exhibited unanticipated potency for inducing ERK activation and the generation of MIP-1 $\beta$  and COX-2-dependent PGD<sub>2</sub> by LAD2 cells (Paruchuri et al., 2008). Because both ERK and calcium-dependent transcriptional events are essential for MC activation, we sought to determine the potential contribution of P2Y<sub>12</sub> receptors to the activation responses of LAD2 cells to LTE<sub>4</sub> and to contrast these responses to those elicited by LTD<sub>4</sub>, the most potent CysLT<sub>1</sub>R ligand. Although ineffective for blocking cys-LT-induced calcium flux, 2-MesAMP effectively



**Figure 6. Platelet dependence of the LTE<sub>4</sub> effect on bronchial inflammation and goblet cell metaplasia.** Sensitized mice were treated i.v. with 50  $\mu$ g ( $\sim$ 2  $\mu$ g/g body weight) of a monoclonal rat IgG directed against mouse CD42b (GPIIb $\alpha$ ) or an equal amount of isotype control (both from Cembret Analytics) 48 h before the first dose of LTE<sub>4</sub>. Each antibody was diluted in 50  $\mu$ l of sterile saline. The depletion of platelets was confirmed by automated counting, and some mice were treated with clopidogrel. (A) PAS stains of the lungs of representative mice treated with isotype control (top) or with an anti-CD42 platelet-depleting antibody (bottom). (B) Inflammation (top) and goblet cell metaplasia (bottom) in the lungs of mice treated with the indicated LT and antibody. A cohort of mice was treated with clopidogrel as indicated. Results in B are from a single experiment with five mice per group. A second experiment with an equal number of mice showed similar results. Error bars represent  $\pm$  SEM. (C) Cytofluorographic detection of surface expression of CD62P (P-selectin) by platelets stimulated for 10 min with 100  $\mu$ M ADP or 1  $\mu$ M LTE<sub>4</sub>. Percentages of CD61-positive platelets expressing CD62P are displayed in the top right quadrants. Results are from a single experiment performed three times on different donors. Bars, 100  $\mu$ m.



blocked the  $\text{LTE}_4$ -mediated increment in MIP-1 $\beta$  production (Fig. 2 B) and also reduced the response to the higher concentrations of  $\text{LTD}_4$ , indicating that  $\text{LTD}_4$  at high concentrations can also activate native  $\text{P2Y}_{12}$  receptors, as supported by its actions as an agonist for ERK activation in the  $\text{P2Y}_{12}$  receptor transfectants (Fig. 1). MK571 suppressed MIP-1 $\beta$  generation in response to both ligands (likely reflecting the requirement for calcium flux for chemokine generation) and was additive with 2-MesAMP for the suppression of the response to  $\text{LTD}_4$ . The shRNA-mediated knockdowns of  $\text{CysLT}_1\text{R}$  and  $\text{P2Y}_{12}$  receptors revealed strong dependence of  $\text{LTD}_4$ -mediated activation on  $\text{CysLT}_1\text{R}$ , whereas  $\text{LTE}_4$ -mediated responses were clearly  $\text{P2Y}_{12}$  receptor dependent (Fig. 2 C). Although there is segregation of the receptor requirements for these two related ligands on the same cell, the ability of  $\text{LTE}_4$  to “cross over” and induce some complementarities between the  $\text{CysLT}_1\text{R}$  (via  $\text{G}\alpha_q$  proteins and calcium-induced pathways) and  $\text{P2Y}_{12}$  receptors (via  $\text{G}\alpha_i$  proteins), particularly for chemokine generation. This is analogous to the cooperation by  $\text{G}\alpha_q$ -linked  $\text{P2Y}_1$  receptors with  $\text{P2Y}_{12}$  receptors in regulating ADP responses of platelets (Lova et al., 2002; Woulfe et al., 2002). The  $\text{P2Y}_{12}$ -dependent activation of LAD2 cells does not likely reflect autocrine effects of released ADP because it was resistant to treatment of the cells with the ectonucleotidase apyrase (Fig. S2).

Surprisingly, although  $\text{P2Y}_{12}$  receptors were essential for competition between  $\text{LTE}_4$  with ADP for binding to membranes of LAD2 cells (Fig. S3, A and B), they were not involved in the direct low-affinity binding of  $[3\text{H}]\text{LTE}_4$  to these same membranes (Fig. S3 C). Additionally,  $\text{LTE}_4$  could not compete for binding to  $\text{P2Y}_{12}$  receptors expressed in isolation on COS-7 cells (Fig. S3 D). The fact that  $\text{P2Y}_{12}$  receptors do not directly bind  $\text{LTE}_4$  despite their essential nature implies that they are components of a complex with another  $\text{LTE}_4$ -reactive GPCR, perhaps with the putative  $\text{CysLT}_E\text{R}$  reported in the mouse skin (Maekawa et al., 2008). The facts that  $\text{P2Y}_{12}$  receptors are required for  $\text{LTE}_4$  to activate transfected CHO cells (Fig. 1) and LAD2 cells (Fig. 2), and for competition between  $\text{LTE}_4$  and ADP for binding to LAD2 cells (Fig. S3 B), and that knockdown of  $\text{P2Y}_{12}$  receptors did not reduce direct binding of radiolabeled  $\text{LTE}_4$  (Fig. S3 C) are all consistent with this thesis. Precedents for such complexes on MCs include  $\text{CysLT}_1\text{R}$  and  $\text{CysLT}_2\text{R}$  heterodimers (Jiang et al., 2007) and a functional requirement of  $\text{CysLT}_1\text{R}$  for the uridine diphosphate-reactive  $\text{P2Y}_6$  receptor (Jiang et al., 2009). Our data indicate that presence of  $\text{P2Y}_{12}$  is required for signaling and activation by  $\text{LTE}_4$  in a cell-specific context.

The fact that  $\text{LTE}_4$ , but not  $\text{LTD}_4$ , induces bronchial eosinophilia when administered by inhalation to the airways of individuals with asthma (Gauvreau et al., 2001) argued for the presence of an  $\text{LTE}_4$ -reactive receptor in inflamed lung. In our model, which was designed to study potentiation of submaximal bronchial inflammation in sensitized mice, we found that  $\text{LTE}_4$  exceeded the potency of  $\text{LTD}_4$  for potenti-

ating BAL fluid eosinophilia (Fig. 3 A), cellular infiltration of the bronchovascular bundles (Fig. 3, B and C), and goblet cell metaplasia (Fig. 3, D and E). Thus,  $\text{LTE}_4$  exceeds the efficacy of  $\text{LTD}_4$  for potentiating bronchial inflammation in both mouse and man, a pattern not explicable by the known properties of  $\text{CysLT}_1\text{R}$  or  $\text{CysLT}_2\text{R}$ . Several pieces of evidence link these  $\text{LTE}_4$ -mediated responses in mice to the  $\text{P2Y}_{12}$  receptor. First, treatment of the mice with a highly potent selective antagonist of the  $\text{P2Y}_{12}$  receptor, clopidogrel, completely eliminated the histological response to  $\text{LTE}_4$  (Fig. 4, A and B), which is associated with blockade of  $\text{LTE}_4$ -potentiated expression of IL-13, the Th2 cytokine most closely linked to the development of goblet cell metaplasia (Zhu et al., 1999), and of MUC5AC, a major mucus glycoprotein which is controlled by IL-13 (Fig. 4 C). Second,  $\text{LTE}_4$  fully amplified pulmonary inflammation in *Cysltr1/Cysltr2*<sup>-/-</sup> mice (Fig. 4, G–I). Lastly, the ability of  $\text{LTE}_4$  to potentiate mucosal inflammation and goblet cell metaplasia was nearly completely abrogated in the absence of  $\text{P2Y}_{12}$  receptors (Fig. 4, D–F). Thus,  $\text{LTE}_4$  requires  $\text{P2Y}_{12}$  receptors, but not the classical  $\text{CysLTRs}$ , to amplify the histological signatures of allergen-induced pulmonary inflammation. Because  $\text{LTD}_4$  is converted in vivo to  $\text{LTE}_4$ , it was somewhat surprising that the ligands do not behave interchangeably in potentiating bronchial eosinophilia in mouse or man. In addition to  $\text{P2Y}_{12}$ -dependent effects of  $\text{LTE}_4$ ,  $\text{LTD}_4$  may initiate inhibitory signals through  $\text{CysLT}_2\text{R}$  (Jiang et al., 2007) or other yet-to-be-identified GPCRs that recognize  $\text{LTD}_4$  but not  $\text{LTE}_4$ . The effects of clopidogrel in the model of airway disease induced by the natural allergen Der f without the use of exogenous  $\text{LTE}_4$  (Fig. 5) support the importance of the  $\text{P2Y}_{12}$  pathway in the integrated biology of pulmonary inflammation (although this does not discriminate between effects mediated by  $\text{LTE}_4$  from ADP). The blockade of the response by clopidogrel distinguishes the  $\text{P2Y}_{12}$  receptor-dependent response of the lung to  $\text{LTE}_4$  from the clopidogrel-resistant  $\text{LTE}_4$  response in the skin (Maekawa et al., 2008). Thus, it is likely that different receptors or receptor complexes mediate response to the stable ligand  $\text{LTE}_4$  in distinct anatomical distributions.

Platelets accumulate in the lungs of individuals with asthma (Jeffery et al., 1989) and also are recruited to the lungs of OVA-sensitized and -challenged mice by an IgE-dependent mechanism (Pitchford et al., 2008). Activated platelets generate mediators (serotonin and thromboxane) that can potentiate airway inflammation. The essential nature of  $\text{P2Y}_{12}$  receptors for normal platelet function (Andre et al., 2003) led us to examine the effect of platelet depletion on the response of sensitized challenged mice to exogenous  $\text{LTE}_4$ . Indeed, the response to  $\text{LTE}_4$  was totally abrogated by platelet depletion (Fig. 6, A and B). Whether the requirement for platelets is direct or indirect is less clear. The fact that  $\text{LTE}_4$  did not induce platelet expression of CD62P (an activation marker used as a surrogate for ADP release; Abrams and Shattil, 1991; Fig. 6 C) argues that  $\text{LTE}_4$  does not trigger an ADP-dependent autocrine loop initiated by  $\text{LTE}_4$ -mediated activation of another

receptor. Because platelet activation involves complementary signaling in both Gi and Gq protein-mediated pathways (Jin et al., 2002; Lova et al., 2002; Woulfe et al., 2002) and P2Y<sub>12</sub> provides only the Gi-linked component, LTE<sub>4</sub> (and P2Y<sub>12</sub>) likely synergizes in vivo with a second agonist to facilitate platelet functions in the lung. It is noteworthy that LTE<sub>4</sub>-mediated potentiation of the contractile responses of both guinea pig and human airway smooth muscle is COX dependent (Christie et al., 1992a) and was attributed to secondary generation of thromboxane, a major platelet-derived eicosanoid (Jacques et al., 1991). In retrospect, this finding may have reflected P2Y<sub>12</sub> receptor-dependent signaling on platelets or other cell types. The fact that LTE<sub>4</sub> potentiated inflammation only in sensitized challenged mice may reflect the previously described platelet-mediated pathway for leukocyte recruitment that depends on sensitization and IgE (Pitchford et al., 2008). The complete lack of LTE<sub>4</sub> reactivity in the face of platelet depletion argues against a role for MCs and other P2Y<sub>12</sub>-bearing cell types, at least in this model.

Our findings suggest a potential therapeutic application for P2Y<sub>12</sub> receptor antagonists that may be especially relevant to AERD, which is associated with both high levels of LTE<sub>4</sub> in the urine (Christie et al., 1992b) and selective hyper-responsiveness to LTE<sub>4</sub> (Christie et al., 1993). In AERD, both 5-LO antagonists and CysLT<sub>1</sub>R antagonists provide disease control and attenuate the consequences of aspirin challenge (Israel et al., 1993; Dahlén et al., 1998, 2002; White et al., 2006), in which the reaction is characterized by a marked surge in LTE<sub>4</sub> generation. The fact that the effects of LTE<sub>4</sub> persist in the absence of CysLT<sub>1</sub>R and CysLT<sub>2</sub>R implies that this pathway would be resistant to the available cys-LT receptor antagonists, all of which selectively block CysLT<sub>1</sub>R. We speculate that simultaneous interference with the bronchoconstrictive effects of LTD<sub>4</sub> (via CysLT<sub>1</sub>R) and with pro-inflammatory effects of LTE<sub>4</sub> (by P2Y<sub>12</sub>), or more complete blockade of 5-LO, might improve clinical efficacy. Our study furthermore highlights the biological significance of the structural and functional relationships between the P2Y and cys-LT-reactive classes of GPCRs.

## MATERIALS AND METHODS

**Animals.** All animal protocols were approved by the Dana Farber Cancer Institute Animal Care and Utilization Committee. BALB/c mice lacking both CysLT<sub>1</sub>R and CysLT<sub>2</sub>R (*Cysltr1/Cysltr2*<sup>-/-</sup> mice) and their WT littermate controls were derived as described elsewhere (Maekawa et al., 2008). Mice lacking P2Y<sub>12</sub> receptors (*p2ry12*<sup>-/-</sup> mice) were derived as described elsewhere (Andre et al., 2003) on a mixed C57BL/6-129 background and backcrossed for 10 generations with C57BL/6 mice. WT BALB/c and C57BL/6 mice were purchased from Taconic.

**Cell culture.** The LAD2 line (Kirshenbaum et al., 2003) isolated from the bone marrow of a patient with MC leukemia was a gift from A. Kirshenbaum (National Institutes of Health). These cells were cultured in StemPro-34 (Invitrogen) supplemented with 2 mM L-glutamine (Invitrogen), 100 IU/ml Pen-strep (Invitrogen), and 100 ng/ml stem cell factor (SCF; Thermo Fisher Scientific). Cell culture medium was hemidepleted every week with fresh medium and 100 ng/ml SCF. CHO cells were grown in DMEM/F-12 with 10% FBS and 100 IU/ml Pen-strep (Invitrogen). Primary cord blood MCs were derived in vitro in RPMI 1640 (Invitrogen) supplemented with 10%

fetal bovine serum (Sigma-Aldrich), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 0.2 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 μg/ml gentamycin supplemented with SCF, IL-6, and IL-10 (R&D Systems) as previously described (Ochi et al., 1999).

**Calcium flux.** 0.5–1 × 10<sup>6</sup> cells/sample were washed and labeled with Fura 2-AM for 30 min at 37°C. Cells were stimulated with the indicated concentrations of LTD<sub>4</sub> or LTE<sub>4</sub>, and changes in intracellular calcium concentration were measured using excitation at 340 and 380 nm in a fluorescence spectrophotometer (F-4500; Hitachi; Paruchuri et al., 2008). The relative ratios of fluorescence emitted at 510 nm were recorded and displayed as a reflection of intracellular calcium concentration. In some experiments, cells were preincubated with the 1 μM of the CysLT<sub>1</sub>R antagonist MK571 or with 100 μM of the P2Y<sub>12</sub> receptor antagonist 2-MesAMP for 5 min before the stimulation.

**Real-time quantitative PCR.** The expressions of human P2Y<sub>12</sub> receptor mRNA and mouse IL-13 and MUC5AC RNAs were determined with real-time PCR performed on an ABI PRISM 7700 Sequence detection system (Applied Biosystems). RNA was isolated with an RNeasy mini kit (QIAGEN) and was treated with RNase-free DNase (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized from 1 μg RNA with Superscript II RNase H-RT (Invitrogen). RT was performed using TaqMan RT reagents. Primers and FAM-labeled PCR mix were purchased from SABiosciences.

**shRNA knockdowns.** shRNA constructs targeting human CysLT<sub>1</sub>R and P2Y<sub>12</sub> receptors were purchased from Thermo Fisher Scientific. The constructs were cloned into a lentiviral vector (pLKO1; Thermo Fisher Scientific) and used to generate infectious particles with a lentiviral packaging mix (Virapower; Invitrogen) according to the manufacturer's protocol. The transfections were performed as described previously (Jiang et al., 2007). FACS analysis was used to confirm the knockdowns of CysLT<sub>1</sub>R, whereas quantitative PCR was used to verify the knockdown of the P2Y<sub>12</sub> receptor.

**Generation of stable P2Y<sub>12</sub> receptor transfectants.** A human P2Y<sub>12</sub> receptor cDNA was amplified by 30 cycles of PCR from RT total RNA extracted from primary hMCs. The primer sequences were 5'-CAACAAGAAATGCAAGCCGTCGA-3' and 5'-TACATTGGAGTCTCTTCATTG-3'. The fragment was cloned into a TA vector (Invitrogen). After verifying the nucleotide sequence, the fragment was subcloned into the multiple cloning site of the expression vector pEF1/His B, encoding a C-terminal HIS tag. A plasmid expressing the P2Y<sub>12</sub> receptor construct in the forward orientation was transfected into CHO cells using Eugene HD reagent according to manufacturer's protocol. A construct in the reverse sequence was transfected in parallel as a negative control. Stably expressing clones were selected in the medium containing 1,000 μg/ml G418 (Invitrogen), and expression of the construct was confirmed by FACS analysis of permeabilized cells with a mAb against the HIS tag.

**Cell activation.** LAD2 cells were stimulated with the indicated concentrations of LTD<sub>4</sub> or LTE<sub>4</sub> (Cayman Chemical) in the presence or absence of MK571 (Cayman Chemical) or 2-MesAMP (Sigma-Aldrich) or were passively sensitized with 2 μg/ml of human myeloma IgE (Millipore) overnight and stimulated with 1 μg/ml of rabbit anti-human anti-IgE (Millipore) as detailed elsewhere (Paruchuri et al., 2008). In some experiments, LAD2 cells were incubated with 10 μM apyrase (Sigma-Aldrich) to degrade extracellular nucleotides during the activation. The concentration of MIP-1β was measured by an ELISA (Thermo Fisher Scientific). PGD<sub>2</sub> was quantitated by a PGD<sub>2</sub>-methoxylamine hydrochloride assay (Cayman Chemical).

**SDS PAGE immunoblotting.** After stimulation with the respective agonists, 0.5 × 10<sup>6</sup> LAD2 cells and CHO cells were lysed with lysis buffer (BD) supplemented with protease inhibitor cocktail (Roche) and 1 mM sodium vanadate. Lysates were subjected to 4–12% SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with antibodies against phospho and total ERK (Cell Signaling Technology) in PBS, 5% dry milk, and

0.1% Tween-20 (1:1,000) overnight at 4°C on shaker and then with secondary antibody (peroxidase-conjugated anti-rabbit or anti-mouse). Bands were visualized with enhanced chemiluminescence (Thermo Fisher Scientific).

**Binding assays.** [ $^3\text{H}$ ] LTD<sub>4</sub> ([14,15,19,20- $^3\text{H}$ (N)]<sub>4</sub>; specific activity 100–240 Ci/mmol; PerkinElmer) was converted to [ $^3\text{H}$ ]LTE<sub>4</sub> by the dipeptidases present in the serum. In brief, 100  $\mu\text{l}$  (80 nM) [ $^3\text{H}$ ]LTD<sub>4</sub> was incubated with 100  $\mu\text{l}$  of 10% serum for 2 h at room temperature. The converted product was extracted into 400  $\mu\text{l}$  of methanol, evaporated in the presence of nitrogen, and diluted to the required concentration with the binding buffer for the binding assay. The conversion was confirmed by running an aliquot on high-performance reverse-phase liquid chromatography. The fraction eluting with the LTE<sub>4</sub> peak accounted for >99% of the radioactivity used in the assays. The specific activity of the resultant LTE<sub>4</sub> was consistently 80–100% that of the LTD<sub>4</sub> from which it was converted. [ $^3\text{H}$ ]ADP and [ $^3\text{H}$ ]LTE<sub>4</sub> binding assays were performed using membrane proteins as described earlier (Maekawa et al., 2001). In brief, cells were washed in PBS, resuspended in PBS supplemented with protease inhibitor cocktail, and lysed by sonication for 5 min. The lysed cells were centrifuged at 100,000  $g$  for 45 min and the microsomal pellet was resuspended in 1 ml PBS. Protein concentration was determined using a Protein Assay kit (Bio-Rad Laboratories). 50  $\mu\text{g}$  of membrane protein was incubated for 1 h at room temperature in 250  $\mu\text{l}$  of 10 mM Hepes/KOH, pH 7.4, with various concentrations of radiolabeled ligand and cold competitor. Bound [ $^3\text{H}$ ] was separated from free [ $^3\text{H}$ ] by filtration through GF/C filters (GE Healthcare) and washed twice with 10 mM Hepes/KOH, pH 7.4, containing 0.01% BSA. The residual membrane-associated [ $^3\text{H}$ ] on the filter was determined in 2 ml scintillation fluid by  $\beta$  counter (PerkinElmer). For [ $^3\text{H}$ ]ADP, specific binding was determined by subtracting the amount of [ $^3\text{H}$ ] bound in the presence of 100  $\mu\text{M}$  2-MesADP from total binding. For [ $^3\text{H}$ ]LTE<sub>4</sub>, nonspecific binding was calculated as the residual radioactivity bound in the presence of 10  $\mu\text{M}$  of unlabeled LTE<sub>4</sub>.

**Induction of pulmonary inflammation.** Male BALB/c mice (6–8 wk old) received i.p. injections of 10  $\mu\text{g}$  of reagent-grade chicken egg OVA precipitated with 2.25  $\mu\text{g}$  aluminum hydroxide on days 0 and 7. On days 14–16, the mice received single intranasal doses of 2.2 nmol LTD<sub>4</sub>, LTE<sub>4</sub>, or buffer. 30 min after each dose, the mice were exposed to an aerosol of 0.1% OVA for 30 min delivered by an ultrasonic nebulizer. As a positive control group, some mice received 1% OVA without LTD<sub>4</sub> or LTE<sub>4</sub> pretreatment. The mice were euthanized 24 h after their third and final OVA aerosol challenge. In some experiments, the mice received 500  $\mu\text{g}/\text{ml}$  clopidogrel in drinking water for 3 d before the first intranasal dose of LTs. The treatment was continued throughout the procedure. For Der f-mediated pulmonary inflammation, 6–8-wk-old C57BL/6 mice were lightly anesthetized and received either 3 or 10  $\mu\text{g}$  Der f extract (Greer Laboratories) on days 0, 3, 7, 10, 14, and 17. Some mice received clopidogrel throughout the duration of the experiment. Mice were euthanized 24 h after the last intranasal instillation.

**Platelet depletion.** Sensitized mice were treated i.v. with 50  $\mu\text{g}$  ( $\sim 2 \mu\text{g}/\text{g}$  body weight) of a monoclonal rat IgG directed against mouse CD42b (GPIIb $\alpha$ ) or an equal amount of isotype control (both from Emfret Analytics; Nieswandt et al., 2000). Each antibody was diluted in 50  $\mu\text{l}$  of sterile saline. The depletion of platelets was confirmed by automated counting.

**Histological assessment.** The left lungs were fixed for at least 8 h in 4% paraformaldehyde and embedded in glycol methacrylate, as described previously (Kim et al., 2006). Then, 2.5- $\mu\text{m}$ -thick glycol methacrylate sections were stained with H&E for general morphological examination. For histological study of the mucus-secreting cells of the epithelium (goblet cells) of the airways, lung sections were stained with PAS. The extent of cellular infiltration in the bronchovascular bundles was evaluated without knowledge of the particular treatment and was categorized arbitrarily into the following grades: 0, no inflammation; 1, mild inflammation; 2, moderate inflammation; and 3, severe inflammation. 15 bronchovascular bundles of each mouse were evaluated. The stained goblet cells were enumerated in at least four in-

dependent bronchovascular bundles from the lung sections obtained for each animal in the different experimental groups. The length of basal lamina of corresponding bronchus was measured by ImageJ image analysis software (National Institutes of Health). Only the comparable large-caliber preterminal bronchi (diameter 200–220  $\mu\text{m}$ ) were examined because minimal changes occur in terminal bronchioles. The data were expressed as the mean of goblet cell counts stained in each bronchus in each section per millimeter of bronchial basal lamina.

**Statistics.** Data are expressed as mean  $\pm$  SEM from at least three experiments except where otherwise indicated. Significance was determined with the Welch's test for samples of unequal variance. Analysis of variance was used to test differences between multiple groups.

**Online supplemental material.** Fig. S1 shows the dose-dependent effects of LTE<sub>4</sub> and LTD<sub>4</sub> on ERK activation in CHO cells stably transfected with the human P2Y<sub>12</sub> construct. Fig. S2 shows the effect of apyrase treatment on LT-mediated activation of LAD2 cells. Fig. S3 shows competitive ligand binding assays. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20091240/DC1>.

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