Leukotriene E_4–induced pulmonary inflammation is mediated by the $P2Y_{12}$ receptor

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Of the potent lipid inflammatory mediators comprising the cysteinyl leukotrienes (LTs; LTC_4, LTD_4, and LTE_4), only LTE_4 is stable and abundant in vivo. Although LTE_4 shows negligible activity at the type 1 and 2 receptors for cys-LTs ($CysLT_1R$ and $CysLT_2R$), it is a powerful inducer of mucosal eosinophilia and airway hyperresponsiveness in humans with asthma. We show that the adenosine diphosphate (ADP)–reactive purinergic ($P2Y_{12}$) receptor is required for LTE_4-mediated pulmonary inflammation. $P2Y_{12}$ receptor expression permits LTE_4 -induced activation of extracellular signal-regulated kinase in Chinese hamster ovary cells and permits chemokine and prostaglandin D_2 production by LAD2 cells, a human mast cell line. $P2Y_{12}$ receptor expression by LAD2 cells is required for competition between radiolabeled ADP and unlabeled LTE_4 but not for direct binding of LTE_4, suggesting that $P2Y_{12}$ complexes with another receptor to recognize LTE_4. Administration of LTE_4 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 $CysLT_1R$ and $CysLT_2R$ but not in mice lacking $P2Y_{12}$ receptors. The effects of LTE_4 on $P2Y_{12}$ in the airway were abrogated by platelet depletion. Thus, the $P2Y_{12}$ receptor is required for proinflammatory actions of the stable abundant mediator LTE_4 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_4, the parent cys-LT, is synthesized from arachidonic acid, which is liberated by calcium-dependent $cPLA_2$ (cytosolic phospholipase A_2) from membrane phospholipids (Clark et al., 1991). Arachidonic acid is then converted to LTA_4 by 5-lipoxygenase (5-LO) in concert with 5-LO–activating protein (Dixon et al., 1990; Malaviya et al., 1993). LTA_4 is conjugated to reduced glutathione by LTC_4 synthase, a homotrimeric integral nuclear membrane protein (Ago et al., 2007), forming LTC_4. LTC_4 is exported to the extracellular space by a multidrug-resistant protein after synthesis (Robbiani et al., 2000), where it is converted to LTD_4 by γ-glutamyl leukotrienase-mediated removal of glutamic acid (Shi et al., 2001). LTD_4 is then converted to LTE_4 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_4 is the only intracellular cys-LT, and LTD_4 is the most powerful contractile agonist of the airway smooth muscle. The half-life of LTD_4 is short (minutes) because of its rapid conversion to LTE_4, effectively limiting its duration of action in vivo. LTE_4 is stable and excreted in the urine (Sala et al., 1990). The stability of LTE_4 accounts for the fact that it is the dominant cys-LT detected

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in biological fluids. Consequently, 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 ($CysLT_1R$ and $CysLT_2R$), 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 $CysLT_1R$, encoded by a gene on chromosome Xq21.13, is a high-affinity receptor for LTD_4 ($K_d \sim 1$ nM; Lynch et al., 1999), whereas the human $CysLT_2R$ is encoded by a gene on chromosome 13q14 and has equal affinity for LTC_4 and LTD_4 ($K_d \sim 10$ nM; Heise et al., 2000). As neither receptor has significant affinity for LTE_4, the existence of an additional cys-LT receptor with a preference for LTE_4 has long been suspected. Early studies demonstrated that purified LTE_4 is more potent than LTC_4 or LTD_4 for inducing contraction of guinea pig tracheal rings (Lee et al., 1984), whereas LTC_4 and LTD_4 were more potent on peripheral lung. Of the three cys-LTs, only 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). 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 LTE_4, but not of 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 LTE_4 relative to LTC_4 or to histamine when compared with aspirin-tolerant asthmatic individuals (Christie et al., 1993). Mice lacking both $CysLT_1R$ and $CysLT_2R$ (*Cysltr1/Cysltr2*$^{-/-}$ mice) exhibit enhanced skin swelling in response to intracutaneous LTE_4 relative to WT controls, indicating that a putative LTE_4-reactive GPCR (termed $CysLT_ER$) exists in the skin (Maekawa et al. 2008). Thus, the potency of 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 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 $CysLT_1R$ (Knorr et al., 1998) show clinical efficacy in asthma, despite the negligible activity of LTE_4 at $CysLT_1R$ and the fact that zileuton blocks only ~50% of cys-LT generation in vivo (Israel et al., 1996; Liu et al., 1996). Identification of receptors and pathways through which 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 LTE_4 are elevated and/or end-organ reactivity to 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 $CysLT_1R$ (Mellor et al., 2001) and $CysLT_2R$ (Mellor et al. 2003) and that these receptors constitutively form heterodimers on this cell type (Jiang et al., 2007). Stimulation of MCs with LTD_4, the most potent agonist of the $CysLT_1R$, 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). $CysLT_1R$ is required for all of these LTD_4-induced responses, whereas $CysLT_2R$ inhibits them (Jiang et al., 2007). MCs also express several P2Y receptors (Feng et al., 2004). We recently reported that LTE_4 induces ERK activation and COX-2 expression, and causes prostaglandin (PG) D_2 and macrophage inflammatory protein 1β (MIP-1β) 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). LTE_4-mediated production of PGD_2 by LAD2 cells was unaffected by short hairpin RNA (shRNA)–mediated knockdown of either $CysLT_1R$ or $CysLT_2R$ (Paruchuri et al., 2008), supporting the presence of a previously unrecognized LTE_4-reactive receptor on this cell type. A computer model (Nonaka et al., 2005) had predicted that the $P2Y_{12}$ receptor, an adenosine diphosphate (ADP)–reactive GPCR which is the target of the thienopyridine anti-thrombotic drugs (Foster et al., 2001), could be an LTE_4 receptor. In this study, we demonstrate a potent unique proinflammatory function for LTE_4 in the lung, and we demonstrate that $P2Y_{12}$ receptors are required for the functions of LTE_4 in vitro and in the lung in vivo. Our results suggest that $P2Y_{12}$ receptors may be a novel target for the treatment of asthma.

RESULTS

Recombinant human $P2Y_{12}$ receptors convey cellular responses to LTE_4

To determine if $P2Y_{12}$ receptors could mediate responses to LTE_4, we stably transfected Chinese hamster ovary (CHO) cells (which do not natively express classical CysLTRs [Maekawa et al., 2001] or $P2Y_{12}$ receptors) with constructs encoding the human $P2Y_{12}$ receptor protein in forward and reverse orientation. The transfectants were stimulated with exogenous LTC_4, LTD_4, or LTE_4. Fura-2 AM–loaded CHO cells expressing $P2Y_{12}$ receptors failed to flux calcium in response to LTE_4 but did exhibit a response to ADP, reflecting

endogenous $P2Y_1$ receptors (Fig. 1 A). However, CHO cells transfected with the $P2Y_{12}$ receptor construct in forward (but not reverse) orientation responded to LTE_4 (and to LTD_4) 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 $P2Y_{12}$ 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 $CysLT_1R$ and some P2Y receptors (Mamedova et al., 2005). Although ERK activation in the $P2Y_{12}$ receptor transfectants was at least as robust in response to LTE_4 as to LTD_4 (Fig. 1 D), $CysLT_1R$ and $CysLT_2R$ transfectants reacted to LTD_4 in marked preference to LTE_4, even at high (500 nM) ligand concentrations. These responses were resistant to PTX and 2-MesAMP and, in the case of the $CysLT_1R$ transfectants, susceptible to MK571.

$P2Y_{12}$ receptor requirement for LTE_4–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 $LTE_4 > LTD_4$. To determine whether $P2Y_{12}$ receptors were responsible for these responses, Fura-2 AM–loaded LAD2 cells were stimulated with LTD_4 or LTE_4 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_4- or LTE_4-mediated calcium flux in LAD2 cells (Fig. 2 A). We determined the effect of MK571 and 2-MesAMP on MIP-1β generation by LAD2 cells in response to stimulation for 6 h with LTD_4 and LTE_4, using IgE plus anti-IgE as a positive control. LTE_4 at doses of 100 and 500 nM induced the generation of large amounts of MIP-1β, exceeding the amounts generated in response to LTD_4 and to IgE plus anti-IgE (Fig. 2 B). Pretreatment of the LAD2 cells with 2-MesAMP blocked the LTE_4-mediated increment in MIP-1β production by >50% (Fig. 2 B). 2-MesAMP also reduced the response to the higher concentrations of LTD_4. MK571 suppressed the response to both ligands and was additive with 2-MesAMP for the suppression of the response to LTD_4. Neither antagonist altered the production of MIP-1β 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 $P2Y_{12}$ sequence-specific shRNA, a $CysLT_1R$-specific shRNA, or an empty vector control before stimulation. Knockdown of $P2Y_{12}$ receptors decreased the receptor messenger RNA (mRNA) expression by ~90% (Fig. 2 D) and did not alter expression of $CysLT_1R$ or $CysLT_2R$ proteins (not depicted). $P2Y_{12}$ receptor knockdown nearly abrogated MIP-1β production, completely eliminated PGD_2 production in response to LTE_4, and minimally affected the response to LTD_4. The knockdown of $CysLT_1R$ completely blocked MIP-1β and PGD_2 production in response to LTD_4 and slightly (but not significantly) decreased the responses to LTE_4 (Fig. 2 C, bottom). Neither knockdown altered MIP-1β generation or PGD_2 production in response to IgE–anti-IgE (Fig. 2 C). LTE_4-mediated

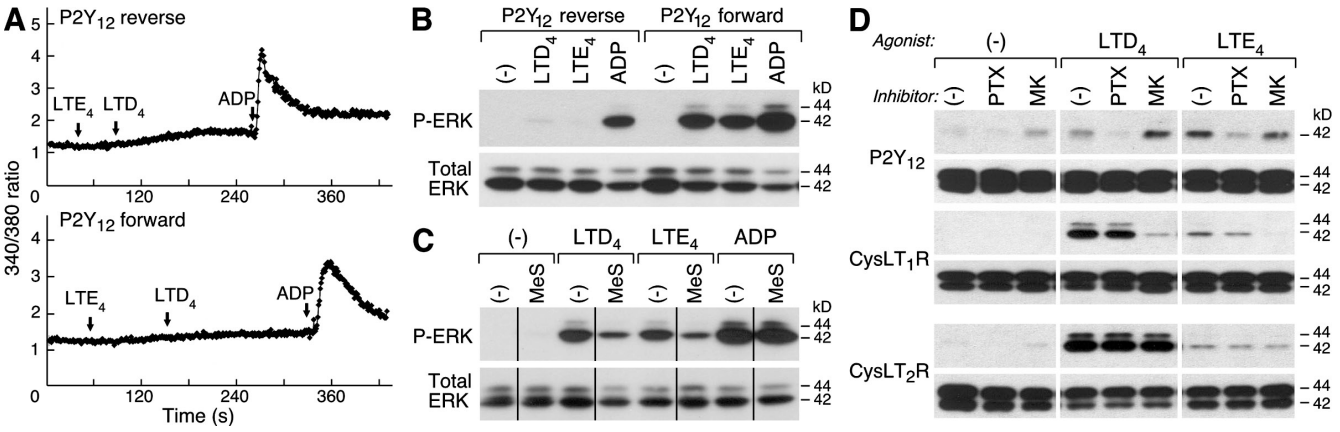

Figure 1. Activation by recombinant human $P2Y_{12}$ receptors in response to cys-LTs. The human $P2Y_{12}$ receptor complementary DNA (cDNA) was cloned in forward and reverse orientation into the expression vector pEF1/His B and transfected into CHO cells using Fugene HD reagent. Stably expressing clones were selected using 1,000 μg/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 LTE_4, 500 nM LTD_4, and 100 μM ADP. Results in a second experiment were identical. (B) SDS-PAGE immunoblots showing phosphorylation of ERK2 by CHO cells stably expressing human $P2Y_{12}$ receptors in reverse (negative control) or forward orientations. Cells were stimulated with 100 μM ADP (positive control ligand), 500 nM LTD_4, or 500 nM LTE_4 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 $P2Y_{12}$ receptor antagonist 2-MesAMP (MeS) on ligand-induced ERK phosphorylation. $P2Y_{12}$ receptor-expressing CHO cells were stimulated with the same doses of agonists used in A, in the absence or presence of 100 μM 2-MesAMP. (D) Comparison of $P2Y_{12}$ receptor-mediated responses to cys-LTs with those of recombinant human $CysLT_1R$ and $CysLT_2R$ expressed in CHO cells, and the effect of 1 μM of the $CysLT_1R$ antagonist MK571. Data in B–D are from individual experiments that were repeated at least three times with similar results.

MIP-1β 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 $P2Y_{12}$ receptors directly mediated LTE_4 binding, membranes were prepared from LAD2 cells with and without $P2Y_{12}$ knockdown. We first performed competitive radioligand binding assays using [^{3}H]ADP (the known natural ligand of $P2Y_{12}$ receptors) and unlabeled LTs as competitors. Unlabeled LTE_4 competed with labeled ADP, blocking 39 ± 9 and 50 ± 9% of specific ADP binding at doses of 0.1 and 1 nM, respectively, and reaching a plateau (60 ± 7%) at 1 μM (mean ± SEM for four separate experiments, as shown for one experiment [Fig. S3 A]). LTE_4 was more efficacious than LTD_4 (Fig. S3 B). Knockdown of $P2Y_{12}$ receptors reduced binding of [^{3}H]ADP by 40–60% and completely eliminated competition by LTE_4 (Fig. S3, A and B, right). LAD2 cell membranes weakly bound [^{3}H]LTE_4 (converted from commercially prepared [^{3}H]LTD_4; Fig. S3 C), but specific binding of [^{3}H]LTE_4 was not altered by the knockdown of $P2Y_{12}$ receptor (Fig. S3 C). To determine whether LTE_4 could block the ADP binding of $P2Y_{12}$ receptors expressed in isolation, the human forward and reverse $P2Y_{12}$ constructs were transiently expressed in COS-7 cells. The membranes from the transfectants expressing the forward construct bound [^{33}P]2-MesADP, a selective $P2Y_{12}$ receptor agonist. In contrast to ADP binding to LAD2 membranes, this binding was not competed by unlabeled LTE_4 or LTD_4 (Fig. S3 D). Additionally, these membranes failed to bind [^{3}H]LTE_4 (unpublished data).

LTE_4 potentiates allergen-induced bronchial inflammation

To determine whether LTE_4 could induce or potentiate histological signatures of pulmonary inflammation in mice, we determined the effect of 2.2 nmol of intranasal LTE_4 administered on three successive days on the lung histology of naive BALB/c mice. We compared the effect to that of LTD_4. 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_4, LTE_4, or a buffer control. A cohort of mice treated

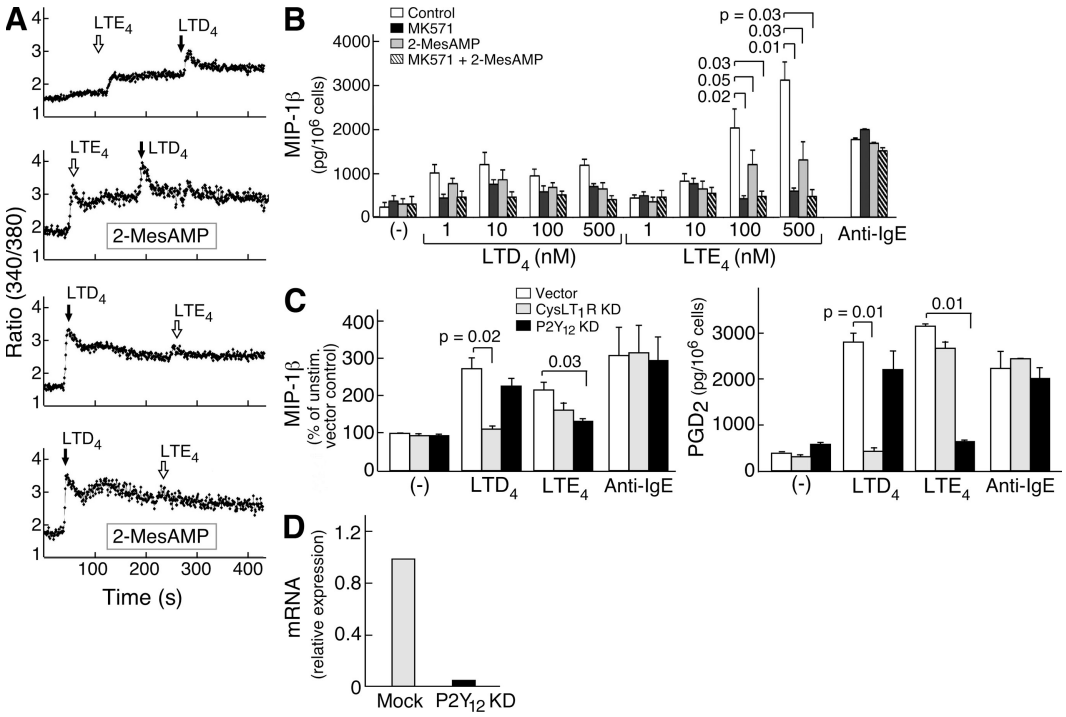

Figure 2. Requirement for $P2Y_{12}$ receptors in LTE_4-mediated MC activation. (A) Calcium flux of LAD2 cells stimulated with 500 nM of the LTs in the presence or absence of 100 μM 2-MesAMP. MK571 completely blocked both LTD_4- and LTE_4-mediated calcium fluxes (not depicted). Results in two additional experiments were similar. (B) Effect of 100 μM 2-MesAMP, 1 μM MK571, or both on MIP-1β generation by LAD2 cells in response to the indicated doses of LTD_4 and LTE_4. Cells sensitized overnight with IgE and challenged with anti-IgE were used as the positive control. (C) Effect of shRNA-mediated knockdowns of $CysLT_1R$ or $P2Y_{12}$ receptors on MIP-1β generation (left) and PGD_2 generation (right) by LAD2 cells stimulated with 100 nM LTD_4 or 100 nM LTE_4. Results in B and C are mean ± SEM from three independent experiments each. Statistical differences were assessed by analysis of variance. (D) Effect of shRNA-mediated knockdown of $P2Y_{12}$ 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 $CysLT_1R$ (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_4 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_4 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_4-treated animals approached the levels of these parameters in the mice treated with 1% OVA.

LTE_4-mediated pulmonary inflammation depends on $P2Y_{12}$ receptors and is independent of classical CysLTRs

To determine whether $P2Y_{12}$ receptors accounted for the LTE_4-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 $P2Y_{12}$ 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_4. Treatment with clopidogrel eliminated LTE_4-induced potentiation of both inflammation and goblet cell metaplasia (Fig. 4, A and B). To determine the effect of $P2Y_{12}$ 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_4 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_4 in the lungs of allergen-sensitized and -challenged mice using a molecular approach. First, we studied the ability of LTE_4 to amplify pulmonary inflammation in OVA-sensitized and -challenged C57BL/6 mice lacking $P2Y_{12}$ receptors (*p2ry12*$^{-/-}$ mice; Andre et al., 2003)

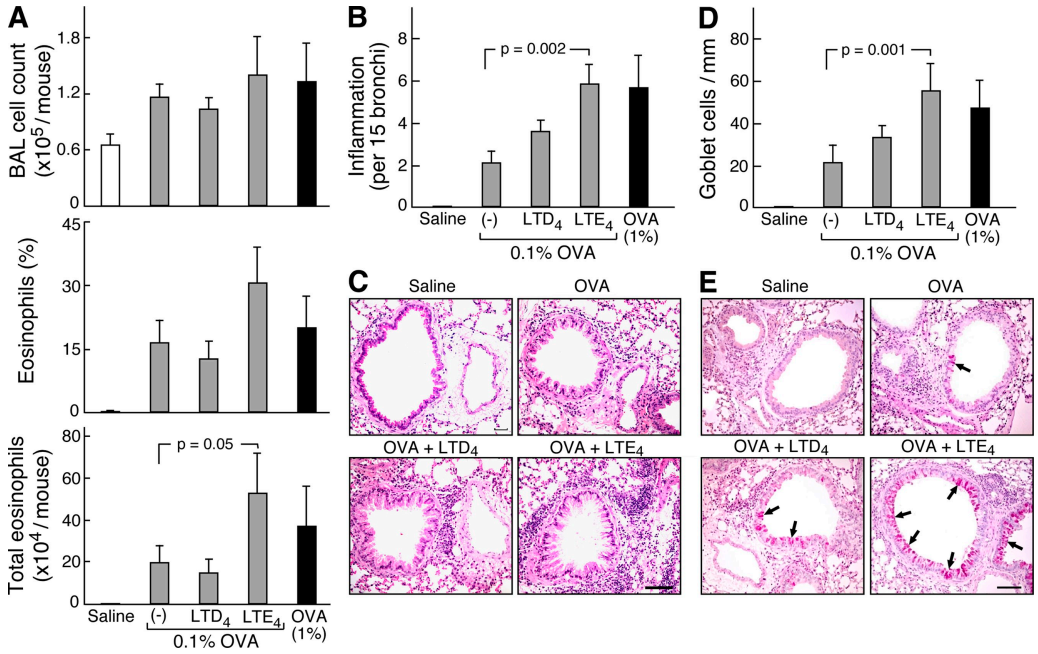

Figure 3. LTE_4-mediated amplification of allergen-induced pulmonary inflammation. Male BALB/c mice (6–8 wk old) were sensitized on days 0 and 7 with i.p. injections of 10 µg Alum-precipitated chicken egg OVA. Mice were challenged with LTs on days 13–15 and OVA on days 14–16 as described in the text. 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-µm-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 ± SEM from eight to nine mice per group. The experiments were repeated three times. Statistical differences were determined by analysis of variance. Bars, 100 µm.

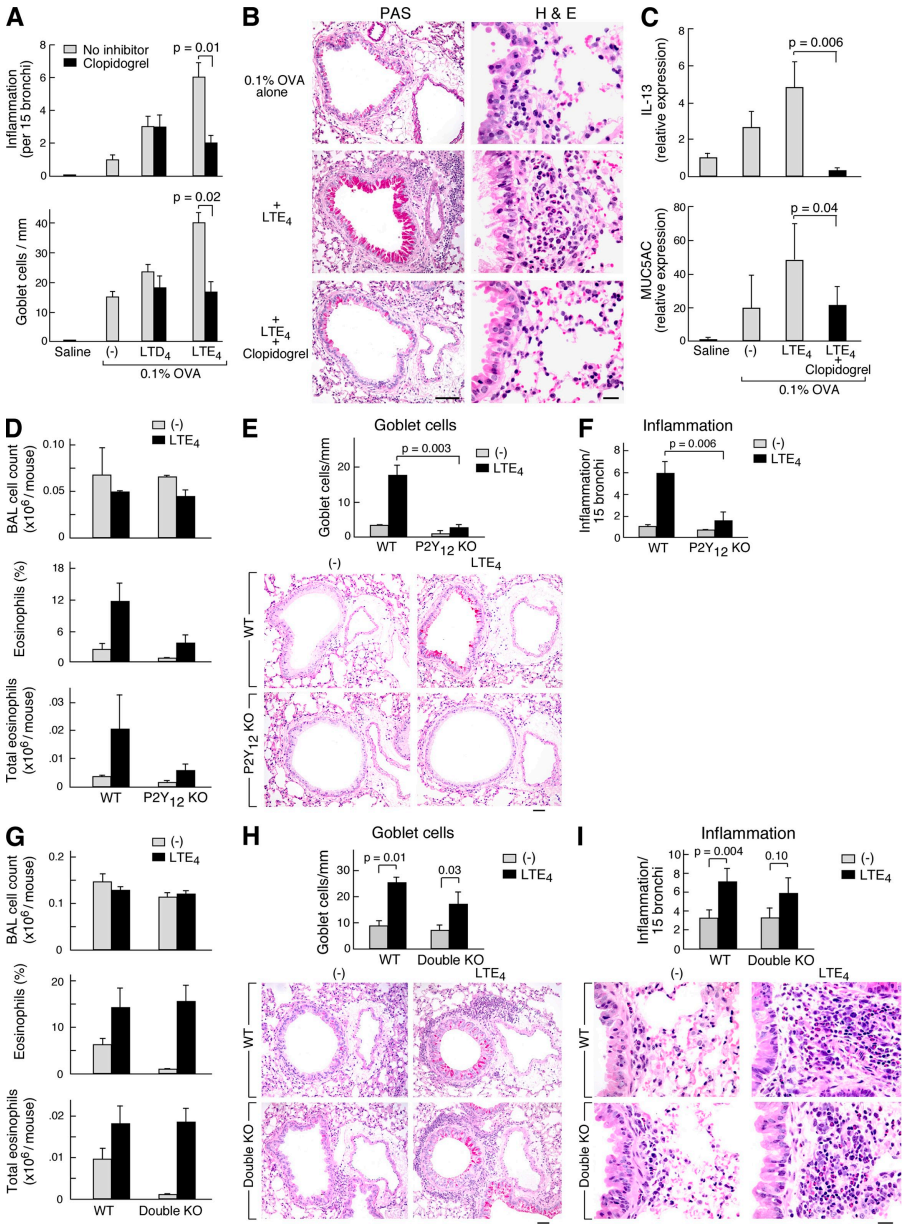

Figure 4. Role of P2Y12 receptors on potentiation of pulmonary inflammation by LTE_4. (A) Bronchovascular inflammation (top) and goblet cell metaplasia in sensitized mice challenged with low-dose OVA with or without the prior administration of 2.2 nmol LTD_4 or LTE_4 30 min before each challenge. 500 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*$^{-/-}$ 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_4 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*$^{-/-}$ 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*$^{-/-}$ mice and age-matched WT controls were subjected to the same protocol as the *p2ry12*$^{-/-}$ 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_4 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*$^{-/-}$ mice (Fig. 4, D–F). To determine whether classical GPCRs for cys-LTs were also required for the LTE_4 effects, BALB/c mice lacking both $CysLT_1R$ and $CysLT_2R$ (*Cysltr1/Cysltr2*$^{-/-}$ 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_4. 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_4 were completely intact in the *Cysltr1/Cysltr2*$^{-/-}$ mice, indicating that LTE_4 was working independently of the known GPCRs for cys-LTs. Thus, the intrapulmonary actions of LTE_4 in vivo require $P2Y_{12}$ receptors but not classical cys-LT–reactive GPCRs.

Blockade of $P2Y_{12}$ receptors blunts pulmonary inflammation induced by house dust mite antigen

To determine whether pharmacologic blockade of $P2Y_{12}$ receptors altered pulmonary inflammatory responses in a more physiological model of pulmonary inflammation, C57BL/6 mice were administered an extract of house dust mite *Dermatophagoides 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).

$LTE_4/P2Y_{12}$ receptor–mediated amplification of pulmonary inflammation requires platelets

Because $P2Y_{12}$ 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_4. Platelets were depleted in sensitized BALB/c mice by the i.v. injection of a rat mAb against mouse CD42b (GPIbα; Nieswandt et al., 2000) or an isotype-matched control IgG 48 h before to the first administration of LTD_4 or LTE_4. 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_4-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_4-mediated expression of mRNA encoding IL-13

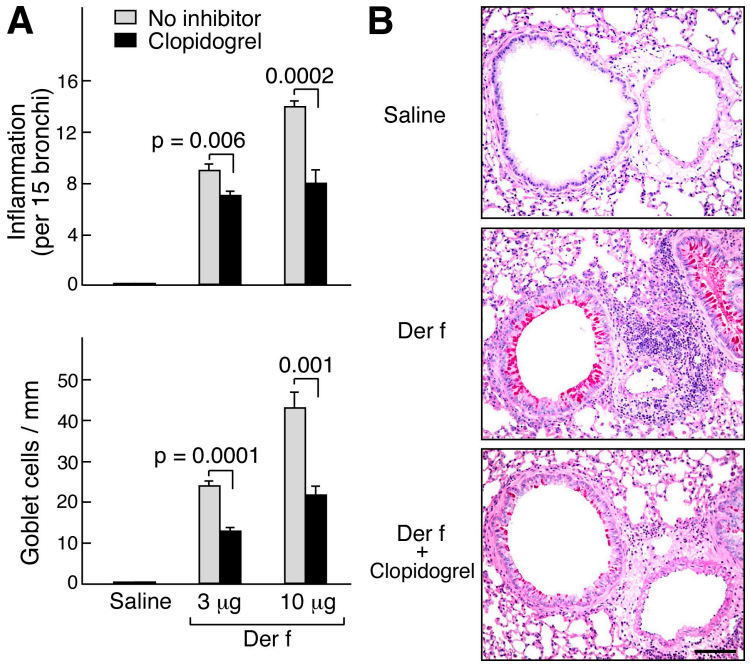

Figure 5. Role of $P2Y_{12}$ receptors in inflammation mediated by dust mite allergen. C57BL/6 mice were treated intranasally with the indicated dose of Der f extract twice weekly for 3 wk. Animals were euthanized 24 h after the last dose. (A) Effect of clopidogrel treatment on bronchovascular inflammation 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 thee 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_4 alone induced platelet activation (leading to ADP release with potential resultant autocrine stimulation of $P2Y_{12}$ receptors), human blood platelets were stimulated with 1 µM LTE_4 or with 100 µM 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_4 (Fig. 6 C).

DISCUSSION

This study establishes that $P2Y_{12}$ receptors are essential for the actions of LTE_4, 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_4 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 $CysLT_1R$ antagonists (Knorr et al., 1998) have clinical efficacy. The cloning and functional characterization of the $CysLT_1R$ (Lynch et al., 1999) and $CysLT_2R$ (Heise et al., 2000) explained the pharmacology of LTC_4 and LTD_4 predicted from studies of contractile tissues (Lee et al., 1984). The finding that neither GPCR showed significant binding or reactivity to LTE_4 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_4 could activate LAD2 cells by a mechanism independent of $CysLT_1R$ and $CysLT_2R$ (Paruchuri et al., 2008), and because LTE_4 was previously identified by an in silico model as a potential surrogate ligand for the $P2Y_{12}$ receptor (Nonaka et al., 2005), we undertook this study to determine whether the $P2Y_{12}$ receptor was a bona fide LTE_4-reactive receptor and to determine its contribution to LTE_4-induced proinflammatory events.

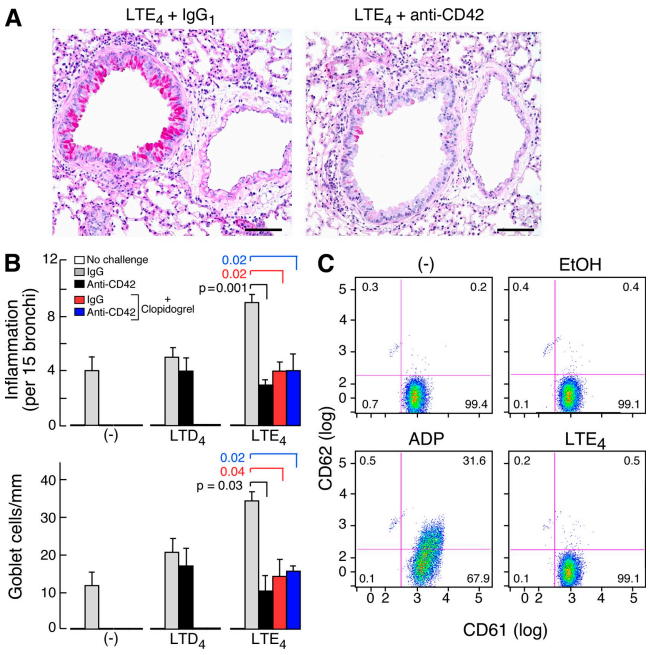

Figure 6. Platelet dependence of the LTE_4 effect on bronchial inflammation and goblet cell metaplasia. Sensitized mice were treated i.v. with 50 µg (~2 µg/g body weight) of a monoclonal rat IgG directed against mouse CD42b (GPIbα) or an equal amount of isotype control (both from Cemfret Analytics) 48 h before the first dose of LTE_4. Each antibody was diluted in 50 µl 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 ± SEM. (C) Cytofluorographic detection of surface expression of CD62P (P-selectin) by platelets stimulated for 10 min with 100 µM ADP or 1 µM LTE_4. Percentages of CD61-postitive platelets expressing CD62P are displayed in the top right quadrants. Results are from a single experiment performed three times on different donors. Bars, 100 µm.

We first established that recombinant human $P2Y_{12}$ receptor protein conveyed activation responses by CHO cells to LTE_4. Unlike most members of the P2Y receptor class, native $P2Y_{12}$ receptors do not couple to Gαq proteins or activate calcium flux; instead, they induce signaling through PTX-sensitive Gαi2 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 $P2Y_{12}$ receptors failed to flux calcium in response to cys-LTs (Fig. 1 A) or that blockade of $P2Y_{12}$ 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 $P2Y_{12}$ receptors responded to LTE_4 with PTX-sensitive ERK activation (Fig. 1). The dose range for this response (Fig. S1) is similar to the LTE_4 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 $P2Y_{12}$-Gα16 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 $P2Y_{12}$ receptors could contribute to an element of cys-LT–driven pathobiology that is relatively selective for LTE_4 and may resist conventional $CysLT_1R$ antagonists. This element may be especially relevant to pathological situations where LTE_4 is abundant as a result of its relative stability.

In our previous study, LTE_4 had exhibited unanticipated potency for inducing ERK activation and the generation of MIP-1β and COX-2–dependent PGD_2 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 $P2Y_{12}$ receptors to the activation responses of LAD2 cells to LTE_4 and to contrast these responses to those elicited by LTD_4, the most potent $CysLT_1R$ ligand. Although ineffective for blocking cys-LT–induced calcium flux, 2-MesAMP effectively

blocked the LTE_4-mediated increment in MIP-1β production (Fig. 2 B) and also reduced the response to the higher concentrations of LTD_4, indicating that LTD_4 at high concentrations can also activate native $P2Y_{12}$ receptors, as supported by its actions as an agonist for ERK activation in the $P2Y_{12}$ receptor transfectants (Fig. 1). MK571 suppressed MIP-1β 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 LTD_4. The shRNA-mediated knockdowns of $CysLT_1R$ and $P2Y_{12}$ receptors revealed strong dependence of LTD_4-mediated activation on $CysLT_1R$, whereas LTE_4-mediated responses were clearly $P2Y_{12}$ 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 LTE_4 to "cross over" and induce some calcium signaling through the $CysLT_1R$ may permit some complementarities between the $CysLT_1R$ (via Gαq proteins and calcium-induced pathways) and $P2Y_{12}$ receptors (via Gαi proteins), particularly for chemokine generation. This is analogous to the cooperation by Gαq-linked $P2Y_1$ receptors with $P2Y_{12}$ receptors in regulating ADP responses of platelets (Lova et al., 2002; Woulfe et al., 2002). The $P2Y_{12}$-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 $P2Y_{12}$ receptors were essential for competition between 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 $[3H]LTE_4$ to these same membranes (Fig. S3 C). Additionally, LTE_4 could not compete for binding to $P2Y_{12}$ receptors expressed in isolation on COS-7 cells (Fig. S3 D). The fact that $P2Y_{12}$ receptors do not directly bind LTE_4 despite their essential nature implies that they are components of a complex with another LTE_4-reactive GPCR, perhaps with the putative $CysLT_ER$ reported in the mouse skin (Maekawa et al., 2008). The facts that $P2Y_{12}$ receptors are required for LTE_4 to activate transfected CHO cells (Fig. 1) and LAD2 cells (Fig. 2), and for competition between LTE_4 and ADP for binding to LAD2 cells (Fig. S3 B), and that knockdown of $P2Y_{12}$ receptors did not reduce direct binding of radiolabeled LTE_4 (Fig. S3 C) are all consistent with this thesis. Precedents for such complexes on MCs include $CysLT_1R$ and $CysLT_2R$ heterodimers (Jiang et al., 2007) and a functional requirement of $CysLT_1R$ for the uridine diphosphate-reactive P2Y6 receptor (Jiang et al., 2009). Our data indicate that presence of $P2Y_{12}$ is required for signaling and activation by LTE_4 in a cell-specific context.

The fact that LTE_4, but not 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 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 LTE_4 exceeded the potency of LTD_4 for potentiating 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, LTE_4 exceeds the efficacy of LTD_4 for potentiating bronchial inflammation in both mouse and man, a pattern not explicable by the known properties of $CysLT_1R$ or $CysLT_2R$. Several pieces of evidence link these LTE_4-mediated responses in mice to the $P2Y_{12}$ receptor. First, treatment of the mice with a highly potent selective antagonist of the $P2Y_{12}$ receptor, clopidogrel, completely eliminated the histological response to LTE_4 (Fig. 4, A and B), which is associated with blockade of 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, LTE_4 fully amplified pulmonary inflammation in *Cysltr1/Cysltr2*$^{-/-}$ mice (Fig. 4, G–I). Lastly, the ability of LTE_4 to potentiate mucosal inflammation and goblet cell metaplasia was nearly completely abrogated in the absence of $P2Y_{12}$ receptors (Fig. 4, D–F). Thus, LTE_4 requires $P2Y_{12}$ receptors, but not the classical CysLTRs, to amplify the histological signatures of allergen-induced pulmonary inflammation. Because LTD_4 is converted in vivo to LTE_4, it was somewhat surprising that the ligands do not behave interchangeably in potentiating bronchial eosinophilia in mouse or man. In addition to $P2Y_{12}$-dependent effects of LTE_4, LTD_4 may initiate inhibitory signals through $CysLT_2R$ (Jiang et al., 2007) or other yet-to-be-identified GPCRs that recognize LTD_4 but not LTE_4. The effects of clopidogrel in the model of airway disease induced by the natural allergen Der f without the use of exogenous LTE_4 (Fig. 5) support the importance of the $P2Y_{12}$ pathway in the integrated biology of pulmonary inflammation (although this does not discriminate between effects mediated by LTE_4 from ADP). The blockade of the response by clopidogrel distinguishes the $P2Y_{12}$ receptor-dependent response of the lung to LTE_4 from the clopidogrel-resistant 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 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 $P2Y_{12}$ 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 LTE_4. Indeed, the response to 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 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 LTE_4 does not trigger an ADP-dependent autocrine loop initiated by 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 $P2Y_{12}$ provides only the Gi-linked component, LTE_4 (and $P2Y_{12}$) likely synergizes in vivo with a second agonist to facilitate platelet functions in the lung. It is noteworthy that LTE_4-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 $P2Y_{12}$ receptor-dependent signaling on platelets or other cell types. The fact that LTE_4 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_4 reactivity in the face of platelet depletion argues against a role for MCs and other $P2Y_{12}$-bearing cell types, at least in this model.

Our findings suggest a potential therapeutic application for $P2Y_{12}$ receptor antagonists that may be especially relevant to AERD, which is associated with both high levels of LTE_4 in the urine (Christie et al., 1992b) and selective hyperresponsiveness to LTE_4 (Christie et al., 1993). In AERD, both 5-LO antagonists and $CysLT_1R$ 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_4 generation. The fact that the effects of LTE_4 persist in the absence of $CysLT_1R$ and $CysLT_2R$ implies that this pathway would be resistant to the available cys-LT receptor antagonists, all of which selectively block $CysLT_1R$. We speculate that simultaneous interference with the bronchoconstrictive effects of LTD_4 (via $CysLT_1R$) and with proinflammatory effects of LTE_4 (by $P2Y_{12}$), 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 $CysLT_1R$ and $CysLT_2R$ (*Cysltr1/Cysltr2*$^{-/-}$ mice) and their WT littermate controls were derived as described elsewhere (Maekawa et al., 2008). Mice lacking $P2Y_{12}$ receptors (*p2ry12*$^{-/-}$ 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. $0.5–1 \times 10^6$ 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_4 or LTE_4, 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 $CysLT_1R$ antagonist MK571 or with 100 μM of the $P2Y_{12}$ receptor antagonist 2-MesAMP for 5 min before the stimulation.

Real-time quantitative PCR. The expressions of human $P2Y_{12}$ 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 $CysLT_1R$ and $P2Y_{12}$ 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 $CysLT_1R$, whereas quantitative PCR was used to verify the knockdown of the $P2Y_{12}$ receptor.

Generation of stable $P2Y_{12}$ receptor transfectants. A human $P2Y_{12}$ 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′-TACATTGGAGTCTCTTCATTTGG-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 $P2Y_{12}$ receptor construct in the forward orientation was transfected into CHO cells using Fugene 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_4 or LTE_4 (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_2 was quantitated by a PGD_2-methoxylamine hydrochloride assay (Cayman Chemical).

SDS PAGE immunoblotting. After stimulation with the respective agonists, 0.5×10^6 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}H] LTD_4 ([14,15,19,20-^{3}H(N)]; specific activity 100–240 Ci/mmol; PerkinElmer) was converted to [^{3}H]LTE_4 by the dipeptidases present in the serum. In brief, 100 µl (80 nM) [^{3}H]LTD_4 was incubated with 100 µl of 10% serum for 2 h at room temperature. The converted product was extracted into 400 µl 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_4 peak accounted for >99% of the radioactivity used in the assays. The specific activity of the resultant LTE_4 was consistently 80–100% that of the LTD_4 from which it was converted. [^{3}H]ADP and [^{3}H]LTE_4 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 µg of membrane protein was incubated for 1 h at room temperature in 250 µl of 10 mM Hepes/KOH, pH 7.4, with various concentrations of radiolabeled ligand and cold competitor. Bound [^{3}H] was separated from free [^{3}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}H] on the filter was determined in 2 ml scintillation fluid by β counter (PerkinElmer). For [^{3}H]ADP, specific binding was determined by subtracting the amount of [^{3}H] bound in the presence of 100 µM 2-MesADP from total binding. For [^{3}H]LTE_4, nonspecific binding was calculated as the residual radioactivity bound in the presence of 10 µM of unlabeled LTE_4.

Induction of pulmonary inflammation. Male BALB/c mice (6–8 wk old) received i.p. injections of 10 µg of reagent-grade chicken egg OVA precipitated with 2.25 µg aluminum hydroxide on days 0 and 7. On days 14–16, the mice received single intranasal doses of 2.2 nmol LTD_4, LTE_4, 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_4 or LTE_4 pretreatment. The mice were euthanized 24 h after their third and final OVA aerosol challenge. In some experiments, the mice received 500 µg/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 anaesthetized and received either 3 or 10 µg 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 µg (~2 µg/g body weight) of a monoclonal rat IgG directed against mouse CD42b (GPIbα) or an equal amount of isotype control (both from Emfret Analytics; Nieswandt et al., 2000). Each antibody was diluted in 50 µl 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-µm-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 independent 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 µm) 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 ± 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_4 and LTD_4 on ERK activation in CHO cells stably transfected with the human $P2Y_{12}$ 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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