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Leukotriene D_4 paradoxically limits LTC_4-driven platelet activation and lung immunopathology

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Abstract

Background: The 3 cysteinyl leukotrienes (cysLTs), leukotriene (LT) C_4 (LTC_4), LTD_4, and LTE_4, have different biologic half-lives, cellular targets, and receptor specificities. $CysLT_2R$ binds LTC_4 and LTD_4 *in vitro* with similar affinities, but it displays a marked selectivity for LTC_4 *in vivo*. LTC_4, but not LTD_4, strongly potentiates allergen-induced pulmonary eosinophilia in mice through a $CysLT_2R$-mediated, platelet-and IL-33–dependent pathway.

Objective: We sought to determine whether LTD_4 functionally antagonizes LTC_4 signaling at $CysLT_2R$.

Methods: We used 2 different *in vivo* models of $CysLT_2R$-dependent immunopathology, as well as *ex vivo* activation of mouse and human platelets.

Results: LTC_4-induced CD62P expression; HMGB1 release; and secretions of thromboxane A_2, CXCL7, and IL-33 by mouse platelets were all were blocked by a selective $CysLT_2R$ antagonist and inhibited by LTD_4. These effects did not depend on $CysLT_1R$. Inhaled LTD_4 blocked LTC_4-mediated potentiation of ovalbumin-induced eosinophilic inflammation; recruitment of platelet-adherent eosinophils; and increases in IL-33, IL-4, IL-5, and IL-13 levels in lung tissue. In contrast, the effect of administration of LTE_4, the preferred ligand for $CysLT_3R$, was additive with LTC_4. The administration of LTD_4 to *Ptges*$^{-/-}$ mice, which display enhanced LTC_4 synthesis similar to that in aspirin-exacerbated respiratory disease, completely blocked the physiologic response to subsequent lysine-aspirin inhalation challenges, as well as increases in levels of IL-33, type 2 cytokines, and biochemical markers of mast cell and platelet activation.

Conclusion: The conversion of LTC_4 to LTD_4 may limit the duration and extent of potentially deleterious signaling through $CysLT_2R$, and it may contribute to the therapeutic properties of desensitization to aspirin in aspirin-exacerbated respiratory disease.

Graphical Abstract

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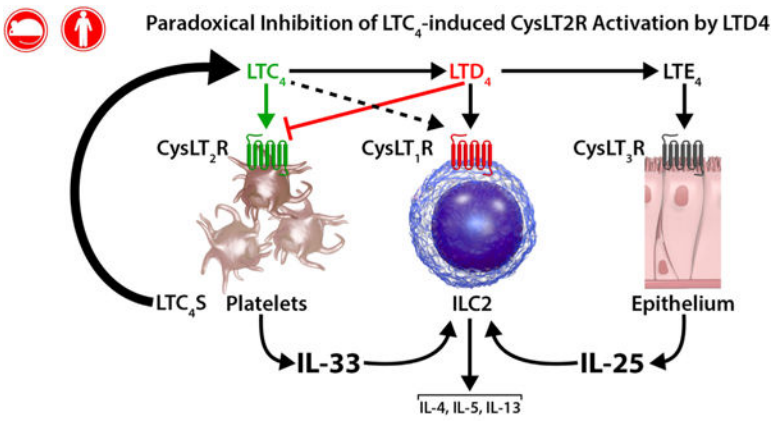

Legend:
ASM- airway smooth muscle
$CysLT_1R$, $CysLT_2R$, $CysLT_3R$- types 1, 2, and 3 receptors for the cysteinyl leukotrienes
ILC2, group 2 innate lymphoid cell
LTC_4S, leukotriene C4 synthase

Keywords

Platelets; leukotrienes; $CysLT_2R$; mast cells; eosinophils; AERD

Cysteinyl leukotrienes (cysLTs) are potent lipid mediators of inflammation that are prominent in asthma and mucosal type 2 immune responses. Inhaled cysLTs cause bronchoconstriction[1] and airway mucous secretion,[2] and they potentiate airway eosinophilia when administered by inhalation to human subjects.[3–5] They are derived from arachidonic acid oxidized by 5-lipoxygenase (5-LO) to generate the unstable precursor leukotriene (LT)A_4.[6] LTA_4 is then conjugated to reduced glutathione by leukotriene C_4 synthase (LTC_4S),[7] forming LTC_4, the parent cysLT. The principal sources of LTC_4 are mast cells (MCs), eosinophils, basophils, macrophages, and platelet-adherent granulocytes.[8] Following release from these cell types, LTC_4 is converted extracellularly to LTD_4 by gamma-glutamyl transferase 5 (GGT5), removing glutamic acid from the glutathione adduct.[9] LTD_4 is converted to the stable metabolite LTE_4 by di-peptidases that remove glycine.[10] The conversion of LTC_4 to LTD_4 occurs rapidly, and the conversion of LTD_4 to LTE_4 occurs even more rapidly, such that the typical ratio of the 3 molecules detected in biologic fluids is as follows: LTE_4 is greater than LTC_4 is much greater than LTD_4.[8] 5-LO activity and consequent synthesis of LTC_4 increase during exacerbations of asthma, as is reflected by increases in the urinary levels of LTE_4.[11] Steady-state LTC_4 synthesis rates are especially high in aspirin-exacerbated respiratory disease (AERD),[12] a phenotypically distinct clinical syndrome characterized by severe eosinophilic asthma and nasal polyps. Administration of aspirin or other COX-1–active drugs to subjects with AERD precipitates pathognomonic clinical reactions typified by changes in lung function and sinonasal obstruction, accompanied by a level of LTC_4 synthesis that is markedly increased above the high baseline.[12] Pharmacologic inhibition of 5-LO[13] or blockade of the type 1 receptor for cysLTs ($CysLT_1R$)[14] can reduce the severity of these reactions; it can also improve baseline lung function and reduce the frequency of asthma exacerbations in aspirin-tolerant patients.

[15,16] These findings verify the role of cysLTs in asthma exacerbations, as well as the idiosyncratic immunopathology of AERD.

The 3 cysLT-selective receptors, termed $CysLT_1R$, $CysLT_2R$, and $CysLT_3R$,[17–19] differ in their ligand binding preferences and sites of expression. $CysLT_1R$ binds LTD_4 with higher affinity (~1 nM) than it binds LTC_4 and LTE_4 (~1 and 2 log-fold lower affinity than LTD_4, respectively). $CysLT_1R$ is expressed by vascular and airway smooth muscle, several leukocyte subsets, and platelets. $CysLT_2R$ binds LTC_4 and LTD_4 with nearly identical affinity (~20 nM), but it exhibits weak or negligible activity for LTE_4.[18] It is expressed by endothelial cells, cardiac Purkinje cells, leukocytes, and platelets. $CysLT_3R$ binds LTE preferentially (~2 nM), but it can also respond to LTC_4 and LTD_4 *in vivo*.[19] It is expressed principally by respiratory mucosal epithelial cells[20] and proximal convoluted tubular cells. Mouse and human receptors have very similar ligand affinities and preferences, and their sequences are highly conserved.[17,21] LTC_4 and LTD_4 are exceedingly potent bronchoconstrictors in human subjects[1,22] and guinea pigs.[23] These responses are sensitive to $CysLT_1R$ antagonists.[24] Although LTE_4 is a weaker constrictor than its precursors,[25,26] subjects with asthma are approximately 10-fold more sensitive to the contractile effects of LTE_4 than are subjects without asthma.[26] Like LTC_4- and LTD_4-medicated bronchoconstrction, LTE_4-mediated bronchoconstriction in human subjects is sensitive to blockade by $CysLT_1R$ antagonists.[4,27] Thus, cysLT-induced contractile effects in human airways are mediated largely, if not exclusively, by $CysLT_1R$.

Whereas $CysLT_1R$ mediates direct contractile responses to cysLTs *in vivo*,[27] the expression of cysLT receptors by epithelial,[28] endothelial,[29] and hematopoietic cells[30,31] suggest additional noncontractile functions of cysLTs relevant to asthma and inflammation. Inhalation of LTE_4, but not inhalation of LTD_4, by human subjects with mild asthma elicits airway wall eosinophilia, basophilia, and neutrophilia when the 2 ligands are administered at doses that elicit equivalent degrees of contraction.[3,5] Inhalation challenge of naive mice with LTE_4 elicits $CysLT_3R$-dependent activation of group 2 innate lymphoid cells and consequent eosinophil recruitment by a mechanism involving IL-25 and epithelial brush cells.[32] Inhaled LTE_4 also activates airway MCs in both humans and mice by a mechanism that is blocked by $CysLT_1R$ antagonists.[27,33] In mice, repetitively inhaled LTC_4 upregulates expression of the type 2 cytokine IL-33 by alveolar type 2 (AT2) cells[34] and also elicits a substantial additional rapid, transient increase in lung IL-33 that requires recruited platelets.[35] $CysLT_2R$ deletion or blockade eliminates both of these LTC_4-inducible pools. The LTC_4/$CysLT_2R$-driven increment in IL-33 synergizes with direct LTC_4/$CysLT_1R$-dependent signaling on group 2 innate lymphoid cells (ILC2s) to promote ILC2 expansion and IL-5 and IL-13 generation, resulting in markedly potentiated airway eosinophilia.[34] Curiously, although LTC_4 and LTD_4 bind to $CysLT_2R$ with nearly equal affinity, LTD_4 potentiates eosinophilia only weakly and does not reproduce the effects of LTC_4 on platelet activation *in vivo* or *ex vivo*; nor does it induce expression of IL-33 or type 2 cytokines in lung tissue.[34] These observations suggest that LTC_4 and LTD_4 elicit qualitatively different signaling at $CysLT_2R$ *in vivo* despite equivalent binding affinities for this receptor *in vitro*.

In the current study, we have demonstrated that rather than activating $CysLT_2R$, LTD_4 functionally antagonizes the effects of LTC_4 at this receptor. LTC_4, but not LTD_4, strongly

activates p38 mitogen-activated protein kinase (MAPK) in platelets, which is blocked by LTD_4. LTD_4 blocks *ex vivo* platelet activation; production of thromboxane A_2 (TXA_2); and release of CXCL7, preformed IL-33, and high mobility box 1 (HMGB1) in response to either exogenous or endogenous LTC_4 in a dose-dependent manner, all of which require p38 activation. The inhibitory effects of LTD_4 neither require the presence of $CysLT_1R$ nor are blocked by montelukast, a $CysLT_1R$-selective antagonist. The intransal administration of LTD_4 to wild-type (WT) mice markedly suppresses LTC_4-elicited potentiation of allergen-induced pulmonary inflammation, upregulation of IL-33 expression, increases in platelet activation markers, recruitment of platelet-adherent eosinophils to the lung, and increases in lung type 2 cytokines. Moreover, LTD_4 administration to C57BL/6 mice lacking prostaglandin E_2 synthase (*Ptges*$^{-/-}$ mice) blocks the AERD-like physiologic response to inhalation challenge with lysine-aspirin (Lys-ASA), including the characteristic $CysLT_2R$- and platelet-dependent increase in lung IL-33, type 2 cytokine expression, and MC activation. Although the conversion of LTC_4 to LTD_4 generates a short-lived potent contractile agonist *in vivo*, it may also limit potential pathology induced by excessive endogenous $CysLT_2R$ receptor signaling and facilitate clinical desensitization in AERD.

METHODS

Reagents

Extract from *Dermatophagoides farinae* (*Df*) was obtained from Greer Laboratories (XPB81D3A25; Lenoir, NC). Ovalbumin (OVA) and PBS were obtained from Sigma-Aldrich (St Louis, Mo). The mMCP-1 EIA kit was purchased from eBiosciences (San Diego, Calif). LTA_4, LTC_4, LTD_4, LTE_4, MK571, and HAMI3379 were obtained from Cayman Chemical (Ann Arbor, Mich). Histamine, thromboxane receptor B_2, PGD_2, and cysLT EIA kits were obtained from Cayman. IL-4, IL-5, IL-13, ICAM-1, and VCAM-1 EIA kits were from R&D Systems (Minneapolis, Minn). The CXCL7 EIA kit was purchased from Abcam (Cambridge, Mass). The HMGB1 EIA kit was from LifeSpan (Providence, RI). The monoclonal goat anti-mouse IL-33 was purchased from R&D Systems (Minneapolis, Minn), and the rat anti-mouse IgG (H1L) secondary antibody, fluorescein isothiocyanate (FITC) anti-mouse CD11c, FITC anti-mouse/human CD11b, FITC anti-mouse IgE, FITC anti-mouse CD3ε, FITC anti-mouse CD19, FITC anti-mouse CD8a, FITC anti-mouse NK-1.1, FITC anti-mouse Ly-6G/Ly-6C (Gr-1), allophycocyanin (APC) anti-mouse CD45, APC/cyanine 7 (Cy7) anti-mouse/human CD44, PerCP/Cy5.5 anti-mouse CD90.2, phycoerythrin (PE) anti-mouse CD278 (inducible costimulatory molecule), APC anti-mouse CD41, PE/Cy7 anti-mouse CD62P, APC anti-human CD61, anti-mouse CD16/32, PE/Cy7 anti-mouse CD45, and PE anti-mouse Siglec F were all obtained from BioLegend (San Diego, Calif).

Mice

The C57BL/6 mice lacking mPGES-1 (*Ptges*$^{-/-}$ mice) were a gift from Dr Shizuo Akira (Osaka University, Japan).[36] All of the mice, including the WT C57BL/6 controls, were housed at Brigham and Women's Hospital's Hale Building for Transformative Medicine (Boston, Mass). Six-to 8-week-old male mice were used. All animal studies were approved

by the Animal Care and Use Committee of Brigham and Women's Hospital (protocol 2016N000294).

Immunization and challenge

To study potentiation of airway inflammation by exogenous cysLTs, mice were sensitized intraperitoneally on days 0 and 5 with alum-precipitated chicken egg OVA (10 μg). On days 16 to 18, the mice received intranasal challenge of 2.2 nmol LTC_4 or vehicle. On days 17 to 19, the mice were challenged by inhalation of 0.1% OVA.[37] Twenty-four hours after the final OVA aerosol challenge, the mice were humanely killed and exsanguinated. The lungs were lavaged 3 times with 0.7 mL of PBS and 5 mM EDTA. Bronchoalveolar lavage (BAL) fluid cells were cytocentrifuged onto slides, stained with Diff-Quick (Fisher Diagnostics, Middletown, Va), and differentially counted.

In the *Ptges*$^{-/-}$ mice, airway inflammation was induced by intranasal administration of *Df* (Greer, 3 μg) as described elsewhere.[38] The mice were challenged with Lys-ASA 24 hours after the last treatment.

Flow cytometry

The mouse lungs (right lobes) were transferred into a 6-well dish, and the tissue was teased apart with forceps. The tissue was then digested at room temperature for 45 minutes in 2 mL of dispase (2 U/mL), after which 0.5 mg of DNAse per mouse was added to the mixture, which was then incubated for 10 minutes at RT with gentle rocking on a shaker to 200 rpm. The cells were filtered through 70-μm nylon mesh and pelleted by centrifugation for 10 minutes at 350 *g* at 4°C. Red blood cell lysis was performed by resuspending the pellet in 2 mL of 1× red blood cell lysis buffer (Biolegend) and incubating it on ice for 4 minutes, after which the reaction was terminated by addition of 13 mL of Dulbecco modified Eagle medium. The cells were centrifuged for 10 minutes at 350 *g* at 4°C and then washed twice with fluorescence-activated cell sorting buffer (0.5% BSA in PBS). Next, 1×10^6 cells were stained with antibodies in 100 μL of fluorescence-activated cell sorting buffer for 20 minutes on ice in the dark. The cells were washed and resuspended in 300 μL of 1% paraformaldehyde in PBS before analysis on a FACSCanto flow cytometer (BD Biosciences). ILC2s were quantitated as $Lin^-CD45^+CD44^+CD278^+CD90.2^+$ cells in the lymphocyte gate.

For analysis of the platelet-adherent eosinophils, lung or BAL cells were stained with PE anti-mouse Siglec F and PE/Cy7 anti-mouse CD45 (to identify eosinophils), as well as with APC anti-mouse CD41 (to identify the platelets), after which the eosinophils were analyzed by flow cytometry. The percentages of eosinophils that were platelet-adherent ($CD41^+$) and the total numbers of platelet-adherent eosinophils were quantified.

Platelets in platelet-rich plasma (PRP) were identified on the basis of size and presence of CD41. The $CD62P^+$ baseline of the $CD41^+$ events was set at 5% for the vehicle control treatment and compared with the agonist treatment. For human samples, healthy volunteer subjects were recruited from the Brigham and Women's Hospital primary care practice for blood donations. The local institutional review board approved the study, and all subjects provided written informed consent. PRP was stimulated with LTA_4, LTC_4, LTD_4, or an equal

volume of ethanol (vehicle control) and processed for flow cytometry for surface $CD62P^+$ on the $CD61^+$ gate. In some of the experiments, supernatants and/or pellets were collected after stimulation to analyze CXCL7 and thromboxane receptor B_2 release by ELISA, or the IL-33 release by Western blot.

MAPK activation

Lysates of washed platelets stimulated with LTC_4 or vehicle in the absence or presence of LTD_4 were used to generate Western blots. Membranes were probed with antibodies specific for phosphorylated and total c-Jun terminal kinase (JNK), p38, and extracellular signal–regulated kinase (ERK) (Cell Signaling Technologies). In some experiments, platelets were stimulated in the presence of selective inhibitors of JNK (SP600125, Sigma), p38 (SB203580, InVivogen), or ERK (UO126 InVivogen) (10 μM each) for 30 minutes before activation and measurement of mediator release or flow cytometry assays.

Measurement of airway resistance

Airway resistance (R_L) in response to Lys-ASA was assessed with an Invasive Pulmonary Function Device (Buxco, Sharon, Conn). Briefly, the mice were anesthetized 24 hours after the last *Df* challenge, and tracheotomy was performed. After allowing for R_L to reach a stable baseline, Lys-ASA (12 μL of 100 mg/mL) was delivered to the lung via a nebulizer, and R_L was recorded for 45 minutes. The results were expressed as the percentage of change in R_L from baseline.

Statistical analysis

Data are expressed as means plus or minus SEMs from at least 10 mice from at least 2 experiments, except where otherwise indicated. Analyses were performed with Prism software (GraphPad Software, La Jolla, Calif). Differences between 2 treatment groups were assessed by using the Student *t* test, and differences among multiple groups were assessed by using 1-way ANOVA and the Bonferroni *post hoc* test. *P* values less than .05 were considered statistically significant.

RESULTS

LTD_4 blocks LTC_4-induced $CysLT_2R$-mediated platelet activation *ex vivo*

Although mouse and human platelets express both $CysLT_1R$ and $CysLT_2R$,[37,39] their *ex vivo* activation in response to LTC_4 is entirely $CysLT_2R$-dependent and is not reproduced by either LTD_4 or LTE_4.[37] To determine whether LTD_4 or LTE_4 interfered with $CysLT_2R$-dependent platelet activation, mouse PRP was stimulated with LTC_4 in the absence or presence of various concentrations of LTD_4. Some samples were stimulated with LTA_4, which platelets convert to LTC_4 that can activate platelets in an autocrine manner.[35] CD62P surface expression was monitored on $CD41^+$ platelets as an index of activation. LTD_4 inhibited LTC_4-induced CD62P expression in a dose-dependent manner (Fig 1, A [representative plots shown in Fig 1, E]), completely blocking its effect when provided at an equimolar dose, and it also blocked LTA_4-induced CD62P (Fig 1, D). This effect was not reproduced by LTE_4 (not shown). To determine whether the inhibitory effect of LTD_4 on LTC_4-induced CD62P expression required the presence of its preferred receptor $CysLT_1R$,

we conducted studies using the selective $CysLT_1R$ antagonist MK571 and performed experiments using PRP from *Cysltr1*$^{-/-}$ mice. The inhibitory effect of LTD_4 on LTC_4-mediated CD62P expression resisted $CysLT_1R$ blockade of WT platelets (Fig 1, B), and it was equally efficacious on platelets lacking $CysLT_1R$ and on WT platelets (Fig 1, C). LTD_4 also blocked platelet activation by LTA_4, but it did not alter activation induced by the thromboxane receptor (TP receptor) agonist U-46619 (Fig 1, D).

To determine whether LTD_4 blocked the secretion of soluble inflammatory mediators by LTC_4-stimulated platelets, we measured thromboxane receptor B_2 (as a surrogate for TXA_2 generation) and for the platelet-associated chemokine CXCL7 in supernatants of washed LTC_4-activated mouse platelets. LTC_4 elicited the release of these products, whereas LTD_4 blocked their LTC_4-mediated release (Fig 2, A). LTC_4-induced TXA_2 production and CXCL7 release by mouse platelets were blocked by the $CysLT_2R$-selective antagonist HAMI-3379, but not by MK571. MK571 did not reverse the LTD_4-induced inhibition of LTC_4-induced TXA_2 synthesis and CXCL7 release. To verify that LTD_4 could also interfere with LTC_4-dependent activation of human platelets, we stimulated washed platelets from healthy human donors with LTC_4 in the absence or presence of LTD_4. Neither LTC_4 nor LTD_4 elicited CD62 expression by human platelets (not shown). LTC_4, but not LTD_4, induced the release of CXCL7 and TXA_2 production from human platelets. LTD_4 completely blocked these responses of human platelets to LTC_4 without inducing platelet activation (Fig 2, B). In contrast to the activation response of mouse platelets, both MK571 and HAMI-3379 blocked the activation response of human platelets to LTC_4.

LTC_4 elicits the release of preformed IL-33 from mouse platelets by a $CysLT_2R$-dependent mechanism that depends on signal amplification from endogenous HMGB1 and receptor for advanced glycation end products (RAGE).[35] To determine whether LTD_4 blocked this response to LTC_4, we monitored the surface expression of HMGB1 and performed Western blotting for IL-33 on supernatants and pellets from platelets activated by LTC_4 in the absence or presence of LTD_4. LTC_4 induced both HMGB1 surface expression (see Fig E1, A in this article's Online Repository at www.jacionline.org) and release of IL-33 into the supernatants, accompanied by a decrement in IL-33 in the platelet lysate (see Fig E1, B). LTD_4 blocked both of these responses to LTC_4 (see Fig E1, A and B).

To identify mechanisms responsible for the differential responses of $CysLT_2R$ to LTC_4 versus to LTD_4, we performed signaling assays on platelets. Neither LTC_4 nor LTD_4 caused calcium flux in platelets (not shown). LTC_4, but not LTD_4, elicited phosphorylation of JNK, extracellular signal regulated kinase (ERK), and p38 kinase in platelets (see Fig E2, A in this article's Online Repository at www.jacionline.org). The concomitant administration of LTD_4 blocked these responses (see Fig E2, A and B). A selective inhibitor of p38 (SB203580), but not inhibitors of ERK (uo126) or JNK (SP600125), blocked LTC_4-induced release of IL-33 (see Fig E2, C), secretion of TXA_2 and CXCL7 (see Fig E2, D), and surface induction of CD62P and HMGB1 (see Fig E2, E) in response to LTC_4.

LTD_4 inhibits $CysLT_2R$-dependent immunopathology induced by LTC_4, but not by $CysLT_3R$-dependent responses to LTE_4

Given its ability to block LTC_4-induced platelet activation *ex vivo* via $CysLT_2R$, we sought to determine whether LTD_4 blocked the induction of eosinophilic inflammation, platelet activation markers, and type 2 cytokine expression by exogenous LTC_4 or LTE_4 *in vivo*, which depend on $CysLT_2R$ and $CysLT_3R$, respectively.[34] LTD_4 (2.2 nM) was administered intranasally on 3 successive days to WT mice that had been sensitized to OVA; it was administered concomitantly with equal quantities of either LTC_4 or LTE_4. In some experiments, the LTD_4 was administered 1 hour after the LTC_4 or LTE_4 to determine whether it dampened inflammation even after the initial receptor stimulations. Twenty-four hours after each dose of cysLTs, the mice were challenged with low-dose OVA (0.1% for 30 minutes) (Fig 3, A). Twenty-four hours after the last challenge, the mice were humanely killed, BAL fluid and lung lysates were collected, and single-cell suspensions were prepared for analysis by flow cytometry. As expected, intranasal cysLTs increased the numbers of total BAL fluid cells and eosinophils with a rank order of LTC_4 greater than LTE_4 much greater than LTD_4 (Fig 3, B). Each cysLT also increased the numbers and percentages of ILC2s in single–lung cell suspensions over control mice not receiving cysLTs, with a rank order of potency that paralleled that of the BAL fluid cell counts (Fig 3, C). LTD_4 sharply decreased the numbers of BAL fluid total cells and eosinophils induced by LTC_4 when the 2 ligands were administered together (Fig 3, B), reaching the levels observed with LTD_4 alone. In contrast, LTD_4 did not reduce the numbers of lung ILC2s induced by LTC_4 inhalation (Fig 3, C). Delayed administration of LTD_4 had no effect on LTC_4-mediated amplification of BAL fluid eosinophilia (see Fig E3 in this article's Online Repository at www.jacionline.org). LTD_4 had no effect on LTE_4/$CysLT_3R$-mediated increases in BAL fluid cellularity or eosinophilia. In contrast to the suppressive effects of LTD_4, the effects of LTE_4 on total BAL fluid cell count and eosinophil count (Fig 3, B) were additive to those of LTC_4, and LTE_4 did not suppress LTC_4-elicited ILC2 expansion (Fig 3, C). LTC_4 increased the extent of bronchovascular cellular infiltrates and markedly increased the numbers of PAS^+ goblet cells. These increases were significantly attenuated by treatment of the mice with LTD_4 (Fig 3, D and *E*). Total serum IgE measurements in all OVA-treated mouse groups increased significantly over those in the unsensitized controls, and they were modestly increased by inhaled LTC_4 (see Fig E4 in this article's Online Repository at www.jacionline.org).

LTD_4 blocks LTC_4-induced upregulation of lung IL-33 expression, platelet activation markers, and adhesion receptors

The amplification of OVA-induced immunopathology by exogenous LTC_4 requires platelet-dependent upregulation of the endothelial adhesion receptor expression[40] that is necessary for recruitment of eosinophils, as well as for the release of platelet HMGB1 and signaling through RAGE.[35] LTC_4 also elicits an increase in intranuclear IL-33 protein associated with AT2 cells.[34] LTC_4/$CysLT_2R$-elicited IL-33 synergizes with direct $CysLT_1R$-mediated stimulation to expand ILC2s and induce their production of IL-5 and IL-13.[34] Depletion of platelets or blockade of $CysLT_2R$ in this model eliminates LTC_4-induced eosinophilia, platelet activation markers, and increments in IL-5 and IL-13, the latter of which are also eliminated by ILC2 depletion. To determine whether the $CysLT_2R$-dependent processes

were specifically blocked by LTD_4 *in vivo*, we examined whole lung lysates from OVA plus LTC_4–challenged mice for IL-33 protein and downstream type 2 cytokines (IL-4, IL-5, and IL-13), as well as from BAL fluid for markers of platelet activation (CXCL7 and HMGB1) and endothelial activation (VCAM-1, ICAM-1). As expected, intranasal LTC_4 increased the content of IL-33, IL-4, IL-5, and IL-13 proteins in the lung lysates (Fig 4, A) 24 hours after the final dose. LTC_4 increased the BAL fluid content of CXCL7 and HMGB1 (Fig 4, B), as well as the content of soluble ICAM-1 and VCAM-1 (Fig 4, C). All of these were blocked by the concomitant administration of LTD_4. LTE_4 only modestly increased lung IL-33 levels (Fig 4, A), substantially potentiated IL-5 but not IL-4 or IL-13 levels, and had no effect on platelet or endothelial activation markers (Fig 4, B and *C*). None of the LTE_4 effects were blocked by the concomitant administration of LTD_4.

Although LTC_4-induced increases in lung IL-33 principally reflect its expression by AT2 cells at 24 hours after the final challenge, LTC_4 inhalation also elicits rapid and transient platelet recruitment to the lungs of sensitized mice, resulting in an additional platelet-dependent increment in BAL fluid eosinophils and a marked additional increment in lung IL-33 protein via $CysLT_2R$.[35] To determine the potential impact of LTD_4 on these rapid LTC_4/$CysLT_2R$-induced responses, we administered a fourth dose of LTC_4 with or without LTD_4 to OVA-challenged mice and humanely killed the mice 1 hour later (Fig 5, A). As expected, the additional dose of LTC_4 caused rapid, additional increases in lung levels of BAL fluid total cells (a 50% increase) and eosinophils (a 2-fold increase) (Fig 5, B). LTC_4 also sharply increased the percentages and numbers of platelet-adherent ($CD41^+$) eosinophils that are present in the BAL fluid (Fig 5, C [as shown for a representative experiment]) and dispersed lung tissue (Fig 5, D), as well as the increases in ILC2s (Fig 5, E). Compared with control mice that received vehicle, the mice that received the additional dose of LTC_4 also displayed significant increases in lung lysate levels of IL-33 and BAL fluid concentrations of HMGB1, CXCL7, and soluble endothelial adhesion receptors (ICAM-1 and VCAM-1) (Fig 5, F).[34,35] The concomitant administration of LTD_4 nearly completely blocked the rapid LTC_4-induced increases in total BAL fluid cells, total eosinophils, and platelet-adherent eosinophils in the BAL fluid and lung tissue, and it also eliminated the rapid additional increases in ILC2s, IL-33, HMGB1, and platelet and endothelial activation markers (Fig 5, B–F).

LTD_4 prevents AERD-like reactions to Lys-ASA in *Ptges*$^{-/-}$ mice

We sought to determine whether exogenous LTD_4 could interfere with AERD-like responses to inhaled Lys-ASA challenges of *Ptges*$^{-/-}$ mice, an acute physiologic event in the airways that depends on endogenously generated LTC_4.[38] We administered LTD_4 to *Df*-primed *Ptges*$^{-/-}$ mice before inhalation challenge with Lys-ASA. These mice exhibited sharply increased BAL fluid levels of cysLTs, accompanied by cysLT-dependent recruitment and activation of platelets, release of platelet-derived IL-33, IL-33-dependent activation of MCs and ILC2s, and increases in airway resistance (R_L). *Df*-primed *Ptges*$^{-/-}$ mice received intranasal LTD_4 before Lys-ASA challenge (Fig 6, A). Compared with vehicle-challenged controls, the Lys-ASA–challenged mice treated with LTD_4 displayed a lower peak R_L value (Fig 6, B); exhibited sharply reduced BAL fluid levels of the MC-derived mediators mMCP-1, PGD_2, and histamine (Fig 6, C); and reduced BAL fluid levels of CXCL7 and HMGB1 and IL-33

in the lung (Fig 6, D). Lys-ASA challenges also rapidly increased the levels of IL-4, IL-5, and IL-13 in the lung lysates (Fig 6, E). These increases were eliminated by the administration of exogenous LTD_4.

The absence of $CysLT_2R$ converts LTD_4 into a potent inducer of eosinophilic inflammation

To verify that the ability of LTD_4 to block LTC_4-elicited increases in indices of lung inflammation were due to blockade of $CysLT_2R$, we examined the effects of each of the 3 cysLTs on potentiation of OVA-induced eosinophilia and associated production of mediators in WT mice and *Cysltr2*$^{-/-}$ mice (see Fig E5, A in this article's Online Repository at www.jacionline.org). The absence of $CysLT_2R$ eliminated potentiation of OVA-induced BAL eosinophilia and all associated features of inflammation (ILC2 expansion and increases in IL-33 and platelet activation markers) (see Fig E5, B–D in this article's Online Repository at www.jacionline.org) induced by LTC_4, while only minimally affecting the responses to LTE_4. Notably, LTD_4 behaved as a stronger agonist for BAL fluid eosinophilia, ILC2 expansion, and IL-33 induction in *Cysltr2*$^{-/-}$ mice than in the WT controls.

DISCUSSION

The synthesis and release of LTC_4 is followed by successive metabolism to LTD_4 and LTE_4, effectively providing 3 distinct extracellular ligands with different respective functional properties in inflammation. Whereas some of these functions differ between mice and humans (eg, the direct $CysLT_1R$-dependent bronchoconstriction elicited by cysLT inhalation in humans[1,22] is not displayed by naive mice[41]), some are conserved (eg, airway eosinophilia[3–5,32,42] and MC activation elicited by inhaled LTE_4[27,33,43]). Although $CysLT_2R$-dependent upregulation of lung IL-33 expression by LTC_4 has not been examined in humans, LTC_4 can directly induce IL-33 release from freshly surgically excised human sinonasal tissue.[35] Restriction of LTC_4 synthesis by cyclic AMP and protein kinase A–induced 5-LO phosphorylation,[44] ligand-dependent internalization/desensitization of $CysLT_1R$,[45,46] restriction of $CysLT_1R$ membrane expression, signaling, and/or ligand binding by dimerization with $CysLT_2R$[47] or GPR17,[48] and inhibition of $CysLT_3R$ signaling by coexpression of $CysLT_1R$ and $CysLT_2R$[19,49] may all limit the potentially deleterious effects of excessive cysLT-induced signaling *in vivo*. To date, no described mechanism has limited signaling through $CysLT_2R$, which is frequently expressed by the same cells that express LTC_4S,[30] implying a potential autocrine role for LTC_4/$CysLT_2R$ signaling. The curious observations that the potency of exogenous LTC_4, acting at $CysLT_2R$, far exceeded the potency of LTD_4 for inducing type 2 immunopathology and that LTC_4, but not LTD_4, could activate mouse platelets through $CysLT_2R$[37] are counterintuitive for a receptor known to bind LTC_4 and LTD_4 with equal affinity *in vitro*. These observations prompted this study.

Exogenous LTC_4 upregulates CD62P on mouse platelets in a $CysLT_2R$-dependent manner, permitting their binding to granulocytes and other PSGL-1–expressing leukocytes.[37] Because platelets also express LTC_4S, they can convert granulocyte-derived LTA_4 to LTC_4, an event that also elicits CD62P via an autocrine, $CysLT_2R$-dependent circuit.[35] Although platelets also express $CysLT_1R$, these responses in mice require only $CysLT_2R$, are unaffected by deletion of *Cysltr3*, and cannot be reproduced by either LTD_4 or LTE_4.[37]

Several lines of evidence are consistent with LTD_4 functionally antagonizing platelet $CysLT_2R$. First, the complete blockade of LTC_4-induced CD62P by an equimolar dose of LTD_4 reflects the nearly identical affinities of LTC_4 and LTD_4 for $CysLT_2R$.[21] Second, LTD_4-mediated inhibition of LTC_4-elicited platelet activation is both resistant to the $CysLT_1R$ antagonist MK571 (Fig 1, B) and unaffected by *Cysltr1* deletion (Fig 1, C). Third, LTD_4 also blocked the response to exogenous LTA_4 (Fig 1, D), which depends on $CysLT_2R$ signaling following conversion of LTA_4 to LTC_4 by platelet LTC_4S.[35] Finally, LTD_4 did not affect CD62P expression elicited by the selective agonist of the TP receptor (Fig 1, D), suggesting a CysLT receptor–specific action. LTD_4 also blocked the release of soluble mediators (TXA_2 and CXCL7) (Fig 2, A) and prevented release of the alarmins HMGB1 and IL-33 (see Fig E1). Moreover, LTD_4 blocked the phosphorylation of all 3 MAPKs elicited by an equimolar dose of LTC_4 (see Fig E2), including p38, and inhibition of p38 (like LTD_4) abrogated all indices of LTC_4-induced platelet activation. Thus, although a non–receptor-dependent effect cannot be completely excluded, it seems likely that LTD_4 competes with LTC_4 for binding to $CysLT_2R$.

Although the selective agonist effect of LTC_4 and the blocking effect of LTD_4 observed in mouse platelets was similar for human platelets (Fig 2, B), there were significant interspecies differences. First, LTC_4 induced CXCL7 release and TXA_2 generation by human platelets in quantities nearly identical to the quantities induced in mouse platelets despite its lack of ability to induce CD62P expression. Second, both HAMI-3379 and MK571 blocked LTC_4-induced mediator release by human platelets, whereas only HAMI-3379 blocked the response of mouse platelets (Fig 2, B). A previous study reported that the $CysLT_1R$ antagonist pranlukast blocked cysLT-induced secretion of the chemokine RANTES by human platelets[39]; $CysLT_2R$ function was not assessed. It is possible $CysLT_1R$ displays different degrees of surface expression on human and mouse platelets, or that both receptors are regulated functionally by divergent postreceptor signaling pathways in the 2 species. Nevertheless, whereas the role of $CysLT_1R$ differs, the contrasting effects of LTC_4 and LTD_4, the requirement for $CysLT_2R$ for LTC_4-elicited secretory function, and the functional antagonism exerted by LTD_4 are common to the platelets of both species. This may explain the curious observation that $CysLT_2R$ displays a sharp preference for LTC_4 in certain *in vivo* models,[29,50] and it highlights the fact that G protein–coupled receptor functions are highly modified by the cellular context in which they are expressed.

Both LTC_4 (through $CysLT_2R$) and LTE_4 (through $CysLT_3R$) elicit mild airway eosinophilia when administered to naive mice by inhalation,[32,34] and both significantly amplify eosinophilia and ILC2 expansion when administered to OVA-sensitized mice before OVA challenge with a potency of LTC_4 greater than that of LTE_4. This amplification of the effector phase is not due to a requirement for LTC_4 to induce IgE sensitization (see Fig E4). Although LTD_4 and LTC_4 can both elicit $CysLT_1R$-dependent expansion of lung ILC2s *in vivo*,[34] LTD_4 potentiates allergen-induced pulmonary inflammation only weakly when compared with LTC_4 and LTE_4,[34] and unlike LTC_4, it does not elicit $CysLT_2R$-dependent IL-33 expression and platelet activation. Several lines of evidence suggest that LTD_4 instead actively suppresses $CysLT_2R$ signaling to block inflammation elicited in response to LTC_4 *in vivo*. First, LTD_4 suppressed all features of this model that depend exclusively on signaling through $CysLT_2R$ (potentiation of BAL fluid eosinophilia [Fig 3, B], induction of

bronchovascular inflammation [Fig 3, D and E], increases in IL-33 protein content and level of downstream type 2 cytokines [Fig 4, A], increases in HMGB1 and CXCL7 [Fig 4, B], and upregulation of endothelial adhesion receptors [Fig 4, C]). Second, the $CysLT_1R$-dependent expansion of ILC2s was modestly induced, rather than suppressed, by LTD_4 (Fig 3, C). Third, LTD_4 failed to alter the activity of LTE_4, which induces eosinophilic pathology through epithelium-associated $CysLT_3R$ and the platelet-associated purinergic receptor $P2Y_{12}$[34,42] (Fig 3, B and C). These findings support a surprising degree of functional segregation of cysLT-driven immunologic effects despite predicted crossover of these ligands on the same receptors based on studies using the recombinant proteins. The fact that the antagonism of LTC_4 effects by LTD_4 *in vivo* required that the 2 ligands be administered simultaneously (see Fig E3) implies that the mechanism likely involves direct competition for the same binding site on platelets or other relevant effectors.

Activated platelets adhere avidly to circulating eosinophils in asthma[51] and especially in AERD,[52] likely priming them for adhesion and migration to the respiratory tissue. Platelets store IL-33 in their cytosol,[53] and they release IL-33 in response to LTC_4 by a mechanism involving $CysLT_2R$-driven mobilization of cytosolic HMGB1 and autocrine signaling through RAGE.[35] Whereas LTC_4-potentiated IL-33 protein localizes principally to AT2 cells 24 hours after LTC_4 challenge,[34] intransal LTC_4 also elicits rapid additional increments in IL-33, type 2 cytokine generation, and eosinophil recruitment, all of which depend on rapid $CysLT_2R$-dependent intrapulmonary platelet recruitment and activation. LTD_4 completely blocked these rapid platelet-and $CysLT_2R$-dependent events (Fig 5, B–D and F), which is consistent with its ability to prevent LTC_4-induced release of IL-33 from platelets *ex vivo* (see Fig E1). Notably, virtually the entire increment in eosinophils observed in this short time frame was due to the platelet-adherent fraction, suggesting another platelet-related effect of LTC_4. It is possible that eosinophils provide a vehicle to transport platelet-associated IL-33 to the lung tissue to elicit a rapid amplification of type 2 cytokine generation from ILC2s and potentially additional cell types. Notably, LTC_4 potently synergizes with IL-33 to induce ILC2 activation *ex vivo*,[54] and platelet/eosinophil complexes could provide both of these ligands following their recruitment. Additionally, platelet-associated IL-33 could also directly activate eosinophils.[55]

CysLT-dependent respiratory reactions to aspirin and other COX-1–active drugs are the pathognomonic feature of AERD,[13] a disease that involves both cysLToverproduction and selective hyperresponsiveness to LTE_4-induced bronchoconstriction.[12,56] *Ptges*$^{-/-}$ mice display both of these AERD-like properties,[33,38] whereas WT mice show negligible cysLT-specific airway responses.[33,41] Our previous studies demonstrated that several features of AERD-like responses of *Df*-primed *Ptges*$^{-/-}$ mice to inhaled Lys-ASA were blocked by HAMI-3379, including the rapid recruitment and activation of platelets in the lung, IL-33–driven MC activation, and changes in airway physiology,[35] as well as rapid increases in IL-5 and IL-13 protein levels. Accordingly, LTD_4 administered by inhalation before Lys-ASA challenge prevented all HAMI-3379–sensitive features of the reaction to Lys-ASA (Fig 6). We cannot exclude an additional significant contribution from ligand-induced downregulation of $CysLT_1R$ expression *in vivo*, a function that limits signaling in response to LTD_4 in transfected cells,[45,57] and this may account for the sharp reduction in $CysLT_1R$ expression by leukocytes in nasal tissue biopsy specimens from subjects with AERD after

therapeutic desensitization to aspirin.[46] Nonetheless, our findings suggest that LTD_4 *in vivo* likely terminates signaling through $CysLT_2R$ as an adaptive feature leading to a loss of end-organ responsiveness to endogenous cysLTs. This process may be especially relevant to AERD, in which aspirin-induced reactions are followed by refractoriness to cysLT-induced bronchoconstriction[58] and in which high-dose aspirin produces a clinical benefit despite increasing the levels of cysLT generation.[59]

Although $CysLT_2R$ is essential to the agonistic effects of LTC_4 on platelets[37] and dermal fibroblasts,[50] it also suppresses $CysLT_1R$-dependent signaling responses to LTD_4 in dendritic cells[60,61] and MCs.[47] As expected, deletion of $CysLT_2R$ eliminated the potentiation of OVA-induced BAL fluid eosinophilia elicited by LTC_4, but it markedly potentiated the response to LTD_4, suggesting a loss of $CysLT_2R$-mediated restraint of $CysLT_1R$ signaling on 1 or more key cell types. Because responses to LTE_4 were not altered, it seems likely that $CysLT_2R$ does not regulate the LTE_4-$CysLT_3R$ pathway that elicits airway inflammation. Notably, whereas the absence of $CysLT_2R$ markedly inhibited the induced expression of IL-33 in response LTC_4, it sharply increased this response to LTD_4, implying that at least 1 intrapulmonary cell type is competent to drive IL-33 induction when the restraining effects of $CysLT_2R$ on $CysLT_1R$ signaling are removed. Thus, depending on the cellular context, $CysLT_2R$ may act to suppress or induce functions relevant to type 2 inflammation. Notably, variants of $CysLT_2R$ that affect expression or ligand binding are associated with asthma and allergic sensitization, and they could be important determinants of whether LTD_4 acts to induce or suppress inflammation and cellular functions.[62,63]

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Abbreviations used

AERD	Aspirin-exacerbated respiratory disease
APC	Allophycocyanin
AT2	Alveolar type 2
BAL	Bronchoalveolar lavage
CysLT	Cysteinyl leukotriene
$CysLT_1R$	Type 1 receptor for cysLT
$CysLT_2R$	Type 2 receptor for cysLT
$CysLT_3R$	Type 3 receptor for cysLT
Df	Extract from *Dermatophagoides farina*

ERK	Extracellular signal–regulated kinase
FITC	Fluorescein isothiocyanate
5- ILC2	Group 2 innate lymphoid cell
HMGB1	High-mobility box 1
JNK c	Jun terminal kinase
LO	5-Lipoxygenase
LT	Leukotriene
LTC_4S	Leukotriene C_4 synthase
Lys	-ASA Lysine-aspirin
MAPK	Mitogen-activated protein kinase
MC	Mast cell
NFAT	Nuclear factor of activated T
OVA	Ovalbumin
PE	Phycoerythrin
PRP	Platelet-rich plasma
PTGES	Prostaglandin E_2 synthase
RAGE	Receptor for advanced glycation end products
R_L	Lung resistance
TXA_2	Thromboxane A_2
WT	Wild-type

REFERENCES

1. Weiss JW, Drazen JM, Coles N, McFadden ER Jr, Weller PF, Corey EJ, et al. Bronchoconstrictor effects of leukotriene C in humans. Science 1982;216:196–8. [PubMed: 7063880]
2. Marom Z, Shelhamer JH, Bach MK, Morton DR, Kaliner M. Slow-reacting substances, leukotrienes C4 and D4, increase the release of mucus from human airways in vitro. Am Rev Respir Dis 1982;126:449–51. [PubMed: 7125334]
3. Laitinen LA, Laitinen A, Haahtela T, Vilkka V, Spur BW, Lee TH. Leukotriene E4 and granulocytic infiltration into asthmatic airways. Lancet 1993;341:989–90. [PubMed: 8096945]
4. Laitinen A, Lindqvist A, Halme M, Altraja A, Laitinen LA. Leukotriene E:-induced persistent eosinophilia and airway obstruction are reversed by zafirlukast in patients with asthma. J Allergy Clin Immunol 2005;115:259–65. [PubMed: 15696079]
5. Gauvreau GM, Parameswaran KN, Watson RM, O'Byrne PM. Inhaled leukotriene E(4), but not leukotriene D(4), increased airway inflammatory cells in subjects with atopic asthma. Am J Respir Crit Care Med 2001;164:1495–500. [PubMed: 11704602]

6. Reid GK, Kargman S, Vickers PJ, Mancini JA, Leveille C, Ethier D, et al. Correlation between expression of 5-lipoxygenase-activating protein, 5-lipoxygenase, and cellular leukotriene synthesis. J Biol Chem 1990;265:19818–23. [PubMed: 2174053]
7. Lam BK, Penrose JF, Freeman GJ, Austen KF. Expression cloning of a cDNA for human leukotriene C4 synthase, an integral membrane protein conjugating reduced glutathione to leukotriene A4. Proc Natl Acad Sci U S A 1994;91:7663–7. [PubMed: 8052639]
8. Austen KF, Maekawa A, Kanaoka Y, Boyce JA. The leukotriene E4 puzzle: finding the missing pieces and revealing the pathobiologic implications. J Allergy Clin Immunol 2009;124:406–14. [PubMed: 19647860]
9. Shi ZZ, Han B, Habib GM, Matzuk MM, Lieberman MW. Disruption of gamma-glutamyl leukotrienase results in disruption of leukotriene D: synthesis in vivo and attenuation of the acute inflammatory response. Mol Cell Biol 2001;21: 5389–95. [PubMed: 11463821]
10. Lee CW, Lewis RA, Corey EJ, Austen KF. Conversion of leukotriene D4 to leukotriene E4 by a dipeptidase released from the specific granule of human polymorphonuclear leucocytes. Immunology 1983;48:27–35. [PubMed: 6293969]
11. Drazen JM, O'Brien J, Sparrow D, Weiss ST, Martins MA, Israel E, et al. Recovery of leukotriene E4 from the urine of patients with airway obstruction. Am Rev Respir Dis 1992;146:104–8. [PubMed: 1320817]
12. Christie PE, Tagari P, Ford-Hutchinson AW, Charlesson S, Chee P, Arm JP, et al. Urinary leukotriene E4 concentrations increase after aspirin challenge in aspirin-sensitive asthmatic subjects. Am Rev Respir Dis 1991;143:1025–9. [PubMed: 1850964]
13. Israel E, Fischer AR, Rosenberg MA, Lilly CM, Callery JC, Shapiro J, et al. The pivotal role of 5-lipoxygenase products in the reaction of aspirin-sensitive asthmatics to aspirin. Am Rev Respir Dis 1993;148:1447–51. [PubMed: 8256883]
14. White A, Ludington E, Mehra P, Stevenson DD, Simon RA. Effect of leukotriene modifier drugs on the safety of oral aspirin challenges. Ann Allergy Asthma Immunol 2006;97:688–93. [PubMed: 17165280]
15. Liu MC, Dube LM, Lancaster J. Acute and chronic effects of a 5-lipoxygenase inhibitor in asthma: a 6-month randomized multicenter trial. Zileuton Study Group. J Allergy Clin Immunol 1996;98:859–71. [PubMed: 8939149]
16. Israel E, Chervinsky PS, Friedman B, Van BJ, Skalky CS, Ghannam AF, et al. Effects of montelukast and beclomethasone on airway function and asthma control. J Allergy Clin Immunol 2002;110:847–54. [PubMed: 12464949]
17. Lynch KR, O'Neill GP, Liu Q, Im DS, Sawyer N, Metters KM, et al. Characterization of the human cysteinyl leukotriene CysLT1 receptor. Nature 1999;399:789–93. [PubMed: 10391245]
18. Heise CE, O'Dowd BF, Figueroa DJ, Sawyer N, Nguyen T, Im DS, et al. Characterization of the human cysteinyl leukotriene 2 receptor. J Biol Chem 2000;275: 30531–6. [PubMed: 10851239]
19. Kanaoka Y, Maekawa A, Austen KF. Identification of GPR99 protein as a potential third cysteinyl leukotriene receptor with a preference for leukotriene E4 ligand. J Biol Chem 2013;288:10967–72. [PubMed: 23504326]
20. Bankova LG, Lai J, Yoshimoto E, Boyce JA, Austen KF, Kanaoka Y, et al. Leukotriene E4 elicits respiratory epithelial cell mucin release through the G-protein-coupled receptor, GPR99. Proc Natl Acad Sci U S A 2016;113:6242–7. [PubMed: 27185938]
21. Hui Y, Yang G, Galczenski H, Figueroa DJ, Austin CP, Copeland NG, et al. The murine cysteinyl leukotriene 2 (CysLT2) receptor. cDNA and genomic cloning, alternative splicing, and in vitro characterization. J Biol Chem 2001;276:47489–95. [PubMed: 11591709]
22. Griffin M, Weiss JW, Leitch AG, McFadden ER Jr, Corey EJ, Austen KF, et al. Effects of leukotriene D on the airways in asthma. N Engl J Med 1983;308:436–9. [PubMed: 6823253]
23. Lee TH, Austen KF, Corey EJ, Drazen JM. Leukotriene E4-induced airway hyperresponsiveness of guinea pig tracheal smooth muscle to histamine and evidence for three separate sulfidopeptide leukotriene receptors. Proc Natl Acad Sci U S A 1984;81:4922–5. [PubMed: 6087352]
24. Aharony D Pharmacology of leukotriene receptor antagonists. Am J Respir Crit Care Med 1998;157:S214–8, discussion S8–9, S47-S48.

25. Davidson AB, Lee TH, Scanlon PD, Solway J, McFadden ER Jr, Ingram RH Jr, et al. Bronchoconstrictor effects of leukotriene E4 in normal and asthmatic subjects. Am Rev Respir Dis 1987;135:333–7. [PubMed: 3028218]
26. Arm JP, O'Hickey SP, Hawksworth RJ, Fong CY, Crea AE, Spur BW, et al. Asthmatic airways have a disproportionate hyperresponsiveness to LTE4, as compared with normal airways, but not to LTC4, LTD4, methacholine, and histamine. Am Rev Respir Dis 1990;142:1112–8. [PubMed: 2173457]
27. Lazarinis N, Bood J, Gomez C, Kolmert J, Lantz AS, Gyllfors P, et al. Leukotriene E4 induces airflow obstruction and mast cell activation through the cysteinyl leukotriene type 1 receptor. J Allergy Clin Immunol 2018;142:l1080–9.
28. Corrigan C, Mallett K, Ying S, Roberts D, Parikh A, Scadding G, et al. Expression of the cysteinyl leukotriene receptors cysLT(1) and cysLT(2) in aspirin-sensitive and aspirin-tolerant chronic rhinosinusitis. J Allergy Clin Immunol 2005;115: 316–22. [PubMed: 15696087]
29. Uzonyi B, Lotzer K, Jahn S, Kramer C, Hildner M, Bretschneider E, et al. Cysteinyl leukotriene 2 receptor and protease-activated receptor 1 activate strongly correlated early genes in human endothelial cells. Proc Natl Acad Sci U S A 2006;103:6326–31. [PubMed: 16606835]
30. Figueroa DJ, Borish L, Baramki D, Philip G, Austin CP, Evans JF. Expression of cysteinyl leukotriene synthetic and signalling proteins in inflammatory cells in active seasonal allergic rhinitis. Clin Exp Allergy 2003;33:1380–8. [PubMed: 14519144]
31. Zhu J, Qiu YS, Figueroa DJ, Bandi V, Galczenski H, Hamada K, et al. Localization and upregulation of cysteinyl leukotriene-1 receptor in asthmatic bronchial mucosa. Am J Respir Cell Mol Biol 2005;33:531–40. [PubMed: 16123393]
32. Bankova LG, Dwyer DF, Yoshimoto E, Ualiyeva S, McGinty JW, Raff H, et al. The cysteinyl leukotriene 3 receptor regulates expansion of IL-25-producing airway brush cells leading to type 2 inflammation. Sci Immunol 2018;3:eaat9453. [PubMed: 30291131]
33. Liu T, Kanaoka Y, Barrett NA, Feng C, Garofalo D, Lai J, et al. Aspirin-exacerbated respiratory disease involves a cysteinyl leukotriene-driven IL-33-mediated mast cell activation pathway. J Immunol 2015;195:3537–45. [PubMed: 26342029]
34. Liu T, Barrett NA, Kanaoka Y, Yoshimoto E, Garofalo D, Cirka H, et al. Type 2 cysteinyl leukotriene receptors drive IL-33-dependent type 2 immunopathology and aspirin sensitivity. J Immunol 2018;200:915–27. [PubMed: 29282304]
35. Liu T, Barrett NA, Kanaoka Y, Buchheit K, Laidlaw TM, Garofalo D, et al. Cysteinyl leukotriene receptor 2 drives lung immunopathology through a platelet and high mobility box 1-dependent mechanism. Mucosal Immunol 2019;12: 679–90. [PubMed: 30664709]
36. Uematsu S, Matsumoto M, Takeda K, Akira S. Lipopolysaccharide-dependent prostaglandin E(2) production is regulated by the glutathione-dependent prostaglandin E(2) synthase gene induced by the Toll-like receptor 4/MyD88/NF-IL6 pathway. J Immunol 2002;168:5811–6. [PubMed: 12023384]
37. Cummings HE, Liu T, Feng C, Laidlaw TM, Conley PB, Kanaoka Y, et al. Cutting edge: leukotriene C4 activates mouse platelets in plasma exclusively through the type 2 cysteinyl leukotriene receptor. J Immunol 2013; 191:5807–10. [PubMed: 24244016]
38. Liu T, Laidlaw TM, Katz HR, Boyce JA. Prostaglandin E2 deficiency causes a phenotype of aspirin sensitivity that depends on platelets and cysteinyl leukotrienes. Proc Natl Acad Sci U S A 2013;110:16987–92. [PubMed: 24085850]
39. Hasegawa S, Ichiyama T, Hashimoto K, Suzuki Y, Hirano R, Fukano R, et al. Functional expression of cysteinyl leukotriene receptors on human platelets. Platelets 2010;21:253–9. [PubMed: 20433311]
40. Liu T, Garofalo D, Feng C, Lai J, Katz H, Laidlaw TM, et al. Platelet-driven leukotriene C4-mediated airway inflammation in mice is aspirin-sensitive and depends on T prostanoid receptors. J Immunol 2015;194:5061–8. [PubMed: 25904552]
41. Martin TR, Gerard NP, Galli SJ, Drazen JM. Pulmonary responses to bronchoconstrictor agonists in the mouse. J Appl Physiol 1988;64:2318–23. [PubMed: 2457008]

42. Paruchuri S, Tashimo H, Feng C, Maekawa A, Xing W, Jiang Y, et al. Leukotriene E4-induced pulmonary inflammation is mediated by the P2Y12 receptor. J Exp Med 2009;206:2543–55. [PubMed: 19822647]
43. Paruchuri S, Jiang Y, Feng C, Francis SA, Plutzky J, Boyce JA. Leukotriene E4 activates peroxisome proliferator-activated receptor gamma and induces prostaglandin D2 generation by human mast cells. J Biol Chem 2008; 283:16477–87. [PubMed: 18411276]
44. Luo M, Jones SM, Flamand N, Aronoff DM, Peters-Golden M, Brock TG. Phosphorylation by protein kinase a inhibits nuclear import of 5-lipoxygenase. J Biol Chem 2005;280:40609–16. [PubMed: 16230355]
45. Naik S, Billington CK, Pascual RM, Deshpande DA, Stefano FP, Kohout TA, et al. Regulation of cysteinyl leukotriene type 1 receptor internalization and signaling. J Biol Chem 2005;280:8722–32. [PubMed: 15590629]
46. Sousa AR, Parikh A, Scadding G, Corrigan CJ, Lee TH. Leukotriene-receptor expression on nasal mucosal inflammatory cells in aspirin-sensitive rhinosinusitis. N Engl J Med 2002;347:1493–9. [PubMed: 12421891]
47. Jiang Y, Borrelli LA, Kanaoka Y, Bacskai BJ, Boyce JA. CysLT2 receptors interact with CysLT1 receptors and down-modulate cysteinyl leukotriene dependent mitogenic responses of mast cells. Blood 2007;110:3263–70. [PubMed: 17693579]
48. Maekawa A, Xing W, Austen KF, Kanaoka Y. GPR17 regulates immune pulmonary inflammation induced by house dust mites. J Immunol 2010;185: 1846–54. [PubMed: 20574000]
49. Maekawa A, Kanaoka Y, Xing W, Austen KF. Functional recognition of a distinct receptor preferential for leukotriene E4 in mice lacking the cysteinyl leukotriene 1 and 2 receptors. Proc Natl Acad Sci U S A 2008;105:16695–700. [PubMed: 18931305]
50. Oyoshi MK, He R, Kanaoka Y, ElKhal A, Kawamoto S, Lewis CN, et al. Eosinophil-derived leukotriene C4 signals via type 2 cysteinyl leukotriene receptor to promote skin fibrosis in a mouse model of atopic dermatitis. Proc Natl Acad Sci U S A 2012;109:4992–7. [PubMed: 22416124]
51. Johansson MW, Han ST, Gunderson KA, Busse WW, Jarjour NN, Mosher DF. Platelet activation, P-selectin, and eosinophil beta1-integrin activation in asthma. Am J Respir Crit Care Med 2012;185:498–507. [PubMed: 22227382]
52. Laidlaw TM, Kidder MS, Bhattacharyya N, Xing W, Shen S, Milne GL, et al. Cysteinyl leukotriene overproduction in aspirin-exacerbated respiratory disease is driven by platelet-adherent leukocytes. Blood 2012;119:3790–8. [PubMed: 22262771]
53. Takeda T, Unno H, Morita H, Futamura K, Emi-Sugie M, Arae K, et al. Platelets constitutively express IL-33 protein and modulate eosinophilic airway inflammation. J Allergy Clin Immunol 2016;138:1395–403. [PubMed: 27056266]
54. von MJ, O'Leary CE, Barrett NA, Kanaoka Y, Austen KF, Locksley RM. Leukotrienes provide an NFAT-dependent signal that synergizes with IL-33 to activate ILC2s. J Exp Med 2017;214:27–37. [PubMed: 28011865]
55. Cherry WB, Yoon J, Bartemes KR, Iijima K, Kita H. A novel IL-1 family cytokine, IL-33, potently activates human eosinophils. J Allergy Clin Immunol 2008;121: 1484–90. [PubMed: 18539196]
56. Christie PE, Schmitz-Schumann M, Spur BW, Lee TH. Airway responsiveness to leukotriene C4 (LTC4), leukotriene E4 (LTE4) and histamine in aspirin-sensitive asthmatic subjects. Eur Respir J 1993;6:1468–73. [PubMed: 8112440]
57. Capra V, Ravasi S, Accomazzo MR, Citro S, Grimoldi M, Abbracchio MP, et al. CysLT1 receptor is a target for extracellular nucleotide-induced heterologous desensitization: a possible feedback mechanism in inflammation. J Cell Sci 2005;118:5625–36. [PubMed: 16306225]
58. Arm JP, O'Hickey SP, Spur BW, Lee TH. Airway responsiveness to histamine and leukotriene E4 in subjects with aspirin-induced asthma. Am Rev Respir Dis 1989; 140:148–53. [PubMed: 2546469]
59. Cahill KN, Cui J, Kothari P, Murphy K, Raby BA, Singer J, et al. Unique effect of aspirin therapy on biomarkers in aspirin-exacerbated respiratory disease: a prospective trial. Am J Respir Crit Care Med 2019;200:704–11. [PubMed: 30978291]

60. Barrett NA, Maekawa A, Rahman OM, Austen KF, Kanaoka Y. Dectin-2 recognition of house dust mite triggers cysteinyl leukotriene generation by dendritic cells. J Immunol 2009;182:1119–28. [PubMed: 19124755]

61. Barrett NA, Fernandez JM, Maekawa A, Xing W, Li L, Parsons MW, et al. Cysteinyl leukotriene 2 receptor on dendritic cells negatively regulates ligand-dependent allergic pulmonary inflammation. J Immunol 2012;189:4556–65. [PubMed: 23002438]

62. Thompson MD, Storm van's GK, Galczenski H, Burnham WM, Siminovitch KA, Zamel N, et al. A cysteinyl leukotriene 2 receptor variant is associated with atopy in the population of Tristan da Cunha. Pharmacogenetics 2003;13: 641–9. [PubMed: 14515063]

63. Pillai SG, Cousens DJ, Barnes AA, Buckley PT, Chiano MN, Hosking LK, et al. A coding polymorphism in the CYSLT2 receptor with reduced affinity to LTD4 is associated with asthma. Pharmacogenetics 2004;14:627–33. [PubMed: 15475736]

Key messages

- Leukotriene D_4 paradoxically blocks leukotriene C_4–induced platelet activation and accumulation of platelet-adherent eosinophils.
- Leukotriene D_4 may act to terminate reactions to aspirin by preventing pathogenic leukotriene C_4–induced signaling at $CysLT_2R$.

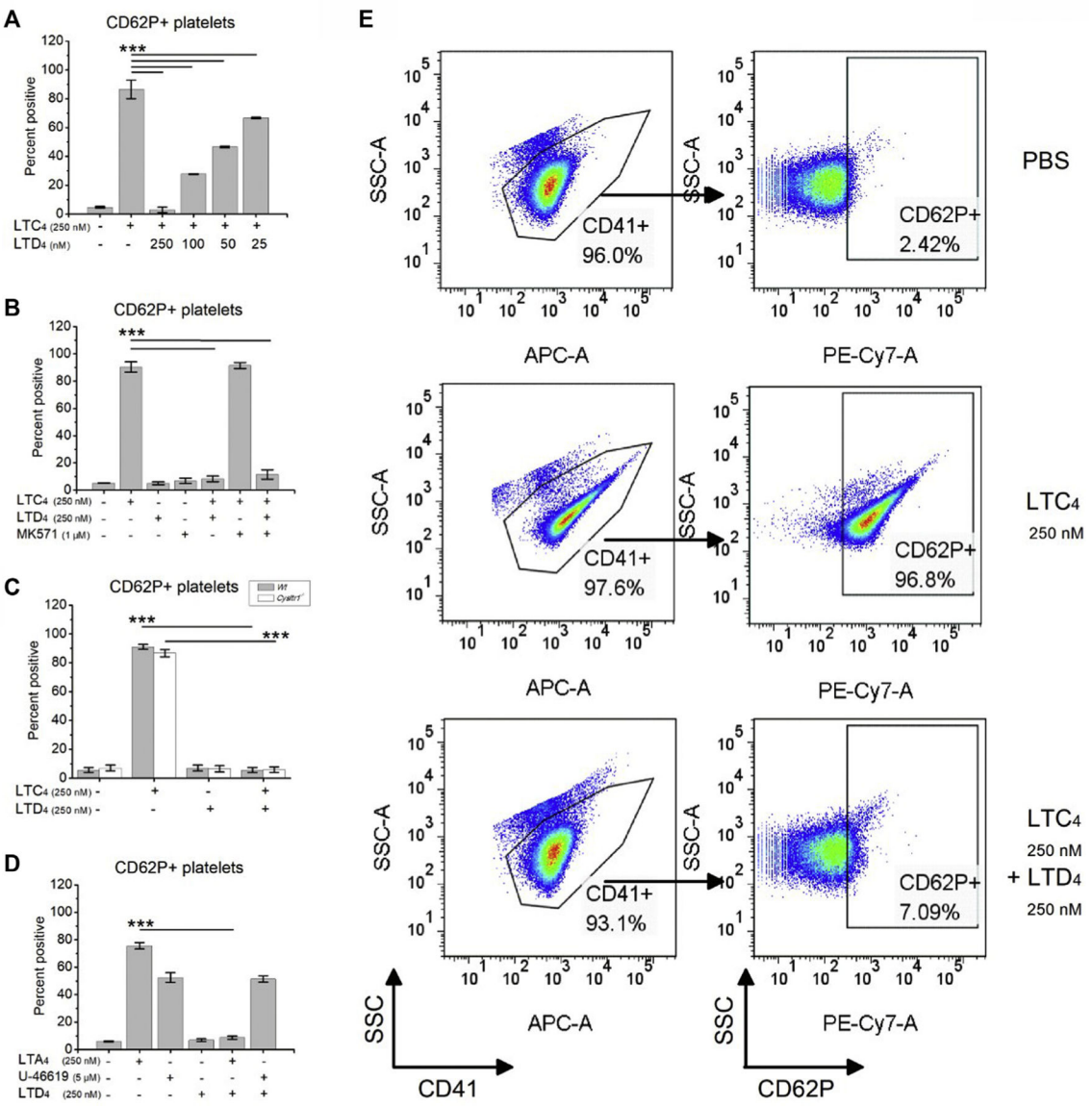

FIG 1.

LTD_4 blocks LTC_4/$CysLT_2R$-dependent platelet activation. PRP from the indicated mouse strains was stimulated with LTC_4 (250 nM) for 30 minutes with or without the simultaneous addition of the indicated agonists and antagonists. Surface expression of CD62P was monitored by flow cytometry. **A**, Dose-dependent effect of LTD_4 on LTC_4-elicited expression of CD62P. **B**, Lack of effect of the $CysLT_1R$-selective antagonist MK571 on LTC_4-elicited CD62P expression and its suppression by LTD_4. **C**, Lack of effect of *Cysltr1* deletion on LTC_4-elicited CD62P expression. **D**, Effect of LTD_4 on CD62P expression elicited by the indicated agonists. **E**, A representative plot showing the effect of LTC_4 on CD62P expression and the blocking effect of LTD_4. Results in (**A**) to (**D**) are the means ± SDs from 3 separate experiments. ***$P < .001$. *APC-A*, Allaphycocyanin; *SSC-A*, side angle light scatter.

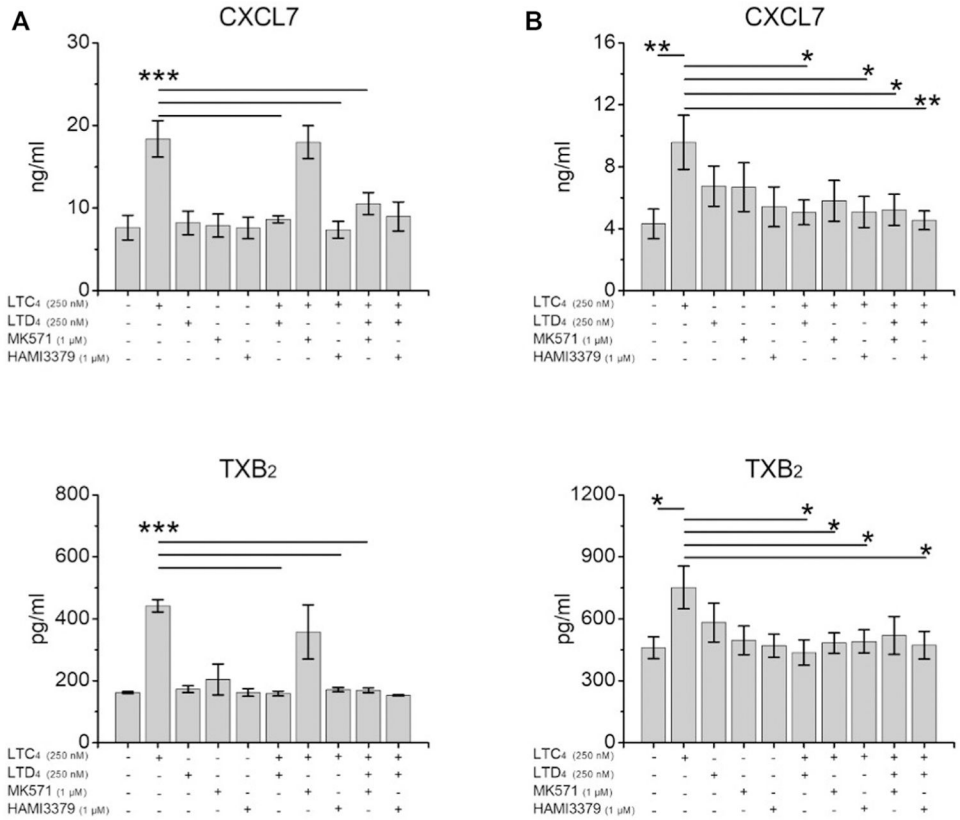

FIG 2.

LTD_4 blocks the release of soluble mediators from mouse and human platelets activated by LTC_4. Washed platelets from WT mice (**A**) or healthy human volunteers (**B**) were stimulated for 30 minutes with LTC_4 in the absence or presence of the indicated agonists or antagonists. CXCL7 and thromboxane receptor B_2 (TXB_2) (as a surrogate for TXA_2 production) were measured in the supernatants by ELISA. The data in (**A**) are means 6 SDs from 3 experiments. The data in (**B**) are from 10 experiments using platelets from different donors. ***$P < .001$; **$P < .01$; *$P < .05$.

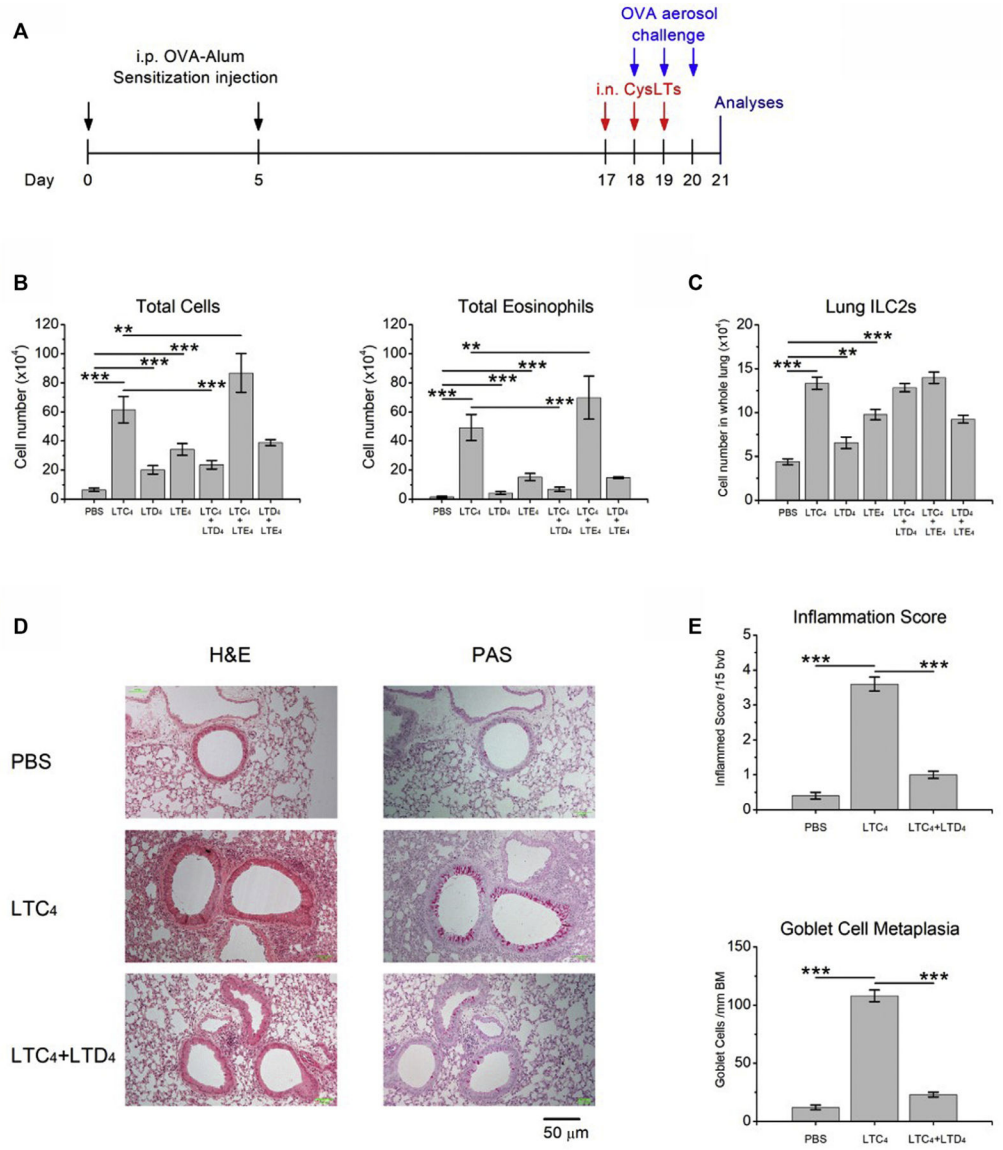

FIG 3.

LTD_4 blocks amplification of OVA-induced pulmonary inflammation induced by LTC_4 but not by LTE_4. **A**, Time line of the experiments. WT mice were sensitized on days 0 and 7 with OVA/alum intraperitoneally. On days 14 to 16, the mice received single intranasal doses (2.2 nmol) of the indicated cysLTs, followed 24 hours later by inhaled OVA (0.1% for 30 minutes). BAL fluid and lung tissue were collected 24 hours after the last dose of OVA. **B**, Total BAL fluid cell counts (*left*) and eosinophil counts (*right*). **C**, Numbers of ILC2s in dispersed lung tissue from the indicated groups. **D**, Hematoxylin and eosin (H&E) and periodic acid–Schiff (PAS) stains from the lungs of the indicated groups of mice. **E**, Quantification of inflammation score and PAS^+ goblet cells. Results in (**B-D**) are from 3 separate experiments with 10 to 15 mice/group.

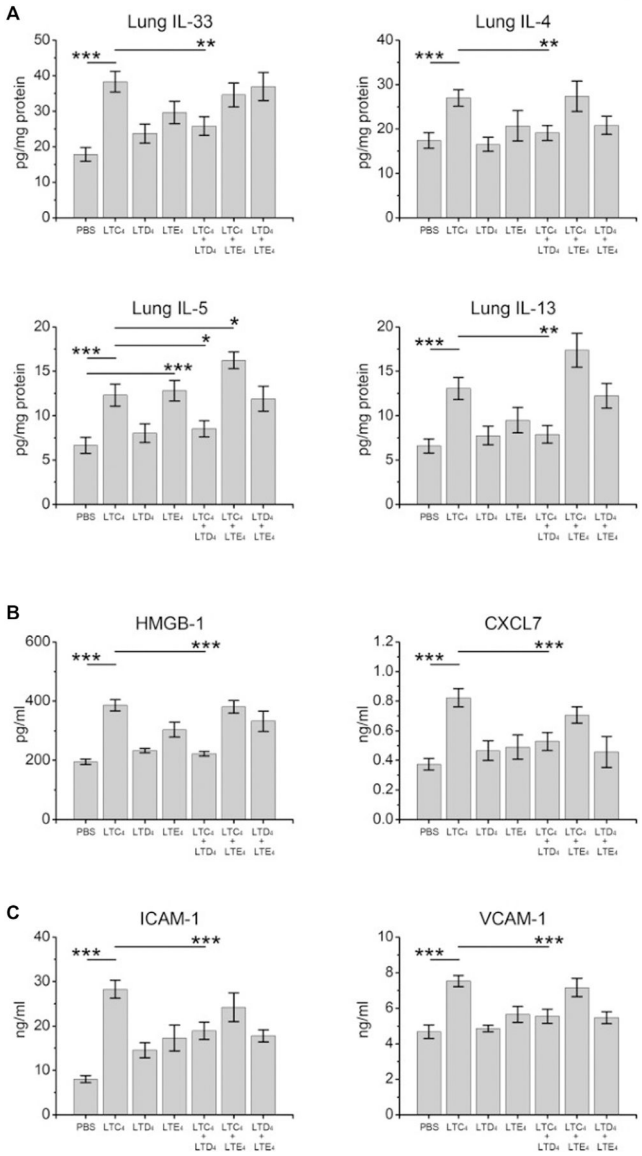

FIG 4.

Effect of LTD_4 on lung lysate cytokines and BAL fluid mediators induced by LTC_4 and LTE_4. A, ELISA measurements of IL-33, IL-4, IL-5, and IL-13 in whole lung lysates from WT OVA-sensitized and challenged mice receiving the indicated cysLTs. Measurements of BAL fluid HMGB1 and CXCL7 (**B**) and soluble adhesion receptors (**C**) from the same mice as in (**A**). Results are from 10–15 mice/group. ***$P < .001$; **$P < .01$; *$P < .05$.

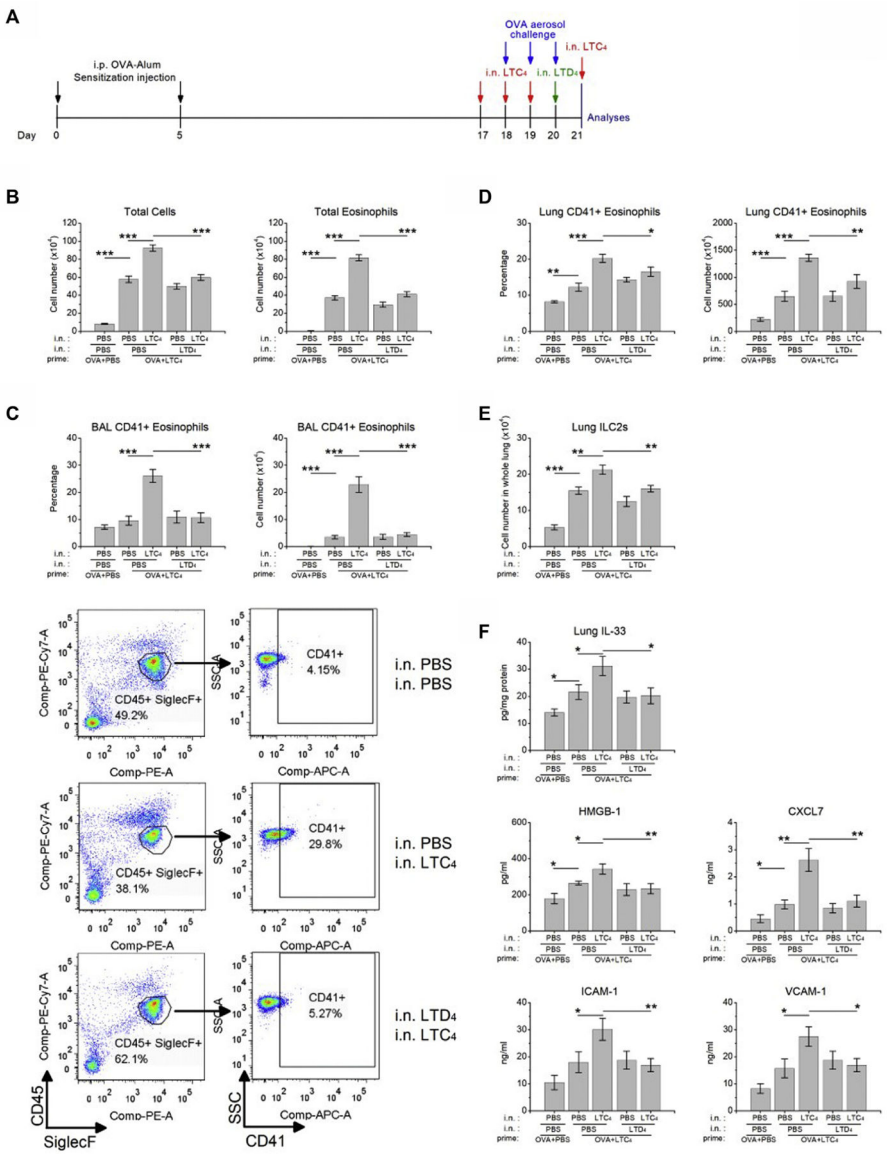

FIG 5.

LTC_4 rapidly elicits incremental lung recruitment of platelet-adherent eosinophils and IL-33, which are blocked by LTD_4. **A**, Time line for the experiments. WT OVA-sensitized mice that were challenged with 3 doses of LTC_4 and OVA were challenged 24 hours later with either LTC_4 (2.2 nmol) or PBS. BAL and lung tissue were collected 30 minutes afterward. **B**, Total numbers of cells (*left*) and eosinophils (*right*) in BAL fluids collected from the indicated groups of mice. **C**, Percentages (*left*) and total numbers (*right*) of platelet-adherent ($CD41^1$) eosinophils identified in BAL fluid cells by flow cytometry. **D**, Percentages (*left*) and numbers (*right*) of platelet-adherent eosinophils identified in single–lung cell suspensions. Representative plots showing platelet-adherent eosinophils in the BAL fluid from mice of the indicated treatment groups. **E**, Numbers of ILC2s in dispersed lung tissues from the indicated groups. **F**, Whole lung levels of IL-33 and platelet and endothelial activation

markers from the same mice as in (**B**). Results are from 10 to 15 mice/group. ***$P < .001$; **$P < .01$; *$P < .05$. *Comp.*, Complete; *i.n.*, intranasal; *SSC*, side scatter.

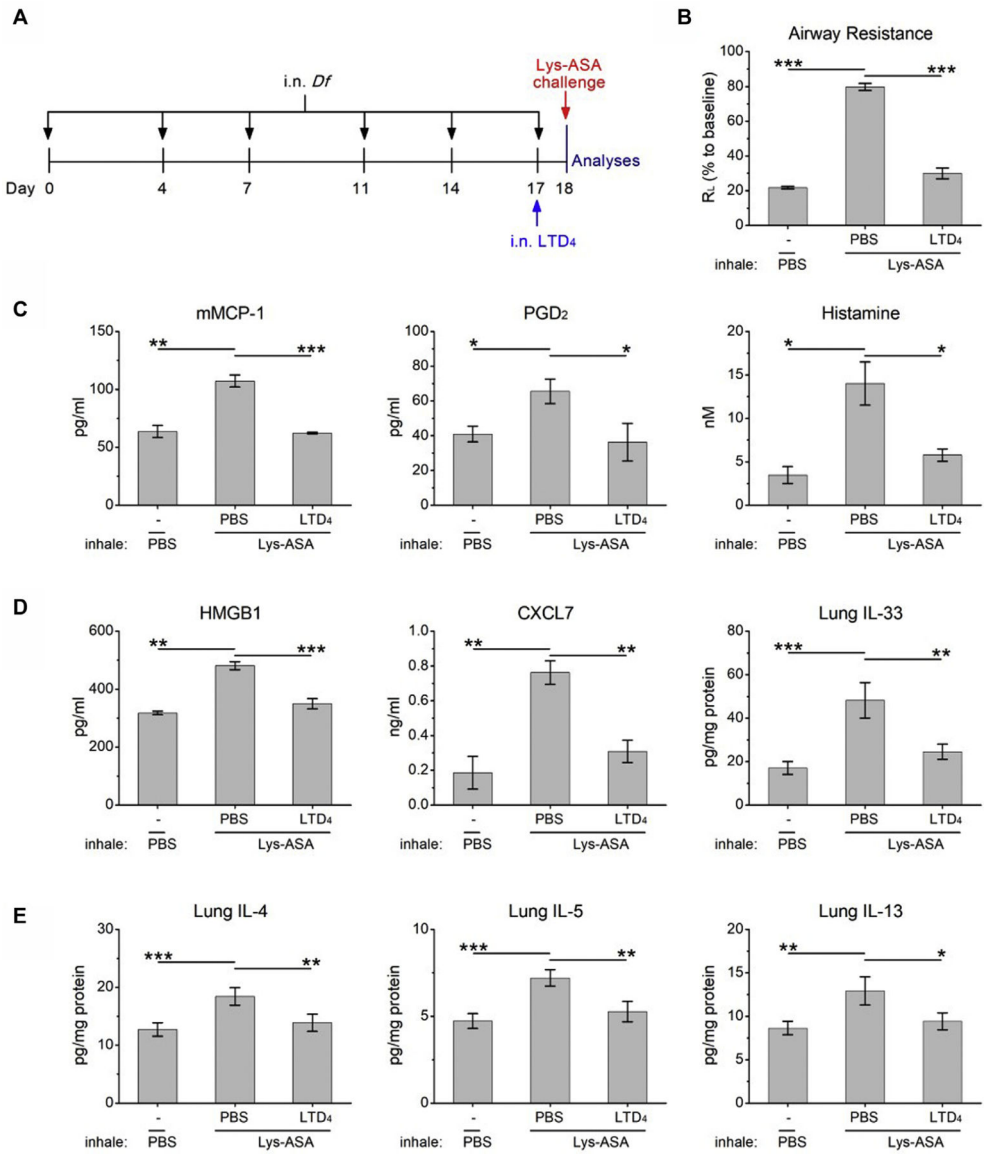

FIG 6.

Inhaled LTD_4 blocks AERD-like reactions to lysine aspirin challenges of *Ptges*$^{-/-}$ mice. **A**, Time line for the experiments. *Df*-primed *Ptges*$^{-/-}$ mice were challenged by inhalation of PBS or Lys-ASA. Some mice received a single inhaled dose of LTD_4 30 minutes before challenge. **B**, Maximum percentage of change in R_L monitored continuously for 45 minutes after the administration of Lys-ASA or PBS. **C**, Levels of MC activation markers (mMCP-1, histamine, and PGD_2). **D**, Platelet activation markers (CXCL7 and HMGB1) in BAL fluids from the indicated groups of mice. Whole lung levels of IL-33 from the same mice are shown. **E**, Whole lung levels of IL-4, IL-5, and IL-13 from the indicated groups. Results are from 10 mice per group. ***$P < .001$; **$P < .01$; *$P < .05$.

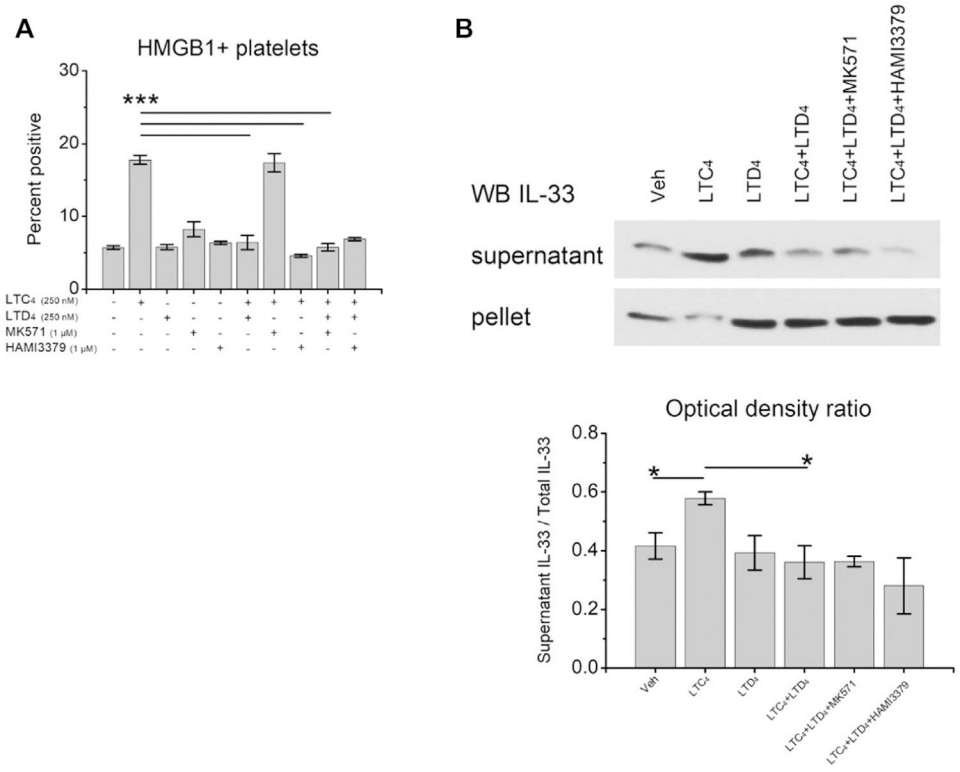

FIG E1.

Effect of LTD_4 on LTC_4-elicited HMGB1 and IL-33 release by platelets. **A**, PRP from WT mice was stimulated with the indicated combinations of agonists and antagonists for 30 minutes. HMGB1 release was detected on the basis of its surface expression. Results are from 3 independent experiments. **B**, Washed platelets were stimulated with the indicated agonists and antagonists for 30 minutes. Supernatants and pellets were collected, and the proteins were resolved by SDS-PAGE. The resultant Western blot (*top*) is representative of 3 separate experiments. Quantitative densitometry showing percentage of release of IL-33 (*bottom*). *Veh*, Vehicle.

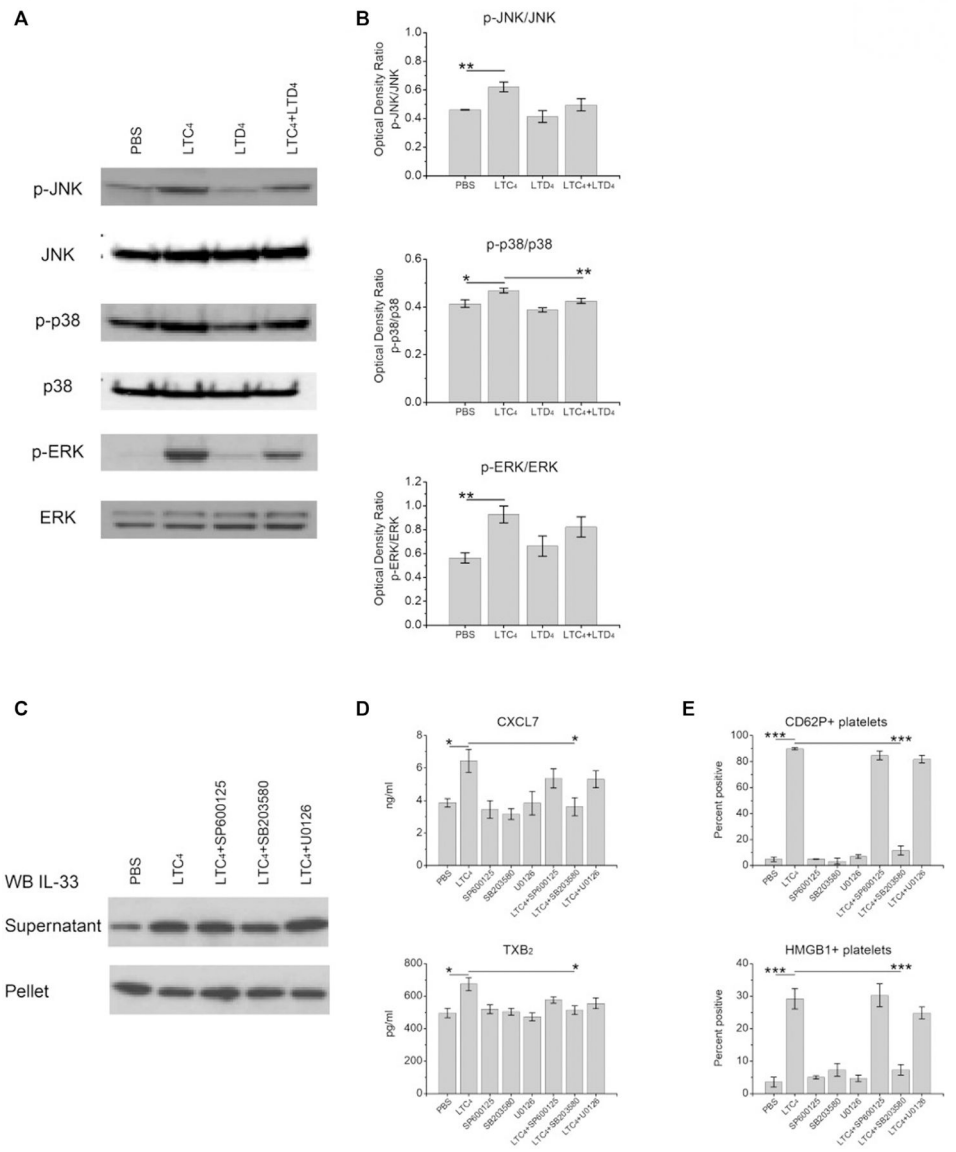

FIG E2.

Differential effects of LTC_4 on and LTD_4 on MAPK activation. **A**, PRP from WT mice was stimulated with the indicated cysLTs for 10 minutes. Western blots were probed for phospho-specific and total JNK, p38, and ERK. **B**, Quantitative densitometry. Effect of specific inhibitors of the indicated MAPK on IL-33 release (**C**), soluble mediator release (**D**), and platelet surface inductions of CD62P and HMGB1 (**E**). Data in (**B**), (**D**), and (**E**) are from 3 experiments. ***$P < .001$; **$P < .01$; *$P < .05$. *p*, Phosphorylated; *WB*, Western blot.

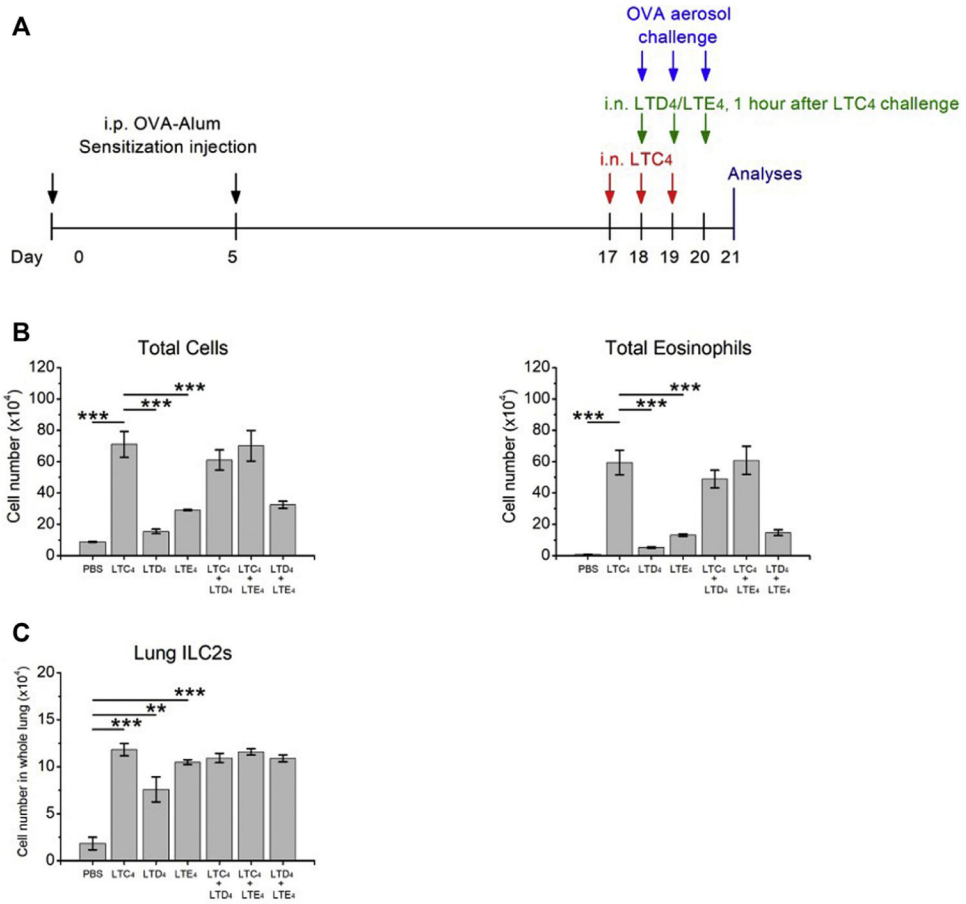

FIG E3.

Delayed administration of LTD_4 does not reverse of the effects of LTC_4. **A**. Time line for the experiments. **B**. Total BAL fluid cell counts (*left*) and eosinophils (*right*). **C**. Lung ILC2s. Results are from 5 mice/group. ***$P < .001$; **$P < .01$. *i.n.*, Intranasal; *i.p.*, intraperitoneal.

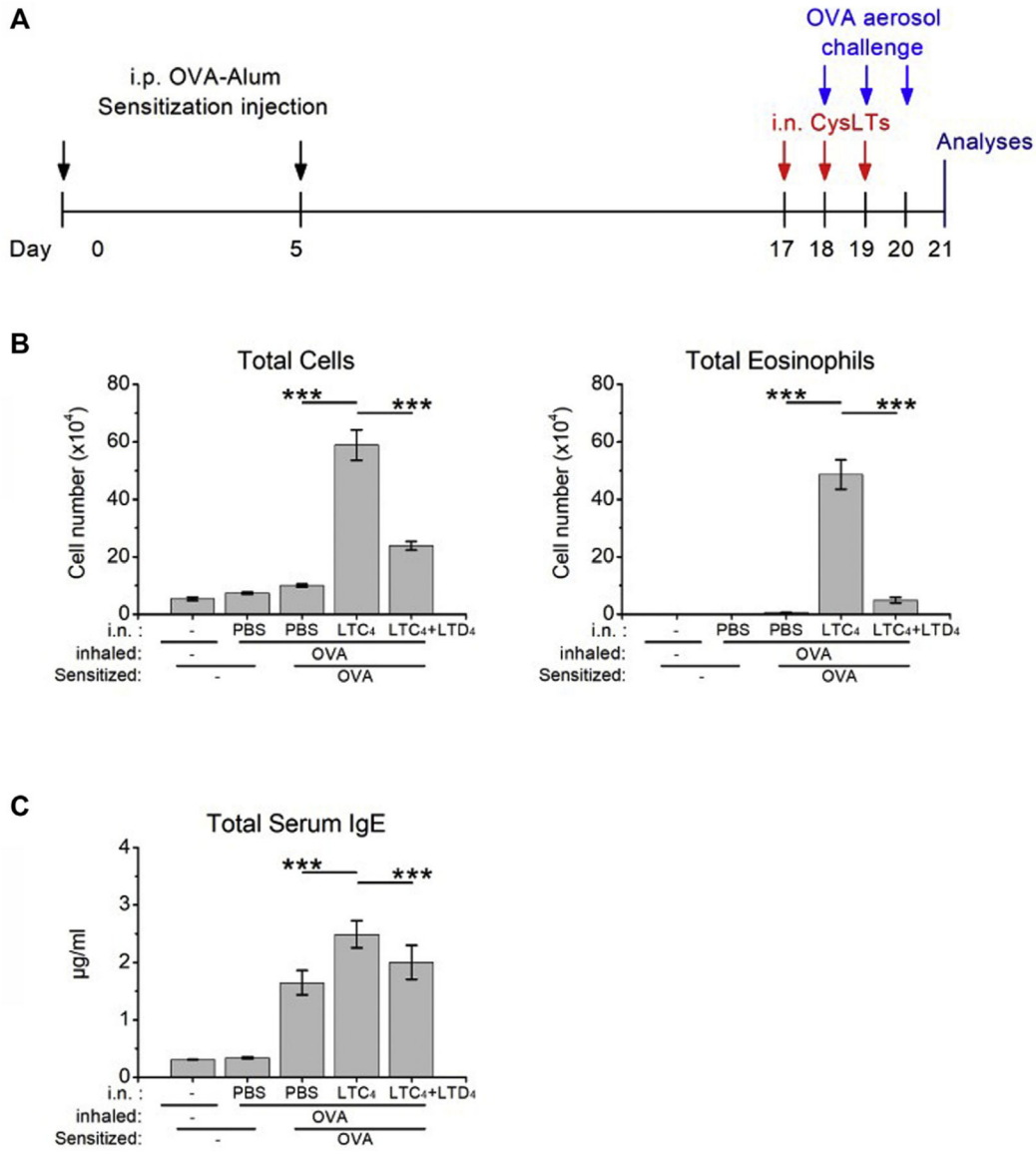

FIG E4.

LTC_4 amplifies lung inflammation without altering IgE sensitization. **A**, Time line for the experiments. **B**, BAL fluid total cells (*left*) and eosinophils (*right*). **C**, Total serum IgE for the indicated groups of mice. Results are from 5 mice/group. ***$P < .001$. *i.n.*, Intranasal; *i.p.*, intraperitoneal.

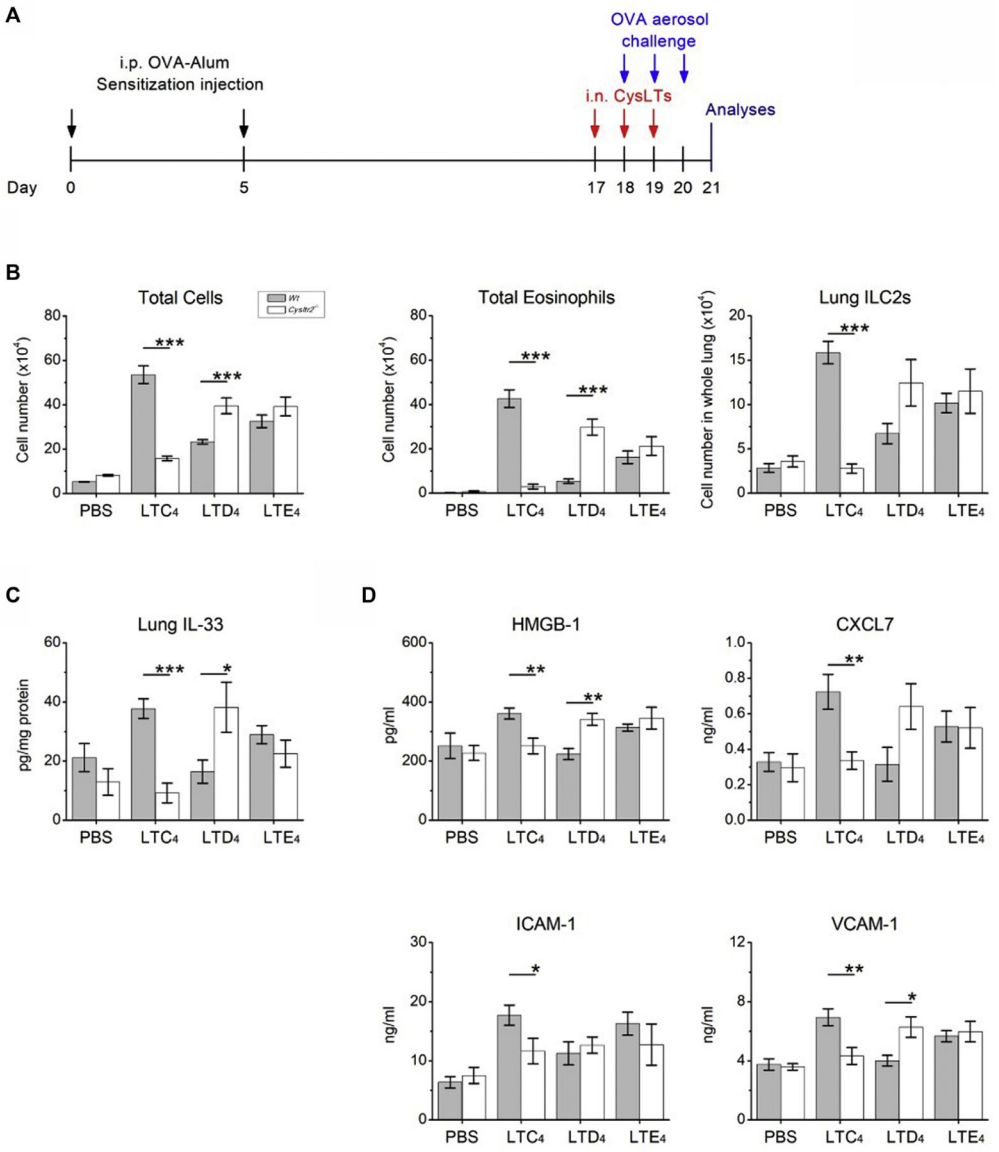

FIG E5.

Deletion of $CysLT_2R$ eliminates potentiation of OVA-induced inflammation by LTC_4 but converts LTD_4 into a potent agonist. WT and *Cysltr2*$^{-/-}$ mice were sensitized with OVA/alum and challenged with the indicated inhaled cysLTs on 3 successive days, with each challenge followed 24 hours later by inhaled OVA. Samples were collected 24 hours after the final OVA challenge. **A**, Time line for the experiments. **B**, BAL fluid total cell and eosinophil counts and lung ILC2s in the indicated groups. **C**, Lung IL-33 levels. **D**, Levels of platelet (CXCL7 and HMGB1) and endothelial (VCAM-1 and ICAM-1) activation markers in BAL fluid from the indicated groups. Results are from 10 mice/group. *i.n.*, Intranasal; *i.p.*, intraperitoneal.