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Leukotriene C_4 Activates Mouse Platelets in Plasma Exclusively Through the Type 2 Cysteinyl Leukotriene Receptor[1]

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Abstract

Leukotriene (LT)C_4 and its extracellular metabolites, LTD_4 and LTE_4, mediate airway inflammation. They signal through three specific receptors ($CysLT_1R$, $CysLT_2R$, and GPR99) with overlapping ligand preferences. Here we demonstrate that LTC_4, but not LTD_4 or LTE_4, activates mouse platelets exclusively through $CysLT_2R$. Platelets expressed $CysLT_1R$ and $CysLT_2R$ proteins. LTC_4 induced surface expression of CD62P by WT mouse platelets in platelet-rich plasma (PRP) and caused their secretion of thromboxane A_2 and CXCL4. LTC_4 was fully active on PRP from mice lacking either $CysLT_1R$ or GPR99, but completely inactive on PRP from $CysLT_2R$-null (*Cysltr2*$^{-/-}$) mice. LTC_4/$CysLT_2R$ signaling required an autocrine ADP-mediated response through $P2Y_{12}$ receptors. LTC_4 potentiated airway inflammation in a platelet- and $CysLT_2R$-dependent manner. Thus, $CysLT_2R$ on platelets recognizes LTC_4 with unexpected selectivity. Nascent LTC_4 may activate platelets at a synapse with granulocytes before it is converted to LTD_4, promoting mediator generation and the formation of leukocyte/platelet complexes that facilitate inflammation.

Introduction

Cysteinyl leukotrienes (cys-LTs) play a validated role in asthma (1). After 5-lipoxygenase (5-LO) oxidizes arachidonic acid to LTA_4 (2), eosinophils, basophils, mast cells and monocytes conjugate LTA_4 to reduced glutathione via leukotriene C_4 synthase (LTC_4S)(3), forming LTC_4. After export, LTC_4 is converted to LTD_4 (4), a smooth muscle spasmogen, and then to LTE_4 (5), a stable metabolite. Three G protein coupled receptors, termed the type 1 cys-LT receptor ($CysLT_1R$) (6,7), type 2 cys-LT receptor ($CysLT_2R$) (8,9), and GPR99 (10), mediate the effects of cys-LTs. $CysLT_1R$ is a high affinity LTD_4 receptor with lower affinity for LTC_4 (6,7). $CysLT_2R$ binds LTC_4 and LTD_4 with equal affinity (8,9), and GPR99 exhibits a preference for LTE_4 (10). $CysLT_1R$-selective antagonists are widely

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prescribed for asthma (11). Although $CysLT_2R$ inhibits dendritic cell priming for T helper type 2 immune responses (12) and GPR99 mediates LTE_4-induced skin edema (10), our understanding of the therapeutic applicability of these receptors is limited. Moreover, since many cell types express more than one cys-LT receptor, assignment of receptor-specific functions through in vitro approaches is challenging.

Platelets play an important role in asthma (13) and vascular inflammation (14). Platelets adhere to granulocytes by a CD62P (P-selectin)-P-selectin glycoprotein-1 (PSGL-1)-dependent mechanism. Adherent platelets upregulate leukocyte integrin avidity (15), and permit transcellular metabolism of arachidonic acid (16). Platelets contain LTC_4S and convert granulocyte-derived LTA_4 to LTC_4 through a transcellular pathway, amplifying the production of cys-LTs (13). Human platelets express both $CysLT_1R$ and $CysLT_2R$ (17). To date, however, no study has definitively addressed whether cys-LTs influence platelet functions, or determined which receptors are most essential.

We now report that LTC_4, but not LTD_4 or LTE_4, activates mouse platelets entirely through $CysLT_2R$. LTC_4 induces expression of platelet CD62P. This response requires $CysLT_2R$, but not $CysLT_1R$ or GPR99. LTC_4 induces platelets to release inflammatory mediators, and to augment allergen-induced airway inflammation. $CysLT_2R$-dependent platelet activation requires amplification from $P2Y_{12}$ receptors and ADP. LTC_4 may facilitate local activation of platelets in a synapse with leukocytes, in turn amplifying inflammatory responses. This function is distinct from those of its extracellular metabolites. Moreover, $CysLT_2R$ can function as an LTC_4 receptor with high specificity despite its ability to bind LTD_4 in transfected cells (8).

Methods

Animals

Tbxa2r–/– mice were obtained from Dr. Thomas Coffman (Duke University, Durham, NC) (18). *P2ry12*–/– mice were from Portola Pharmaceuticals (San Francisco, CA) (19). *Cysltr1*–/–, *Cysltr2*–/–, and *Gpr99*–/– mice were generated in our institution (10,20,21). Mice were sensitized I.P. on days 0 and 7 with Alum-precipitated chicken egg ovalbumin (OVA, Sigma, 10 μg) and challenged by inhalation of 0.1% OVA with or without intranasal cys-LTs as described (22). Platelets were depleted by an I.P. injection of an anti-CD41 antibody (clone MWReg30, Biolegend) or an isotype control (23).

Platelet isolation

Blood was obtained by cardiac puncture using a 21G needle into 4% sodium citrate (Sodium Citrate Enzyme Grade, Fisher Scientific, Pittsburgh, PA). Platelet Rich Plasma (PRP) was obtained by slow spin centrifugation of whole blood at 1000 rpm/900xg for 15 minutes. PRP was incubated with $CaCl_2$ (Fischer) ([final]= 5mM) at 37°C for 10 minutes.

Platelet activation

Aliquots of PRP (50 μl) were stimulated with thrombin (50U/mL, Sigma Aldrich, Saint Louis, MO), LTC_4, LTD_4, or LTE_4 (25–250nM, Cayman Chemical, Ann Arbor, MI) at 37°C for 30 minutes. Samples were stained with PE anti-mouse CD41 (Clone: MWReg30, Biolegend, San Diego, CA) and FITC rat Anti- mouse CD62P (Clone: RB40.34, BD Pharmingen, San Diego, CA) for analysis of CD62P expression on $CD41^+$ mouse platelets. PE Rat IgG1κ and FITC Rat IgG1λ were used for isotype controls (BD Pharmingen). Cells were fixed overnight in 1% paraformaldehyde in PBS (Affymetrix®, Cleveland, OH). Some aliquots of PRP were stimulated with at 37°C for 30 minutes for analysis of released thromboxane (Thromboxane B_2 EIA Kit, Cayman), Regulated on Activation, Normal T cell

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Expressed and Secreted (RANTES) (eBiosciences, San Diego, CA) and CXCL4 (Sigma) by ELISA, or for ADP (Abcam). Some samples were treated with the $CysLT_2R$ antagonists $BayCysLT_2$ and HAMI3379 (Cayman Chemical, 300 nM each). In some experiments, supernatants were analyzed for conversion of LTC_4 to LTD_4 and LTE_4 by high performance liquid chromatography (24).

RESULTS AND DISCUSSION

LTC_4 is synthesized by cells that express both 5-LO and LTC_4S (25), or generated through from granulocyte-derived LTA_4 by adherent LTC_4S-expressing platelets (26). Since extracellular enzymes efficiently convert LTC_4 to LTD_4 and LTE_4, LTC_4 most likely functions in a synapse between the cells of origin and adjacent endothelium or platelets. However, apart from its role as a precursor, no unique functions have been attributed to LTC_4. Human platelets express both $CysLT_1R$ and $CysLT_2R$ (17), as is the case for many hematopoietic cells (25). Given that cell recruitment (27), bronchoconstriction (28), airway inflammation (22), and fibrosis (20) all involve both cys-LTs and platelet activation (13,22,29,30), we sought to determine whether platelets might respond directly to cys-LTs.

We first stimulated platelets from WT mice with various concentrations of LTC_4, LTD_4, and LTE_4. Only LTC_4 elicited an increase in surface CD62P expression (Fig. 1), and was active at the lowest dose tested (25 nM). The response to LTC_4 at 250 nM was ~60% of that elicited by thrombin (Fig. 1). PRP did not convert LTC_4 to LTD_4 or LTE_4 (not shown). The induction of CD62P by LTC_4, and the lack of any response to LTD_4 and LTE_4 at physiologic ranges, suggests that LTC_4 has specific functions in the formation of platelet-leukocyte complexes, which depend on induction of CD62P and its interaction with PSGL-1 on the leukocyte surface (26).

Given that $CysLT_1R$ and $CysLT_2R$ each bind LTC_4 and LTD_4 at low nM ranges (6,9), the response limited to LTC_4 was unexpected. To identify the responsible receptors, we stimulated PRP obtained from mice lacking $CysLT_1R$ (*Cysltr1*–/– mice), $CysLT_2R$ (*Cysltr2*–/– mice), and GPR99 (*Gpr99*–/– mice). Platelets from *Cysltr2*–/– mice were unresponsive to LTC_4 (Fig. 2A), whereas platelets from the *Cysltr1*–/– (Fig. 2B) and *Gpr99*–/– strains (Fig. 2C) were fully responsive. Platelets from all three strains responded to thrombin, and none reacted to LTD_4 or LTE_4 (Fig. 2A–C). Platelets from WT mice expressed both $CysLT_1R$ and $CysLT_2R$ proteins, as did human platelets (Fig. 2D). Thus, while recombinant $CysLT_2R$ has equal binding affinities for LTC_4 and LTD_4 (8,9), natively expressed $CysLT_2R$ on mouse platelets exhibits a preference for activation by LTC_4. Moreover, despite the presence of $CysLT_1R$ on platelets, $CysLT_2R$ is the dominant effector of responses to LTC_4 in this cell type. In mast cells (31) and dendritic cells (12), $CysLT_1R$ signaling dominates and $CysLT_2R$ serves an inhibitory function. Cell-specific variations in receptor stoichiometry, relative abundances, localization, or G protein-coupling may account for these functional differences.

Endogenous ADP can amplify platelet activation through $P2Y_1$ and $P2Y_{12}$ receptors (32). $P2Y_{12}$ receptors are implicated in cellular responses to cys-LTs (particularly LTE_4) (22,33), but do not bind cys-LTs (22), suggesting an indirect functional relationship to cys-LT receptors. LTC_4-mediated induction of CD62P was markedly impaired in *P2ry12*–/– platelets (Fig. 3A). Treatment of WT platelets with apyrase attenuated their responses to LTC_4 (Fig. 3B) while depleting extracellular ADP (Fig. 3C). While the doses of LTE_4 used in this study may exceed those required to demonstrate activity at $P2Y_{12}$, only LTC_4 caused platelets to release ADP; this response required $CysLT_2R$ (Fig. 3C). $P2Y_{12}$-targeted thienopyridine drugs, which prevent cardiovascular events (34), may interfere with the LTC_4/$CysLT_2R$-dependent pathway of platelet activation in vivo.

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Activated platelets generate TXA_2, a potent inflammatory mediator, and secrete chemokines (35). Human platelets released RANTES when stimulated with cys-LTs in a prior study (17). In our study, LTC_4 induced mouse platelets to release large quantities of TXA_2, as well as CXCL4 and, to a lesser extent, RANTES (Supplemental Fig. 1A–C) in a $CysLT_2R$- and $P2Y_{12}$ receptor-dependent manner. Two $CysLT_2R$ antagonists, $BayCysLT_2$ and HAMI3379 (300 nM each) suppressed TXA_2 release by WT platelets (Supplemental Fig. 1D). Studies using platelets from *Tbxa2r*–/– mice revealed that TXA_2 was not necessary for LTC_4-induced activation, although there was a trend toward less activation at the lowest LTC_4 doses (Supplemental Fig. 2).

Intrapulmonary administration of LTE_4 to sensitized mice challenged with low-dose OVA potentiates eosinophil recruitment in a platelet- and $P2Y_{12}$-dependent manner (36). We treated sensitized mice intranasally with LTC_4 (2 nmol) on three consecutive days before low-dose (0.1%) OVA challenges. LTC_4 markedly potentiated the recruitment of eosinophils to the BAL fluid. This response depended on $CysLT_2R$, $P2Y_{12}$ (Fig. 4A), and platelets (Fig. 4B). LTC_4 may therefore contribute to platelet activation in asthma, aspirin exacerbated respiratory disease (13), myocardial infarction (37), and stroke (38). Moreover, this pathway likely resists blockade by the available antagonists, which do not target $CysLT_2R$, but may be sensitive to $P2Y_{12}$ receptor-active drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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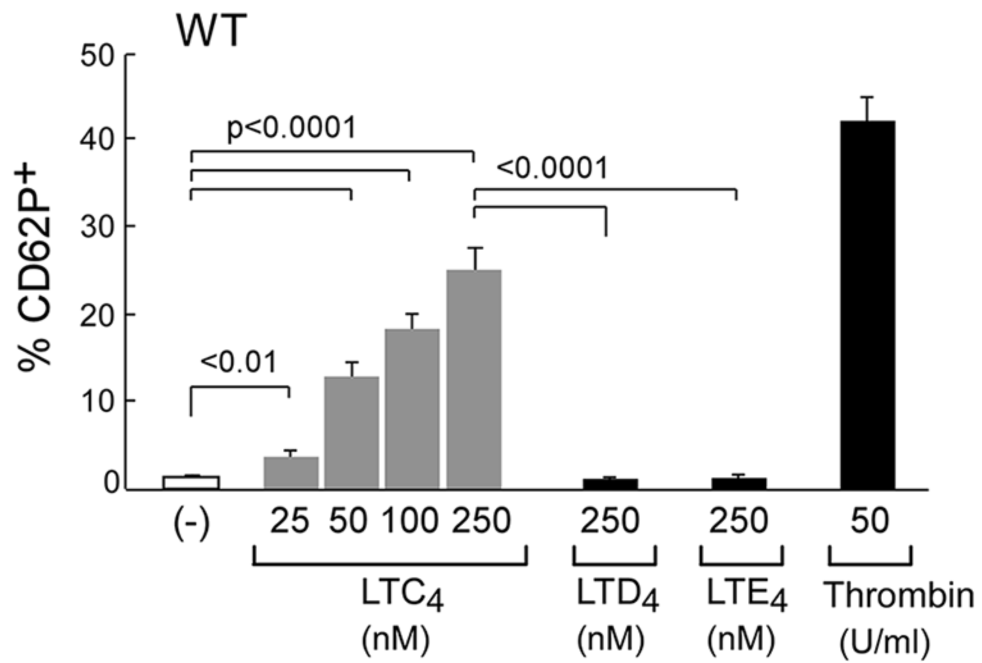

Figure 1.

Platelet activation by cys-LTs. PRP from WT mice was stimulated with the indicated agonists. CD62P was assessed by flow cytometry. Results are mean ± SD from 5–10 separate experiments using platelets from one mouse/strain.

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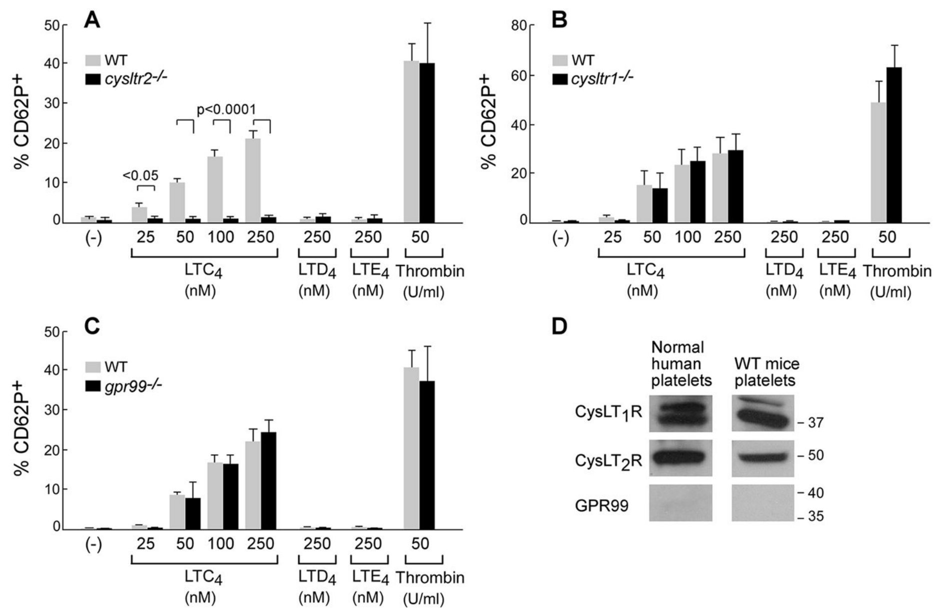

Figure 2.

Cys-LT receptors involved in LTC_4-induced platelet activation. PRP from mice of the indicated genotypes was stimulated with various concentrations of cys-LTs, or with thrombin as a positive control. **A.** Effect of $CysLT_2R$ deletion. **B.** Effect of $CysLT_1R$ deletion. **C.** Effect of GPR99 deletion. **D.** Western blot of proteins from human and WT mouse platelets showing bands corresponding to the anticipated molecular sizes of $CysLT_1R$ and $CysLT_2R$. Results in A-C are mean ± SD from 3–5 separate experiments.

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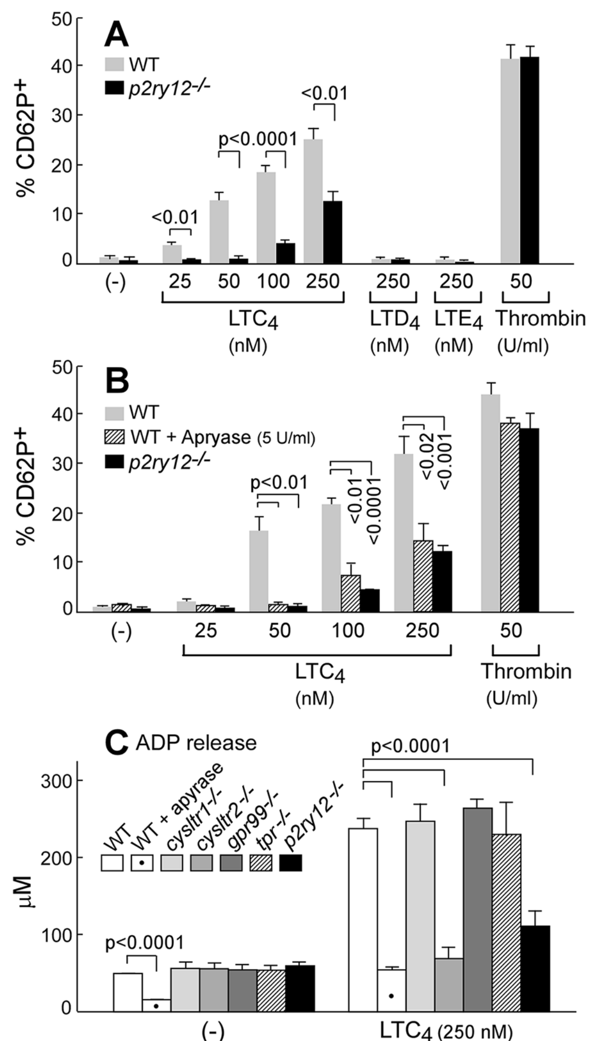

Figure 3.

Involvement of $P2Y_{12}$ receptors and extracellular nucleotides in $CysLT_2R$-mediated platelet activation. **A.** Platelets from WT or *P2ry12*−/− micewere stimulated with the indicated concentrations of cys-LTs or thrombin. CD62P induction was assessed by flow cytometry. **B**. WT platelets were stimulated with cys-LTs or thrombin in the absence or presence of apyrase. PRP from *P2ry12*−/− mice was included as a control. **C.** Release of ADP by stimulated platelets and effects of apyrase and genotypes. Results mean ± SD from 3 separate experiments.

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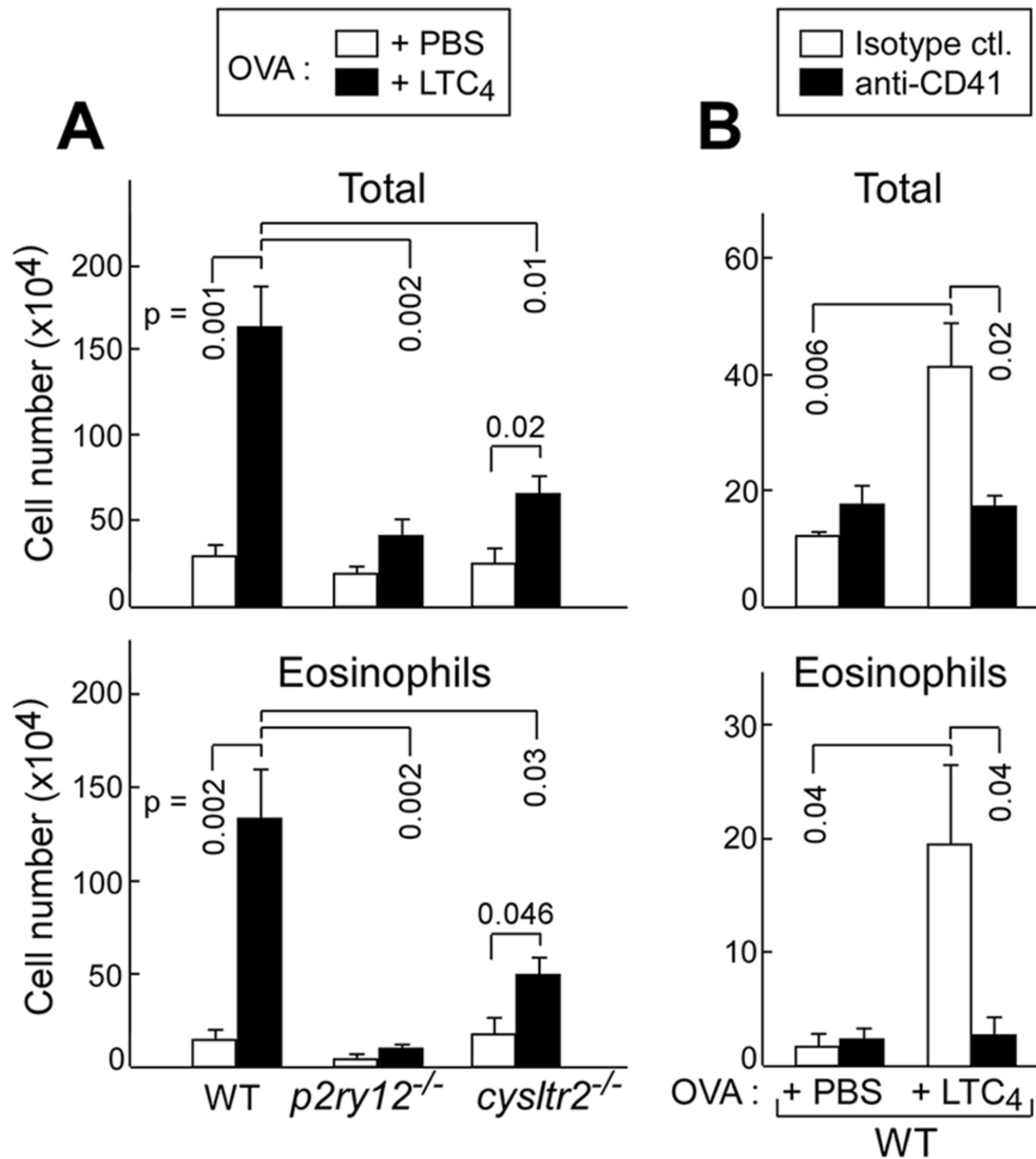

Figure 4.

LTC_4 amplifies allergen-induced pulmonary inflammation in a platelet, $CysLT_2R$ and $P2Y_{12}$-dependent manner. Mice were sensitized intraperitoneally with OVA/Alum and challenged 3x with 0.1% OVA with or without intranasal LTC_4 (2 nmol). **A.** BAL fluid total cell counts (top) and eosinophil counts (bottom) from mice of the indicated genotypes. **B.** Effect of platelet depletion (using anti-CD41 vs. an isotype control) of WT mice challenged with OVA ± LTC_4 on BAL fluid cell counts and eosinophil counts. Results are mean ± SEM from a single experiment using 5–10 mice/group. Results from a second experiment were similar.