Cutting Edge: Leukotriene C₄ Activates Mouse Platelets in Plasma Exclusively through the Type 2 Cysteinyl Leukotriene Receptor

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Cutting Edge: Leukotriene C4 Activates Mouse Platelets in Plasma Exclusively through the Type 2 Cysteinyl Leukotriene Receptor

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Leukotriene C4 (LTC4) and its extracellular metabolites, LTD4 and LTE4, mediate airway inflammation. They signal through three specific receptors (type 1 cyst-LT receptor [CysLT1R], CysLT2R, and GPR99) with overlapping ligand preferences. In this article, we demonstrate that LTC4, but not LTD4 or LTE4, activates mouse platelets exclusively through CysLT2R. Platelets expressed CysLT1R and CysLT2R proteins. LTC4 induced surface expression of CD62P by wild-type mouse platelets in platelet-rich plasma (PRP) and caused their secretion of thromboxane A2 and CXCL4. LTC4 was fully active on PRP from mice lacking either CysLT1R or GPR99, but completely inactive on PRP from CysLT2R-null (Cysltr2−/−) mice. LTC4/CysLT2R signaling required an autocrine ADP-mediated response through P2Y12 receptors. LTC4 potentiated airway inflammation in a platelet- and CysLT2R-dependent manner. Thus, CysLT2R on platelets recognizes LTC4 with unexpected selectivity. Nascent LTC4 may activate platelets at a synapse with granulocytes before it is converted to LTD4, promoting mediator generation and the formation of leukocyte–platelet complexes that facilitate inflammation. *The Journal of Immunology, 2013, 191: 5807–5810.

Cysteinyl leukotrienes (cys-LTs) play a validated role in asthma (1). After 5-lipoxygenase oxidizes arachidonic acid to LTA4 (2), eosinophils, basophils, mast cells, and monocytes conjugate LTA4 to reduced glutathione via leukotriene (LT) C4 synthase (LTC4S) (3), forming LTC4. After export, LTC4 is converted to LTD4 (4), a smooth muscle spasmogen, and then to LTE4 (5), a stable metabolite. Three G protein–coupled receptors, termed the type 1 cys-LT receptor (CysLT1R) (6, 7), type 2 cys-LT receptor (CysLT2R) (8, 9), and GPR99 (10), mediate the effects of cys-LTs. CysLT1R is a high-affinity LTD4 receptor with lower affinity for LTC4 (6, 7). CysLT2R binds LTC4 and LTD4 with equal affinity (8, 9), and GPR99 exhibits a preference for LTE4 (10). CysLT1R-selective antagonists are widely prescribed for asthma (11). Although CysLT2R inhibits dendritic cell priming for Th2 immune responses (12) and GPR99 mediates LTE4-induced skin edema (10), our understanding of the therapeutic applicability of these receptors is limited. Moreover, because 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–dependent mechanism. Adherent platelets upregulate leukocyte integrin avidity (15) and permit transcellular metabolism of arachidonic acid (16). Platelets contain LTC4S and convert granulocyte-derived LTA4 to LTC4 through a transcellular pathway, amplifying the production of cys-LTs (13). Human platelets express both CysLT1R and CysLT2R (17). To date, however, no study has definitively addressed whether cys-LTs influence platelet functions or determined which receptors are most essential.

We report that LTC4, but not LTD4 or LTE4, activates mouse platelets entirely through CysLT2R. LTC4 induces expression of platelet CD62P. This response requires CysLT1R, but not CysLT2R or GPR99. LTC4 induces platelets to release inflammatory mediators, and to augment allergen-induced airway inflammation. CysLT2R-dependent platelet activation requires amplification from P2Y12 receptors and ADP. LTC4 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, CysLT2R can function as an LTC4 receptor with high specificity despite its ability to bind LTD4 in transfected cells (8).

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Materials and 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 OVA (10 μg; Sigma) and challenged by inhalation of 0.1% OVA with or without intranasal cys-LTs as described previously (22). Platelets were depleted by an i.p. injection of an anti-CD41 Ab (clone MWReg30; Biolegend, San Diego, CA) 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/900 × g for 15 min. PRP was incubated with CaCl₂ (Fisher) ([final]= 5 mM) at 37°C for 10 min.

Platelet activation

Aliquots of PRP (50 μl) were stimulated with thrombin (50 U/ml; Sigma Aldrich, St. Louis, MO), LTC₄, LTD₄, or LTE₄ (25–250 nM; Cayman Chemical, Ann Arbor, MI) at 37°C for 30 min. Samples were stained with PE anti-mouse CD41 (clone MWReg30; Biolegend) 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 IgG1 and FITC rat IgG1 were used for isotype controls (BD Pharmingen). Cells were fixed overnight in 1% paraformaldehyde in PBS (Affymetrix, Cleveland, OH).

Results and Discussion

LTC₄ is synthesized by cells that express both 5-lipoxygenase and LTC₄S (24), or generated through granulocyte-derived LTA₄ by adherent LTC₄S-expressing platelets (25). Because extracellular enzymes efficiently convert LTC₄ to LTD₄ and LTE₄, LTC₄ 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 LTC₄. Human platelets express both CysLT₁R and CysLT₂R (17), as is the case for many hematopoietic cells (24). Given that cell recruitment (26), bronchoconstriction (27), airway inflammation (22), and fibrosis (20) all involve both cys-LTs and platelet activation (13, 22, 28, 29), we sought to determine whether platelets might respond directly to cys-LTs.

We first stimulated platelets from wild-type (WT) mice with various concentrations of LTC₄, LTD₄, and LTE₄. Only LTC₄ elicited an increase in surface CD62P expression (Fig. 1) and was active at the lowest dose tested (25 nM). The response to LTC₄ at 250 nM was ~60% of that elicited by}

![FIGURE 1](http://www.jimmunol.org/)

**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 1 mouse/strain.

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** Cys-LT receptors involved in LTC₄-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 CysLT₁R deletion. (B) Effect of CysLT₁R 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 CysLT₁R and CysLT₂R. Results in (A)–(C) are mean ± SD from three to five separate experiments.
thrombin (Fig. 1). PRP did not convert LTC₄ to LTD₄ or LTE₄ (not shown). The induction of CD62P by LTC₄, and the lack of any response to LTD₄ and LTE₄ at physiologic ranges, suggests that LTC₄ has specific functions in the formation of platelet–leukocyte complexes, which depend on induction of CD62P and its interaction with (P-selectin)–P-selectin glycoprotein-1 on the leukocyte surface (25).

Given that CysLT₁R and CysLT₂R each bind LTC₄ and LTD₄ at low nanomolar ranges (6, 9), the response limited to LTC₄ was unexpected. To identify the responsible receptors, we stimulated PRP obtained from mice lacking CysLT₁R (Cysltr₁²⁻/² mice), CysLT₂R (Cysltr₂²⁻/² mice), and GPR99 (Gpr99²⁻/² mice). Platelets from Cysltr₂²⁻/² mice were unresponsive to LTC₄ (Fig. 2A), whereas platelets from the Cysltr₁²⁻/² (Fig. 2B) and Gpr99²⁻/² strains (Fig. 2C) were fully responsive. Platelets from all three strains responded to thrombin, and none reacted to LTD₄ or LTE₄ (Fig. 2A–C). Platelets from WT mice expressed both CysLT₁R and CysLT₂R proteins, as did human platelets (Fig. 2D). Thus, whereas recombinant CysLT₂R has equal binding affinities for LTC₄ and LTD₄ (8, 9), natively expressed CysLT₂R on mouse platelets exhibits a preference for activation by LTC₄. Moreover, despite the presence of CysLT₁R on platelets, CysLT₂R is the dominant effector of responses to LTC₄ in this cell type. In mast cells (30) and dendritic cells (12), CysLT₁R signaling dominates and CysLT₂R 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 P2Y₁ and P2Y₁₂ receptors (31). P2Y₁₂ receptors are implicated in cellular responses to cysteiny1 leukotrienes (particularly LTE₄) (22, 32), but do not bind cysteiny1 leukotrienes (22), suggesting an indirect functional relationship to cysteiny1 leukotriene receptors. LTC₄-mediated induction of CD62P was markedly impaired in P2ry₁₂⁻/⁻ platelets (Fig. 3A). Treatment of WT platelets with apyrase attenuated their responses to LTC₄ (Fig. 3B) while depleting extracellular ADP (Fig. 3C). Although the doses of LTE₄ used in this study may exceed those required to demonstrate activity at P2Y₁₂, only LTC₄ caused platelets to release ADP; this response required CysLT₁R (Fig. 3C). P2Y₁₂-targeted thienopyridine drugs, which prevent cardiovascular events (33), may interfere with the LTC₄/CysLT₂R-dependent pathway of platelet activation in vivo.

Activated platelets generate thromboxane A₂ (TXA₂), a potent inflammatory mediator, and secrete chemokines (34). Human platelets released RANTES when stimulated with cysteiny1 leukotrienes in a prior study (17). In our study, LTC₄ induced mouse platelets to release large quantities of TXA₂, as well as CXCL4 and, to a lesser extent, RANTES (Supplemental Fig. 1A–C), in a CysLT₂R- and P2Y₁₂ receptor–dependent manner. Two
CysLT2R antagonists, BayCysLT2 and HAM13379 (300 nM each), suppressed TXA2 release by WT platelets (Supplemental Fig. 1D). Studies using platelets from Tbias2−/− mice revealed that TXA2 was not necessary for LTC4-induced activation, although there was a trend toward less activation at the lowest LTC4 doses (Supplemental Fig. 2).

Intrapulmonary administration of LTE4 to sensitized mice challenged with low-dose OVA potentiates eosinophil recruitment in a platelet- and P2Y12-dependent manner (35). We treated sensitized mice intranasally with LTC4 (2 nmol) on 3 consecutive days before low-dose (0.1%) OVA challenges. LTC4 markedly potentiated the recruitment of eosinophils to the bronchoalveolar lavage (BAL) fluid. This response depended on CysLT2R, P2Y12 (Fig. 4A), and platelets (Fig. 4B). LTC4 may therefore contribute to platelet activation in asthma, aspirin-exacerbated respiratory disease (13), myocardial infarction (36), and stroke (37). Moreover, this pathway likely resists neutralization of the human cysteinyl leukotriene 1 receptor.

Disclosures
The authors have no financial conflicts of interest.

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References
Supplemental Figure 1. Receptor requirements for cys-LT-induced mediator generation by platelets. PRP From mice of the indicated genotypes was stimulated for 30 min with the indicated cys-LTs (100 nM each). A. TXB₂ concentrations measured in the supernatants. B. Levels of CXCL4 and C. RANTES from the same samples as in A. Result are from three separate experiments. D. Effects of CysLT₂R antagonists BayCysLT2 and HAMI3379 (300 nM each) on TXA₂ release. Results are mean ± SD from five mice/group.
**Supplemental Figure 2.** Effect of TP receptor deletion on cys-LT-mediated induction of CD62P. Results are mean ± SD from 5 mice/group.