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*J Immunol* published online 15 November 2013
http://www.jimmunol.org/content/early/2013/11/15/jimmunol.1302187

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/11/18/jimmunol.1302187.DC1

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

Hannah E. Cummings,*†‡§,1 Tao Liu,*†‡§,1 Pamela B. Conley,*§, Y Yoshihide Kanaoka,*†‡, Chunli Feng,*†, Tanya M. Laidlaw,*†‡,§, and Joshua A. Boyce*†‡,§, Address correspondence and reprint requests to Dr. Joshua A. Boyce, Brigham and Women’s Hospital, 1 Jimmy Fund Way, Smith Building, Boston, MA 02115. E-mail address: boyce@rics.bwh.harvard.edu

Leukotriene C₄ (LTC₄) and its extracellular metabolites, LTD₄ and LTE₄, mediate airway inflammation. They signal through three specific receptors (type 1 cyste-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: 000–000.

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 cyst-LT receptor (CysLT₁R) (6, 7), type 2 cyst-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 cyst-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, CysLT₁R can function as an LTC₄ receptor with high specificity despite its ability to bind LTD₄ in transfected cells (8).
Contents:

Materials and Methods

Animals

Tbx2a<sup>-/-</sup> mice were obtained from Dr. Thomas Coffman (Duke University, Durham, NC) (18). P2ry12<sup>-/-</sup> mice were from Portola Pharmaceuticals (San Francisco, CA) (19). Cysltr1<sup>-/-</sup>, Cysltr2<sup>-/-</sup>, and Gpr99<sup>-/-</sup> 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<sup>+</sup> 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). Some aliquots of PRP were stimulated with at 37°C for 30 min for analysis of released thromboxane (Thromboxane B₂ EIA Kit; Cayman), RANTES (eBioscience, San Diego, CA) and CXCL4 (Sigma) by ELISA, or for ADP (Abcam). Some samples were treated with the CysLT₂R antagonists Bay-CysLT₂ and HAMI3379 (300 nM each; Cayman Chemical). In some experiments, supernatants were analyzed for conversion of LTC₄ to LTD₄ and LTE₄ by high-performance liquid chromatography (3).

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 LTD₄ and LTE₄ 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.

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![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 CysLT₂R 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 (Cysltr1−/− mice), CysLT2R (Cysltr2−/− mice), and GPR99 (Gpr99−/− mice). Platelets from Cysltr2−/− mice were unresponsive to LTC4 (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 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 cys-LTs (particularly LTE4) (22, 32), but do not bind cys-LTs (22), suggesting an indirect functional relationship to cys-LT receptors. LTC4-mediated induction of CD62P was markedly impaired in P2y12−/− 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 cys-LTs 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.
CysLT$_2$R antagonists, BayCysLT$_2$ and HAMI3379 (300 nM each), suppressed TXA$_2$ release by WT platelets (Supplementary Fig. 1D). Studies using platelets from Tbxax2r$^{-/-}$ 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 (35). We treated sensitized mice intranasally with LTC$_4$ (2 nmol) on 3 consecutive days before low-dose (0.1%) OVA challenges. LTC$_4$ markedly potentiated the recruitment of eosinophils to the bronchoalveolar lavage (BAL) fluid. This response depended on CysLT$_2$R, 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 (36), and stroke (37). Moreover, this pathway likely resists blockade by the available antagonists, which do not target CysLT$_2$R, but may be sensitive to P2Y$_{12}$-receptor–active drugs.

Disclosures

The authors have no financial conflicts of interest.

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.** TXB$_2$ 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 CysLT$_2$R antagonists BayCysLT2 and HAMI3379 (300 nM each) on TXA$_2$ 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.