Leukotriene D_4 and prostaglandin E_2 signals synergize and potentiate vascular inflammation in a mast cell–dependent manner through cysteinyl leukotriene receptor 1 and E-prostanoid receptor 3

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Background: Although arachidonic acid metabolites, cysteinyl leukotrienes (cys-LTs; leukotriene [LT] C_4, LTD_4, and LTE_4), and prostaglandin (PG) E_2 are generated at the site of inflammation, it is not known whether crosstalk exists between these 2 classes of inflammatory mediators.

Objective: We sought to determine the role of LTD_4-PGE_2 crosstalk in inducing vascular inflammation *in vivo*, identify effector cells, and ascertain specific receptors and pathways involved *in vitro*.

Methods: Vascular (ear) inflammation was assessed by injecting agonists into mouse ears, followed by measuring ear thickness and histology, calcium influx with Fura-2, phosphorylation and expression of signaling molecules by means of immunoblotting, PGD_2 and macrophage inflammatory protein 1β generation by using ELISA, and expression of transcripts by using RT-PCR. Candidate receptors and signaling molecules were identified by using antagonists and inhibitors and confirmed by using small interfering RNA.

Results: LTD_4 plus PGE_2 potentiated vascular permeability and edema, gearing the system toward proinflammation in wild-type mice but not in $Kit^{W\text{-}sh}$ mice. Furthermore, LTD_4 plus PGE_2, through cysteinyl leukotriene receptor 1 ($CysLT_1R$) and E-prostanoid receptor (EP) 3, enhanced extracellular signal-regulated kinase (Erk) and c-fos phosphorylation, inflammatory gene expression, macrophage inflammatory protein 1β secretion, COX-2 upregulation, and PGD_2 generation in mast cells. Additionally, we uncovered that this synergism is mediated through Gi, protein kinase G, and Erk signaling. LTD_4 plus PGE_2–potentiated effects are partially sensitive to $CysLT_1R$ or EP_3 antagonists but completely abolished by simultaneous treatment both *in vitro* and *in vivo*.

Conclusions: Our results unravel a unique LTD_4-PGE_2 interaction affecting mast cells through $CysLT_1R$ and EP_3 involving Gi, protein kinase G, and Erk and contributing to vascular inflammation *in vivo*. Furthermore, current results also suggest an advantage of targeting both $CysLT_1R$ and EP_3 in attenuating inflammation. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: *Mast cells, prostaglandin E_2, leukotriene D_4, $CysLT_1R$, E-prostanoid receptor 3, prostaglandin D_2, c-fos, protein kinase G, extracellular signal-regulated kinase, macrophage inflammatory protein 1β*

Mast cells (MCs) are recognized as critical components of our immune system. They are vital in the initiation and amplification of acute inflammatory responses and play an important role in triggering asthma exacerbations through the elaboration of several soluble inflammatory mediators.[1,2] MCs reside in connective tissues and are located in close proximity to the blood vessels. Activation of MCs stimulate the formation of leukotrienes (LTs) and prostaglandins (PGs), both of which initiate vascular changes.[3] $Kit^{W\text{-}sh/W\text{-}sh}$ mice have the *W-sash* (W^{sh}) inversion mutation and remarkable deficiency in MCs, providing a great model system to analyze MC function *in vivo*.[4] Cysteinyl leukotrienes (cys-LTs; LTC_4, LTD_4, and LTE_4) are arachidonic acid derivatives generated by MCs, eosinophils, basophils, and macrophages[5] through the action of 5-lipoxygenase enzyme. All PGs are derived from PGH_2 and generated through arachidonic acid through the action of PGH synthase (also known as COX). MCs express both COX-1 and COX-2. COX-2 is upregulated by inflammatory stimuli driving PGD_2 generation under inflammatory conditions. In MCs PGH_2 derived from both COX-1 and COX-2 is converted to PGD_2 by a terminal hematopoietic PGD_2 synthase.[6] Although not a product of MCs, PGE_2, a metabolite of PGH_2 through the action of PGE_2 synthase,[7] is the most ubiquitous PG, with prominent and complex functions in inflammation, asthma, and allergic diseases. Remarkably, MCs not only generate cys-LTs but also express corresponding receptors and respond to them.[8] Two known G protein–coupled receptors, termed cysteinyl leukotriene receptor 1 ($CysLT_1R$) and cysteinyl leukotriene receptor 2 ($CysLT_2R$), specifically recognize cys-LTs and mediate their biologic functions. $CysLT_1R$ binds LTD_4 with higher affinity than LTC_4, whereas $CysLT_2R$ has equal affinity for LTD_4 and LTC_4.[5] GPR17, another cys-LT receptor, has been identified and is expressed primarily in the brain,[9] and GPR99 has been recently identified as a cys-LT receptor with a preference for LTE_4.[10] Mice lacking LTC_4 synthase have reduced numbers of MCs in the airway mucosa after sensitization and challenge by

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Supported by National Institutes of Health grant HL098953 and by James Foght Assistant Professor endowed professorship grant (to S.P.).

Disclosure of potential conflict of interest: V. Kondeti and S. Paruchuri have received research support from the National Institutes of Health (HL098953) and the James L. and Martha J. Foght Assistant Professorship. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication January 4, 2015; revised June 18, 2015; accepted for publication June 23, 2015.

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0091-6749/$36.00

http://dx.doi.org/10.1016/j.jaci.2015.06.030

Abbreviations used
cys-LT: Cysteinyl leukotriene
$CysLT_1R$: Cysteinyl leukotriene receptor 1
$CysLT_2R$: Cysteinyl leukotriene receptor 2
EP: E-prostanoid receptor
Erk: Extracellular signal-regulated kinase
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
hMC: Human cord blood–derived mast cell
LT: leukotriene
MC: Mast cell
MIP-1β: Macrophage inflammatory protein 1β
PG: Prostaglandin
PK: Protein kinase
PTX: Pertussis toxin
SCF: Stem cell factor
siRNA: Small interfering RNA

allergen,[11] suggesting the prominence of cys-LTs in MC function. We have previously demonstrated that stimulation of human cord blood–derived mast cells (hMCs), LAD2 cells, or both with LTD_4 potently induces calcium flux and cytokine generation[8] through $CysLT_1R$. Additionally, MC proliferative and inflammatory responses are modulated by LTD_4 and stem cell factor (SCF) signaling interactions.[12] PGs have also been shown to elicit vasodilation and an increase in blood flow. Among PGs, PGE_2 is the most abundantly synthesized PG at the inflammation site and is regarded as an important regulator of inflammation.[13] The decisive effect of PGE_2 is the outcome of specific E-prostanoid receptor (EP) 1 to 4 activation through which the signal is transduced.[14] EP_1 is coupled to intracellular calcium mobilization through Gq; however, EP_2 and EP_4 are coupled to stimulation of adenylyl cyclase through Gs, and EP_3 is coupled to the inhibition of adenylyl cyclase through Gi. Different splice variants are generated by means of alternative splicing of the C-terminal tail of the EP_3 receptor and can couple to different signal transduction pathways. Eight human EP_3 isoforms are known thus far, which are identical, except for their carboxyl termini.[14]

The interactions among various mediator systems that participate in inflammatory responses are complex, and it is difficult to define the unique contribution of any single element. In the current study we show that LTD_4 and PGE_2 synergistically potentiate peripheral inflammation *in vivo* and MC activation *in vitro* through $CysLT_1R$-, EP_3-, Gi-, protein kinase (PK) G–, and extracellular signal-regulated kinase (Erk)–dependent pathways. Furthermore, our results indicate that blocking EP_3 together with $CysLT_1R$ could be a better therapeutic target to control inflammation.

METHODS

Animals

Six- to 8-week-old BALB/c mice, C57BL/6 mice, and $Kit^{W\text{-}sh}$ mice (W-sh) were obtained from Jackson Laboratories and maintained at the Comparative Medicine Unit, Northeast Ohio Medical University. All animal experiments were done in accordance with standard guidelines, as approved by the Animal Care and Use Committee of Northeast Ohio Medical University.

Reagents

LTD_4, PGE_2, MK571, $BayCysLT_2$, iloprost, butaprost, sulprostone, L-798, ONO-871, L-161, and PGD_2 ELISA kits were purchased from Cayman Chemicals (Ann Arbor, Mich). KT5823, PD98059, pertussis toxin (PTX), H7, GF109203X, Rp-cAMPS, and H89 inhibitors were from Tocris Bioscience (Minneapolis, Minn). Fura-2 AM was from Molecular Probes (Eugene, Ore), phospho-specific antibodies were from Cell Signaling Technology (Danvers, Mass), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from Fitzgerald (Acton, Mass). All secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, Pa). Nonspecific small interfering RNA (siRNA) and isoform-specific siRNAs for $CysLT_1R$, EP_3, and PKG were obtained from Dharmacon (Lafayette, Colo), and the macrophage inflammatory protein 1β (MIP-1β) ELISA kit was from R&D Systems (Minneapolis, Minn). Cytokines for hMC cultures were obtained from PeproTech (Rocky Hill, NJ).

Intradermal injection of agonists and assessment of ear edema

Mice anesthetized with ketamine/xylazine received intradermal injections of 0.5 μmol/L LTD_4, PGE_2, and LTD_4 plus PGE_2 (in a 10-μL volume) in the right ear and 10 μL of saline in the left ear in the presence or absence of MK571, L-798, or both. At 0, 30, 60, 120, 240, and 300 minutes after the intradermal injection, ear thickness was measured with a caliper. Mice were killed 60 minutes after the indicated treatment, ear tissues were fixed in 4% paraformaldehyde and embedded in paraffin, and 4-μm-thick sections were cut and stained for hematoxylin and eosin and toluidine blue (to detect MCs). Total (toluidine blue–positive cells that are compact) and degranulated MCs (toluidine-positive cells with no clearly defined cell membrane and diffuse) in the toluidine blue–stained sections were visualized at ×60 magnification and presented in Fig 1, *B*; counted in each section by a blinded observer; and expressed as the number of MCs per millimeter. Representative images of intact and degranulated MCs were shown in Fig 1, *B*.

Cell culture

The LAD2 MC leukemia line[15] was a kind gift from Dr Arnold Kirshenbaum (National Institutes of Health) and cultured as described previously.[8] Primary hMCs were derived from cord blood mononuclear cells cultured for 6 to 9 weeks in RPMI supplemented with SCF, IL-6, and IL-10.[16]

Calcium flux

LAD2 cells (0.5 to 1 × 10^6/sample) were washed and labeled with Fura 2-AM for 30 minutes at 37°C. Cells were stimulated with PGE_2 (0.5 μmol/L) with or without LTD_4 (0.5 μmol/L) priming, and the changes in intracellular calcium levels measured by using excitation at 340 and 380 nm and emission at 510 nm were recorded in a fluorescence spectrophotometer (Hitachi F-4500).[8]

Cell activation

LAD2 cells were stimulated with 0.5 μmol/L of LTD_4, PGE_2, or both for 15 minutes for the phosphorylation of Erk or 1 hour for expression of c-fos, 2 hours for expression of inflammatory gene transcripts, 3 hours for COX-2 protein expression, and 6 hours for measurement of cytokine and PGD_2 levels. LTD_4 responses were dose dependently inhibited by MK571 (with maximum inhibition at 1 μmol/L), and PGE_2 responses were attenuated by L-798 (in a dose-dependent manner, with maximum inhibition at 100 nmol/L). Therefore 1 μmol/L MK571 and 100 nmol/L L-798 were used in all the subsequent experiments. Transfection of isoform-specific siRNA smart pool constructs from Dharmacon (10 nmol/L) were carried out with siLentFect transfection reagent (Bio-Rad Laboratories, Hercules, Calif) for 48 hours, according to the manufacturer's protocol.

Cell lysates and Western blotting

After stimulation with the respective agonists, LAD2 cells, hMCs, or both (0.5 × 10^6) were lysed with lysis buffer (BD Biosciences, San Jose, Calif) supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany) and phosphatase inhibitor cocktail (Pierce, Rockford, Ill). Immunoblotting was performed, as described previously.[17] Western blots were incubated with