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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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Abstract

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

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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.

Keywords

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 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

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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,

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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 ECL, and the bands were visualized with an imager (ProteinSimple, San Jose, Calif) and quantified by using Alpha View SA (ProteinSimple). The blots were stripped and reprobed with GAPDH antibody.

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Real-time quantitative PCR

Expressions of $CysLT_1R$, $CysLT_2R$, EP_1, EP_2, EP_3, EP_4, MIP-1β, TNF-α, IL-8, COX-1, COX-2, and PKG transcripts were determined with real-time PCR performed on the LightCycler 480 (Roche). Total RNA was isolated from LAD2 cells after respective treatment with an RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized with the cDNA synthesis kit from Quanta BioSciences (Gaithersburg, Md). Real-time PCR was performed by using primers mentioned in Table E1 in this article's Online Repository at www.jacionline.org, the levels of respective genes relative to GAPDH were analyzed, and the cycle threshold values relative to control were expressed as the fold change.

ELISA

Concentrations of PGD_2 and MIP-1β in the supernatants were assayed by using the PGD_2-MOX and MIP-1β ELISA kits purchased from Cayman Chemicals (Ann Arbor, Mich) and R&D Systems, respectively, according to the manufacturer's instructions. PGD_2 secreted into medium was first converted into PGD_2-MOX and then assessed by using the PGD_2-MOX ELISA kit.

Statistical analysis

Blots presented are representative of 3 experiments performed, and data are expressed as means ± SEMs from at least 3 experiments, except where otherwise indicated. Significance was determined by using the Student *t* test, as well as 1-way ANOVA, followed by Tukey *post hoc* analysis.

RESULTS

Combined treatment with PGE_2 and LTD_4 synergistically potentiates peripheral vascular inflammation in mice

To elucidate the possible interaction between LTD_4 and PGE_2, we first evaluated the increase in vascular permeability and edema in the mouse ear, a widely used model to assess peripheral vascular inflammation,[18,19] in response to LTD_4 plus PGE_2 compared with agonists alone. LTD_4 or PGE_2 and LTD_4 plus PGE_2 (0.5 μmol/L, a dose at which both the agonists generated maximum response; data not shown) were injected into the ears of BALB/c mice, and tissue edema was assessed. The patterns of ear edema induced by LTD_4, PGE_2, and LTD_4 plus PGE_2 were similar, peaking at 30 minutes and returning to baseline at 300 minutes. However, we observed a significant enhancement in the magnitude of ear edema with LTD_4 plus PGE_2 compared with LTD_4 or PGE_2 alone (Fig 1, *A*). The increase in ear thickness with LTD_4 plus PGE_2 was rapid, transient, and approximately 6-fold higher compared with control values (0.32 ± 0.05 vs 0.05 ± 0.01) in the first half hour. We also observed an increase in ear thickness in response to both LTD_4 (approximately 3-fold, 0.15 ± 0.01) and PGE_2 (approximately 1.6-fold, 0.08 ± 0.01) individually during the first 30 minutes compared with control values. Histologic analysis of ear tissues revealed expansion of the extracellular space correlating with ear thickness (Fig 1, *B*). Interestingly, we found a robust increase in the number of degranulating MCs with LTD_4 plus PGE_2 treatment (Fig 1, *B*, middle and bottom right panel, arrows point to degranulating MCs, and Fig 1, *C*)

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compared with other groups, suggesting that LTD_4 plus PGE_2 can synergistically potentiate peripheral inflammation through their action on MCs. To elucidate the role of MCs, we repeated the above experiment in C57BL/6 and W-sh mice (on C57BL/6 background). Although we observed a similar pattern of ear inflammation in C57BL/6 wild-type mice as in BALB/c mice with all the agonists, the response was more transient and smaller (Fig 1, *D*). LTD_4 plus PGE_2 synergistically enhanced the inflammation in C57BL/6 wild-type mice, which is significantly attenuated in W-sh mice (Fig 1, *D*).

LTD_4 primes PGE_2-dependent calcium flux and potentiates c-fos phosphorylation and MIP-1β production in MCs

Next, we investigated the molecular mechanisms through which LTD_4 and PGE_2 activate MCs and potentiate inflammation. The expression pattern of cys-LT receptor transcript in LAD2 cells is similar to that in hMCs, with high levels of $CysLT_1R$ compared with $CysLT_2R$ (see Fig E1, *C*, in this article's Online Repository at www.jacionline.org). Additionally, LAD2 cells express all 4 EPs ($EP_3 > EP_2 > EP_4 > EP_1$; see Fig E1, *C*). Furthermore, Western blot analysis of LAD2 cells and hMCs confirmed the expression of $CysLT_1R$, $CysLT_2R$, and EP_{1-4} (see Fig E1, *D*). hMCs expressed higher $CysLT_1R$ levels compared with LAD2 cells, and the expression pattern of EP_3 is comparable.

First, we analyzed the ability of LAD2 cells to flux calcium in response to PGE_2 and LTD_4. We observed that PGE_2 induced modest calcium flux (Fig 1, *E*, top panel, and Fig 1, *F*) and LTD_4 priming before PGE_2 stimulation led to an approximately 2.5-fold increase (0.42 $\pm$ 0.05 vs 0.16 $\pm$ 0.04; Fig 1, *E*, bottom panel, and Fig 1, *F*). Furthermore, LTD_4 and PGE_2 together increased phosphorylation and expression of c-fos (Fig 1, *G*) and secretion of MIP-1β (Fig 1, *H*). Importantly, in hMCs also, LTD_4 plus PGE_2 treatment significantly enhanced c-fos phosphorylation and expression (Fig 1, *I*). Stimulating LAD2 cells with a constant dose of LTD_4 (0.5 μmol/L) and varying concentrations of PGE_2 (0.001, 0.01, 0.1, and 0.5 μmol/L), we observed dose-dependent c-fos phosphorylation and expression and MIP-1β secretion (see Fig E1, *A* and *B*).

$CysLT_1R$ inhibition partially attenuates LTD_4-primed PGE_2 responses

We then sought to identify the receptors involved in mediating this synergy by using MK571 ($CysLT_1R$ antagonist[8]) and $BayCysLT_2$ ($CysLT_2R$ antagonist[20]). MK571 pretreatment abolished LTD_4-induced calcium influx but attenuated LTD_4-primed, PGE_2-generated calcium influx by 30% (Fig 2, *A*). LTD_4 plus PGE_2–stimulated c-fos phosphorylation and expression and MIP-1β secretion were reduced with MK571 preincubation (Fig 2, *B-D*), whereas $BayCysLT_2$ had no effect (Fig 2).

Effects of LTD_4 plus PGE_2 are partially mediated through EP_3

We next investigated which of the putative EPs were interacting with $CysLT_1R$ and enhancing PGE_2 responses. Stimulation of LAD2 cells with iloprost (EP_1/EP_3 agonist) and sulprostone (EP_3 agonist) in the presence of LTD_4 enhanced calcium flux (data not shown), c-fos (Fig 3, *A* and *B*), and MIP-1β expression (Fig 3, *C*), which is similar to what is seen with LTD_4 plus PGE_2, suggesting the involvement of EP_3 and a possible involvement of EP_1 in this synergism. The EP_2 agonist butaprost had no effect (Fig 3, *A-C*). Because EP_1 and

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EP_3 act dominantly through Gq- and Gi-mediated signaling pathways, respectively,[14] we used PTX, a Gαi inhibitor, and evaluated LTD_4 plus PGE_2 synergy. Pretreatment of LAD2 cells with PTX (100 ng/mL for 18 hours) completely abolished the enhanced activation of c-fos and MIP-1β by LTD_4 and PGE_2 (see Fig E2, *A-C*, in this article's Online Repository at www.jacionline.org), suggesting the involvement of EP_3. Furthermore, we found that the synergistic responses by LTD_4 plus PGE_2 were partially sensitive to the inhibition of EP_3 by L-798 (EP_3 antagonist, 100 nmol/L; Fig 3, *D-F*), which is similar to what is seen with $CysLT_1R$ inhibition. Neither ONO-8711 (EP_1 antagonist, 2 nmol/L) nor L-161 (EP_4 antagonist, 100 nmol/L) had any effect on LTD_4 plus PGE_2–mediated c-fos expression/ phosphorylation or MIP-1β generation (see Fig E2, *D* and *E*).

Synergistic responses to LTD_4 and PGE_2 were completely attenuated by blocking both $CysLT_1R$ and EP_3 simultaneously

Next, we speculated that blocking both $CysLT_1R$ and EP_3 simultaneously might completely block the synergistic responses. We observed that although the effects mediated by LTD_4 and PGE_2 are partially blocked by $CysLT_1R$ and EP_3 antagonists alone (40% and 35% for c-fos and 58% and 57% for MIP-1β, respectively), combined treatment with MK571 and L-798 completely blocked the effects of LTD_4 plus PGE_2 (78% for c-fos phosphorylation and expression and 92% for MIP-1β; Fig 4, *A-C*). The combination of MK571 and L-798 also attenuated augmentation of c-fos and MIP-1β generation by LTD_4 plus sulprostone (data not shown). Importantly, in hMCs MK571 plus L-798 totally inhibited c-fos phosphorylation and expression (Fig 4, *D* and *E*), suggesting a functional relevance for this interaction and associated signaling.

LTD_4 and PGE_2 treatment upregulated inflammatory genes, PGD_2 generation, and Erk phosphorylation in LAD2 cells

We then investigated whether stimulation of MCs with LTD_4 plus PGE_2 would upregulate any other inflammatory chemokines and cytokines. LTD_4 treatment upregulated MIP-1β, TNF-α, IL-8, and COX-2, whereas PGE_2 upregulated COX-2 alone. LTD_4 plus PGE_2 treatment significantly upregulated MIP-1β (145 ± 30; Fig 5, *A*), TNF-α (90 ± 11; Fig 5, *B*), IL-8 (72 ± 20; Fig 5, *C*), and COX-2 (60 ± 10; Fig 5, *D*) transcripts, and the expression of COX-1 remained unchanged (Fig 5, *E*). Consistently, we observed a significant 3-fold upregulation of COX-2 protein with LTD_4 plus PGE_2 treatment (Fig 5, *F*). Furthermore, LTD_4 plus PGE_2 treatment upregulated PGD_2 secretion compared with control values (1630 ± 51 vs 1000 ± 75 pg/mL), and combined treatment with both MK571 and L-798 inhibited this secretion (Fig 6, *A*). Then we investigated the ability of LTD_4, PGE_2, and a combination of both in inducing Erk phosphorylation. PGE_2 and LTD_4 stimulation enhanced phosphorylation of Erk, and LTD_4 plus PGE_2 further potentiated this effect, which is sensitive to the MEK inhibitor PD989059 (50 μmol/L; Fig 6, *B*).

LTD_4 plus PGE_2 signals operate through PKG and Erk-dependent pathway

We next examined the signaling involved in LTD_4 plus PGE_2 synergism downstream of receptor activation. PKs are known to be activated downstream of $CysLT_1R$ activation.[8,21] A general PK inhibitor, H7 (10 μmol/L), completely blocked LTD_4, as well as LTD_4 plus PGE_2–induced effects (see Fig E3, *A*, *E*, and *F*, in this article's Online Repository at

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www.jacionline.org). However, the general PKC inhibitor GF109203X (2 μmol/L) inhibited LTD_4 signals but modestly blocked enhanced c-fos and MIP-1β effects by LTD_4 plus PGE_2 (see Fig E3, *B*, *E*, and *F*). Furthermore, the PKA blockers Rp-cAMPS and H-89 had no effect on synergistic LTD_4 plus PGE_2 effects (see Fig E3, *C-F*). Interestingly, pretreatment of LAD2 cells with the PKG inhibitor KT5823 (5 μmol/L) or PD98059 attenuated LTD_4 plus PGE_2 synergistic responses (Fig 6, *C-F*). We then knocked down $CysLT_1R$, EP_3, both $CysLT_1R$ and EP_3, and PKG in LAD2 cells by using protein-specific siRNAs (10 nmol/L) and nonspecific siRNAs as a control and analyzed LTD_4 plus PGE_2 effects. Transfection of MCs with $CysLT_1R$, EP_3, and PKG siRNAs significantly and specifically downregulated $CysLT_1R$ (55%), EP_3 (60%), and PKG (64%) expression (see Fig E4 in this article's Online Repository at www.jacionline.org). Importantly, downregulation of $CysLT_1R$ or EP_3 partially inhibited both LTD_4- and LTD_4 plus PGE_2–induced responses, but knockdown of both $CysLT_1R$ and EP_3 completely inhibited LTD_4 plus PGE_2–induced synergy (Fig 7, *A-C*). Also, knockdown of PKG significantly inhibited LTD_4 plus PGE_2–induced effects (Fig 7, *A-C*), whereas the control siRNA did not affect LTD_4- or PGE_2-induced inflammatory responses.

Combined treatment with $CysLT_1R$ and EP_3 antagonists attenuates vascular inflammation induced by LTD_4 plus PGE_2

Next, we determined the effect of blocking both $CysLT_1R$ and EP_3 simultaneously in evoking vascular inflammation. The synergistic ear edema response caused by combined treatment with PGE_2 and LTD_4 was substantially attenuated with MK571 plus L-798 treatment (Fig 7, *D*).

DISCUSSION

Edema formation is a prominent feature of the inflammatory response and serves an important function in local host defense and tissue repair. Inflammatory responses result in the movement of fluid and plasma proteins into the extracellular space from leaky blood vessels in response to various chemical mediators.[22] In the current study, for the first time, we demonstrate that 2 major eicosanoids, LTD_4 and PGE_2, both derived through alternate pathways from arachidonic acid, synergistically enhanced vascular inflammation *in vivo* (edema formation) and MC activation *in vitro*. Although W-sh mice have been well characterized as MC deficient, they have additional defects beyond just MC deficiency, and hence there exists a possibility that the *in vivo* vascular permeability attenuated in W-sh mice could be mediated through other cell types in addition to MCs. A number of mediators have been implicated in the regulation of inflammation in an MC-mediated mechanism.[23] Although MCs are primarily known to be activated in an antigen-dependent manner, evidence also suggests a role for them in antigen-independent activation.[19,22,24] They are regulatory cells throughout the course of acute inflammation, from its initiation to resolution,[25] and they contribute to the development of allergic disease.[26] MC activation in patients with various kinds of inflammatory diseases is significant from a clinical perspective. Translational implications of LTD_4 plus PGE_2 crosstalk provoked us to examine the candidate molecules and signaling mechanisms involved. We identified that LTD_4-PGE_2 synergism was mediated through $CysLT_1R$ and EP_3. It is known that G protein–coupled

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receptors interact with one another, modulating each other's function both positively and negatively.[27,28] Also, PGE_2 relays differential responses depending on the receptor subtype, receptor coupling, and the cell type. For instance, PGE_2 blocks FcεRI-mediated exocytosis of rat peritoneal MCs[29] and human lung MCs[30] *in vitro* through an increase in cyclic AMP levels (EP_2, EP_4, or both) but activates murine bone marrow-derived mast cells through EP_3 *in vitro*.[31] On the contrary, PGE_2 in hMCs did not suppress FcεRI-dependent exocytosis but caused exocytosis on its own when the cells were primed with IL-4.[16] It is likely that the suppressive effects of PGE_2 on MC activation largely reflects EP_2 signaling, and in agreement with this, EP_2 protein expression is downregulated in MCs in the nasal polyp mucosa of patients with aspirin-exacerbated respiratory disease.[32] PGE_2 has been shown to stimulate chemotaxis and adhesion of MCs through EP.[33,34] In a recent report Morimoto et al[19] demonstrated that EP_3 signaling in MCs generated PGE_2-induced vasodilatation and subsequent edema formation and speculated that EP_3-mediated MC activation might be involved in antigen-independent innate immune reactions. Interestingly, we identified that the LTD_4 plus PGE_2 synergism is also EP_3 dependent, strengthening the idea that EP_3 activation might contribute to a proinflammatory role of PGE_2.

LTD_4 plus PGE_2 treatment also upregulated transcripts for inflammatory mediators, such as TNF-α and IL-8, both of which are chemoattractants for neutrophils. Interestingly, we observed synergistic COX-2 induction by LTD_4 plus PGE_2 treatment and PGD_2 generation, revealing another major finding that LTD_4, in concert with PGE_2, can generate proinflammatory metabolite PGD_2 and amplify associated signaling. MCs express both COX-1 and COX-2, and COX-2 is upregulated by inflammatory stimuli, suggesting a mechanism that can amplify PGD_2 generation under inflammatory conditions. Interestingly, PGD_2 has been recently shown to synergize with LTE_4 to stimulate diverse T_H2 functions and T_H2 cell and neutrophil crosstalk.[35,36] It is possible that during inflammation, LTD_4 plus PGE_2 treatment not only activates MCs and initiates inflammation, but also the product of this signaling, PGD_2, can potentially perpetuate inflammation through combined action with LTE_4. Both LTD_4 and PGE_2 have been demonstrated to phosphorylate Erk in MCs.[8,16] In agreement, we noted phosphorylation of Erk by both agonists and observed enhanced Erk phosphorylation with LTD_4 plus PGE_2. Furthermore, we identified that LTD_4-PGE_2 synergy is relayed through PKG and Erk downstream of $CysLT_1R$ and EP_3. Surprisingly, we found that PKC signals, which are vital in various aspects of $CysLT_1R$,[17,21,37] or PKA signals, which mediate EP-induced effects,[16] are dispensable for this crosstalk, but it is dependent on PKG and Erk.

We have recently shown that LTD_4 can influence SCF responses, potentiating MC proliferation.[12] We also found that LTD_4 potentiates endothelial cell adhesion mediated by TNF-α,[20] suggesting that cys-LTs can modulate inflammation by acting in concert with other inflammatory mediators. Indeed, findings from the present study revealed that LTD_4 and PGE_2 produced at the inflammation site can synergistically activate MCs and further enhance inflammation. Notably, this enhanced inflammation by LTD_4 and PGE_2 both *in vitro* and *in vivo* is blocked only by the combined treatment of $CysLT_1R$ and EP_3 antagonists and not by either antagonist alone. This argues for the possibility that the synergy is achieved through the contribution of a specific signaling component from LTD_4 and another component from PGE_2 signaling, rather than crosstalk at the receptor level.

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Although we found PKG and Erk are effector molecules in this signal, the convergence of these signaling events are still elusive.

Studies from EP knockout mice suggest that EP_3 is mainly responsible for PGE_2-induced MC activation and associated proinflammatory signaling pathways.[31] High expression of EP_3 is observed in the brain, and recent findings suggest an injurious role for the PGE_2-EP_3 signaling axis in modulating brain injury and inflammation after intracerebral hemorrhage.[38] Along similar lines, enhanced EP_3 expression and signaling were found in patients with diabetes and have been speculated as a new therapeutic target for β-cell dysfunction in patients with type 2 diabetes.[39]

All the above studies point to the pathologic role of EP_3. Current work alludes to the advantage of blocking $CysLT_1R$ along with EP_3, and it is tempting to speculate that the combined treatment of EP_3 and $CysLT_1R$ antagonists might contribute effectively to targeting inflammation compared with the available $CysLT_1R$ antagonists alone and could open new avenues in clinical approaches for MC-mediated inflammatory diseases. Although PGE_2 alone did not induce significant inflammatory transcripts and MIP-1β generation, it potentiated all the inflammatory readouts in concert with LTD_4, suggesting that cys-LTs could modulate PGE_2 signaling and blocking LTD_4 plus PGE_2 could be a potential therapeutic target to combat initiation and progression of inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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

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MIP-1β	Macrophage inflammatory protein 1β
PG	Prostaglandin
PK	Protein kinase
PTX	Pertussis toxin
SCF	Stem cell factor
siRNA	Small interfering RNA

References

1. Galli SJ, Kalesnikoff J, Grimbaldeston MA, Piliponsky AM, Williams CM, Tsai M. Mast cells as "tunable" effector and immunoregulatory cells: recent advances. Annu Rev Immunol. 2005; 23:749–86. [PubMed: 15771585]
2. Metcalfe DD, Baram D, Mekori YA. Mast cells. Physiol Rev. 1997; 77:1033–79. [PubMed: 9354811]
3. Hart PH. Regulation of the inflammatory response in asthma by mast cell products. Immunol Cell Biol. 2001; 79:149–53. [PubMed: 11264709]
4. Grimbaldeston MA, Chen CC, Piliponsky AM, Tsai M, Tam SY, Galli SJ. Mast cell-deficient W-sash c-kit mutant Kit W-sh/W-sh mice as a model for investigating mast cell biology in vivo. Am J Pathol. 2005; 167:835–48. [PubMed: 16127161]
5. Kanaoka Y, Boyce JA. Cysteinyl leukotrienes and their receptors: cellular distribution and function in immune and inflammatory responses. J Immunol. 2004; 173:1503–10. [PubMed: 15265876]
6. Urade Y, Ujihara M, Horiguchi Y, Igarashi M, Nagata A, Ikai K, et al. Mast cells contain spleen-type prostaglandin D synthetase. J Biol Chem. 1990; 265:371–5. [PubMed: 2403560]
7. Woodward DF, Jones RL, Narumiya S. International Union of Basic and Clinical Pharmacology. LXXXIII: classification of prostanoid receptors, updating 15 years of progress. Pharmacol Rev. 2011; 63:471–538. [PubMed: 21752876]
8. 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]
9. Ciana P, Fumagalli M, Trincavelli ML, Verderio C, Rosa P, Lecca D, et al. The orphan receptor GPR17 identified as a new dual uracil nucleotides/cysteinyl-leukotrienes receptor. EMBO J. 2006; 25:4615–27. [PubMed: 16990797]
10. 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]
11. Kim DC, Hsu FI, Barrett NA, Friend DS, Grenningloh R, Ho IC, et al. Cysteinyl leukotrienes regulate Th2 cell-dependent pulmonary inflammation. J Immunol. 2006; 176:4440–8. [PubMed: 16547282]
12. Al-Azzam N, Kondeti V, Duah E, Gombedza F, Thodeti CK, Paruchuri S. Modulation of mast cell proliferative and inflammatory responses by leukotriene d4 and stem cell factor signaling interactions. J Cell Physiol. 2015; 230:595–602. [PubMed: 25161061]
13. Stables MJ, Gilroy DW. Old and new generation lipid mediators in acute inflammation and resolution. Prog Lipid Res. 2011; 50:35–51. [PubMed: 20655950]
14. Sugimoto Y, Narumiya S. Prostaglandin E receptors. J Biol Chem. 2007; 282:11613–7. [PubMed: 17329241]
15. Kirshenbaum AS, Akin C, Wu Y, Rottem M, Goff JP, Beaven MA, et al. Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcepsilonRI or FcgammaRI. Leuk Res. 2003; 27:677–82. [PubMed: 12801524]

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16. Feng C, Beller EM, Bagga S, Boyce JA. Human mast cells express multiple EPs for prostaglandin E2 that differentially modulate activation responses. Blood. 2006; 107:3243–50. [PubMed: 16357326]
17. Paruchuri S, Hallberg B, Juhas M, Larsson C, Sjolander A. Leukotriene D(4) activates MAPK through a Ras-independent but PKCepsilon-dependent pathway in intestinal epithelial cells. J Cell Sci. 2002; 115:1883–93. [PubMed: 11956320]
18. Maekawa A, Balestrieri B, Austen KF, Kanaoka Y. GPR17 is a negative regulator of the cysteinyl leukotriene 1 receptor response to leukotriene D4. Proc Natl Acad Sci U S A. 2009; 106:11685–90. [PubMed: 19561298]
19. Morimoto K, Shirata N, Taketomi Y, Tsuchiya S, Segi-Nishida E, Inazumi T, et al. Prostaglandin E2-EP3 signaling induces inflammatory swelling by mast cell activation. J Immunol. 2014; 192:1130–7. [PubMed: 24342806]
20. Duah E, Adapala RK, Al-Azzam N, Kondeti V, Gombedza F, Thodeti CK, et al. Cysteinyl leukotrienes regulate endothelial cell inflammatory and proliferative signals through CysLT(2) and CysLT(1) receptors. Sci Rep. 2013; 3:3274. [PubMed: 24253666]
21. Kondeti V, Duah E, Al-Azzam N, Thodeti CK, Boyce JA, Paruchuri S. Differential regulation of cysteinyl leukotriene receptor signaling by protein kinase C in human mast cells. PLoS One. 2013; 8:e71536. [PubMed: 23977066]
22. Kaplan DH, Igyarto BZ, Gaspari AA. Early immune events in the induction of allergic contact dermatitis. Nat Rev Immunol. 2012; 12:114–24. [PubMed: 22240625]
23. Wernersson S, Pejler G. Mast cell secretory granules: armed for battle. Nat Rev Immunol. 2014; 14:478–94. [PubMed: 24903914]
24. Dudeck A, Dudeck J, Scholten J, Petzold A, Surianarayanan S, Kohler A, et al. Mast cells are key promoters of contact allergy that mediate the adjuvant effects of haptens. Immunity. 2011; 34:973–84. [PubMed: 21703544]
25. St John AL, Abraham SN. Innate immunity and its regulation by mast cells. J Immunol. 2013; 190:4458–63. [PubMed: 23606723]
26. Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM. Asthma. From bronchoconstriction to airways inflammation and remodeling. Am J Respir Crit Care Med. 2000; 161:1720–45. [PubMed: 10806180]
27. 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]
28. Laidlaw TM, Boyce JA. Cysteinyl leukotriene receptors, old and new; implications for asthma. Clin Exp Allergy. 2012; 42:1313–20. [PubMed: 22925317]
29. Kaliner M, Austen KF. Cyclic AMP, ATP, and reversed anaphylactic histamine release from rat mast cells. J Immunol. 1974; 112:664–74. [PubMed: 4130694]
30. Peachell PT, MacGlashan DW Jr, Lichtenstein LM, Schleimer RP. Regulation of human basophil and lung mast cell function by cyclic adenosine monophosphate. J Immunol. 1988; 140:571–9. [PubMed: 2447182]
31. Nguyen M, Solle M, Audoly LP, Tilley SL, Stock JL, McNeish JD, et al. Receptors and signaling mechanisms required for prostaglandin E2-mediated regulation of mast cell degranulation and IL-6 production. J Immunol. 2002; 169:4586–93. [PubMed: 12370397]
32. Ying S, Meng Q, Scadding G, Parikh A, Corrigan CJ, Lee TH. Aspirin-sensitive rhinosinusitis is associated with reduced E-prostanoid 2 receptor expression on nasal mucosal inflammatory cells. J Allergy Clin Immunol. 2006; 117:312–8. [PubMed: 16461132]
33. Sakanaka M, Tanaka S, Sugimoto Y, Ichikawa A. Essential role of EP3 subtype in prostaglandin E2-induced adhesion of mouse cultured and peritoneal mast cells to the Arg-Gly-Asp-enriched matrix. Am J Physiol Cell Physiol. 2008; 295:C1427–33. [PubMed: 18815228]
34. Weller CL, Collington SJ, Hartnell A, Conroy DM, Kaise T, Barker JE, et al. Chemotactic action of prostaglandin E2 on mouse mast cells acting via the PGE2 receptor 3. Proc Natl Acad Sci U S A. 2007; 104:11712–7. [PubMed: 17606905]

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35. Xue L, Fergusson J, Salimi M, Panse I, Ussher JE, Hegazy AN, et al. Prostaglandin D2 and leukotriene E4 synergize to stimulate diverse TH2 functions and TH2 cell/neutrophil crosstalk. J Allergy Clin Immunol. 2015; 135:1358–66. e1–11. [PubMed: 25441644]

36. Xue L, Gyles SL, Wettey FR, Gazi L, Townsend E, Hunter MG, et al. Prostaglandin D2 causes preferential induction of proinflammatory Th2 cytokine production through an action on chemoattractant receptor-like molecule expressed on Th2 cells. J Immunol. 2005; 175:6531–6. [PubMed: 16272307]

37. Paruchuri S, Sjolander A. Leukotriene D4 mediates survival and proliferation via separate but parallel pathways in the human intestinal epithelial cell line Int 407. J Biol Chem. 2003; 278:45577–85. [PubMed: 12912998]

38. Leclerc JL, Lampert AS, Diller MA, Dore S. Genetic deletion of the prostaglandin E prostanoid receptor subtype 3 improves anatomical and functional outcomes after intracerebral hemorrhage. Eur J Neurosci. 2015; 41:1381–91. [PubMed: 25847406]

39. Kimple ME, Keller MP, Rabaglia MR, Pasker RL, Neuman JC, Truchan NA, et al. Prostaglandin E2 receptor, EP3, is induced in diabetic islets and negatively regulates glucose- and hormone-stimulated insulin secretion. Diabetes. 2013; 62:1904–12. [PubMed: 23349487]

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Key messages

- LTD_4 synergizes with PGE_2 and activates MCs *in vitro* and vascular inflammation *in vivo* through $CysLT_1R$ and EP_3, which might contribute to MC-mediated allergic inflammation.
- LTD_4-PGE_2 synergism triggers diverse MC inflammatory responses through Gi-, PKG-, and Erk-dependent pathways.
- LTD_4 plus PGE_2 crosstalk is efficiently blocked by simultaneous inhibition of $CysLT_1R$ and EP_3 but not by either of the agonists alone, suggesting that vascular inflammation can be targeted efficiently by combining currently available $CysLT_1R$ antagonists with EP_3 antagonists.

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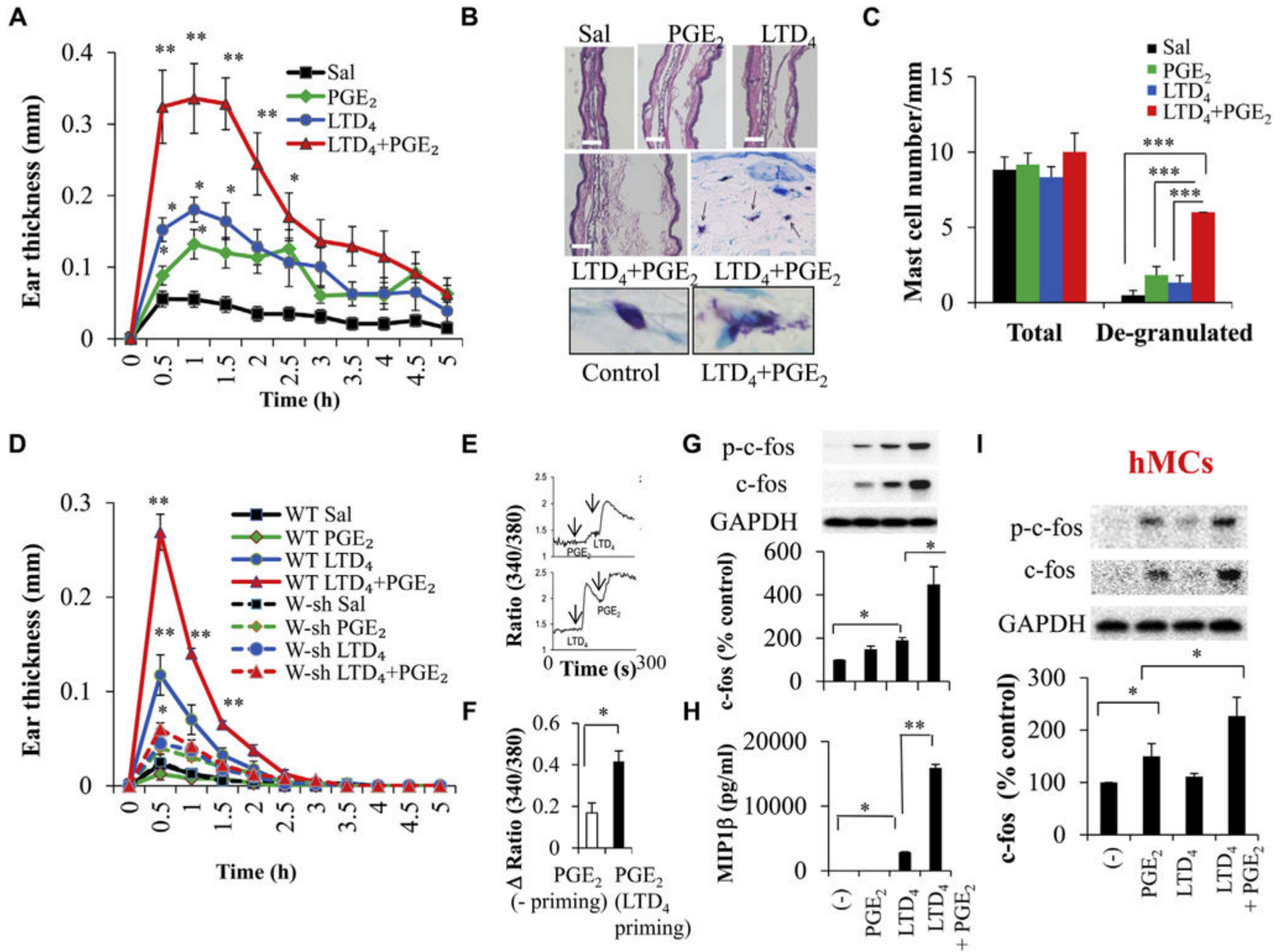


FIG 1.

Effect of LTD_4 and PGE_2 on ear edema in mice *in vivo* and MC activation *in vitro*. Wild-type *(WT)* BALB/c mice were treated with saline *(Sal)*, 0.5 μmol/L LTD_4, 0.5 μmol/L PGE_2, or LTD_4 plus PGE_2. **A**, Ear thickness. **B**, Hematoxylin and eosin staining and toluidine blue staining. **C**, Quantification (blind analysis) of MCs per millimeter. **D**, Ear thickness in C57BL/6 and W-sh mice treated with 0.5 μmol/L LTD_4, PGE_2, and LTD_4 plus PGE_2. Results are means ± SEMs from 4 to 6 mice per group per experiment and 3 experiments performed. **E–I**, LAD2 cells (Fig 1, *E–H*) and hMCs (Fig 1, *I*) were stimulated with 0.5 μmol/L LTD_4, 0.5 μmol/L PGE_2, or both, and calcium flux (Fig 1, *E* and *F*), c-fos (Fig 1, *G* and *I*), and MIP-1β (Fig 1, *H*) were analyzed. *$P < .05$, **$P < .01$, and ***$P < .001$.

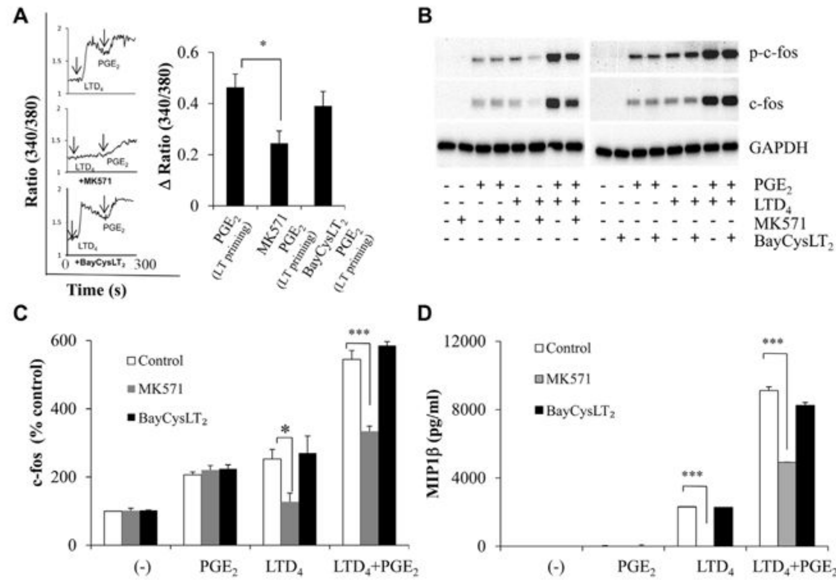
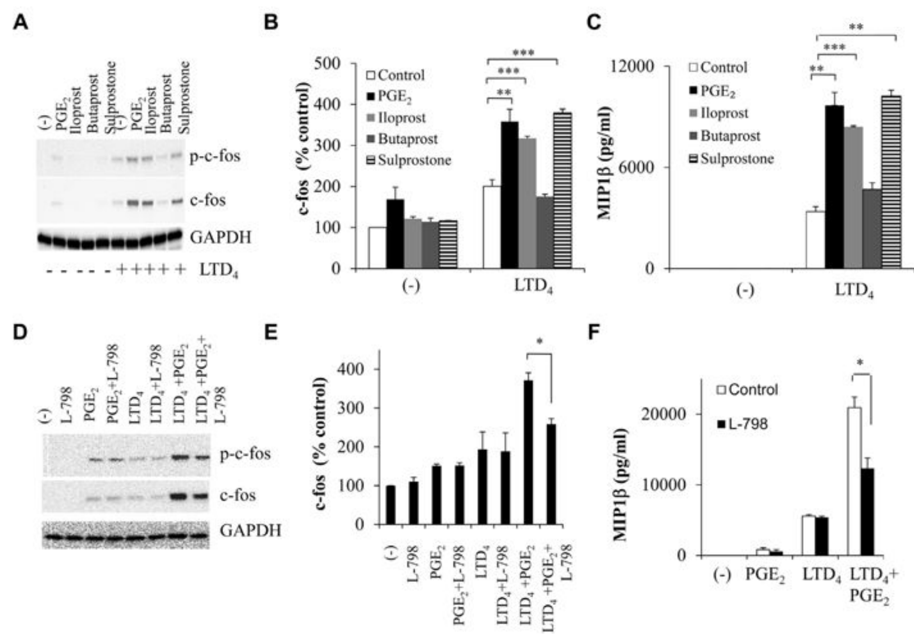


FIG 2. LTD_4-primed and PGE_2-mediated calcium, c-fos, and MIP-1β responses in MCs are partly sensitive to $CysLT_1R$ inhibition. LAD2 cells were stimulated with 0.5 μmol/L LTD_4, 0.5 μmol/L PGE_2, or both in the presence or absence of MK571 (1 μmol/L) and $BayCysLT_2$ (1 μmol/L), and calcium flux **(A)**, c-fos **(B** and **C)**, and MIP-1β **(D)** were analyzed. *P < .05 and ***P < .001.



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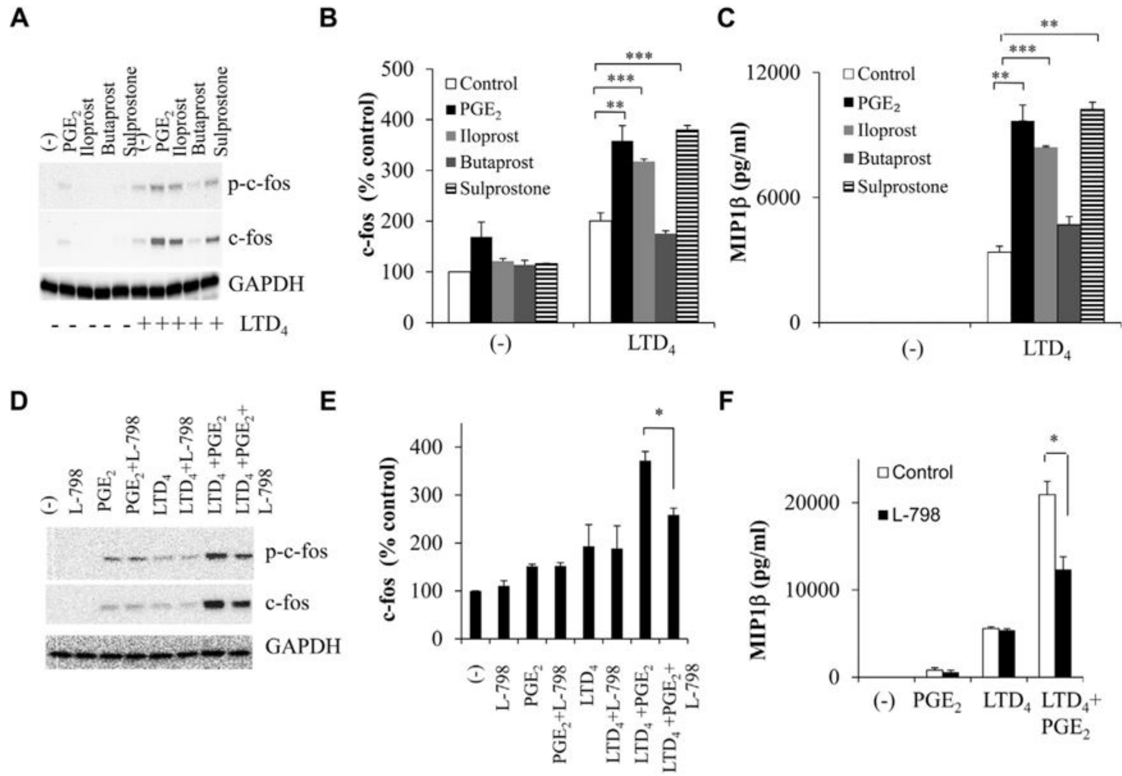

FIG 3.

EP_3 relays synergistic activation of c-fos and MIP-1β in response to PGE_2 and LTD_4. LAD2 cells were treated with LTD_4 (0.5 μmol/L) ± PGE_2 (0.5 μmol/L) or iloprost (10 μmol/L), butaprost (5 μmol/L), or sulprostone (100 nmol/L) ± L-798 (100 nmol/L), and c-fos (**A**, **B**, **D**, and **E**) and MIP-1β (**C** and **F**) were analyzed. *$P < .05$, **$P < .01$, and ***$P < .001$.

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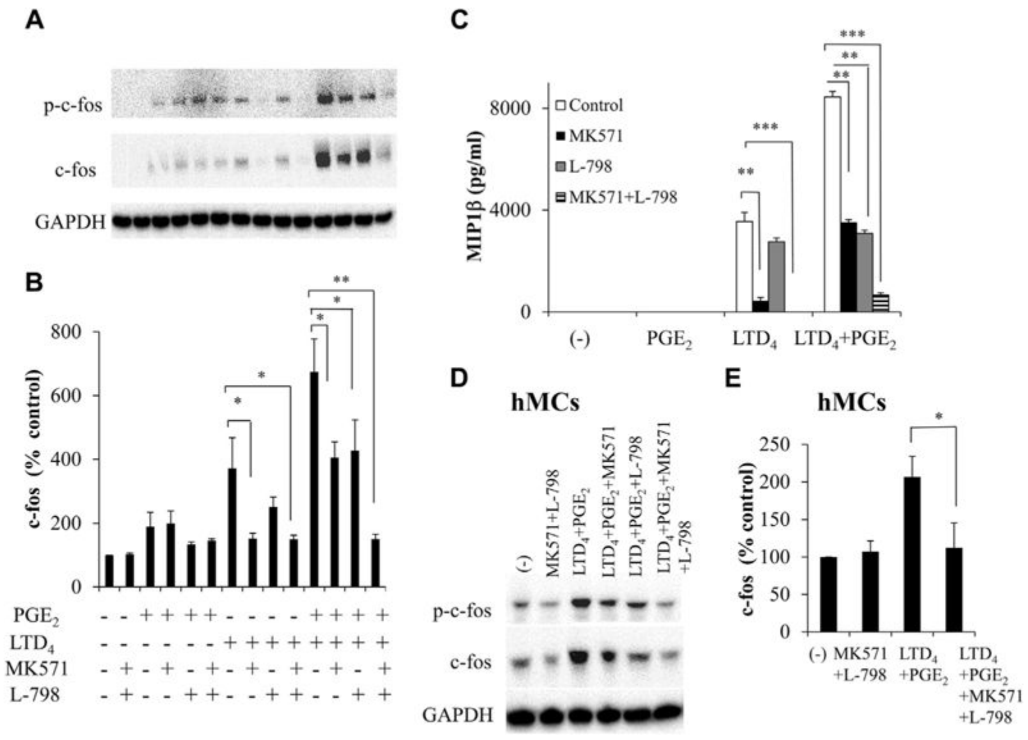

FIG 4.

Combined effect of $CysLT_1R$ antagonist and EP_3 antagonist on synergistic responses to LTD_4 and PGE_2. LAD2 cells (**A–C**) and hMCs (**D** and **E**) were preincubated with MK571 (1 μmol/L) and L-798 (100 nmol/L) separately or in combination and treated with LTD_4 (0.5 μmol/L) ± PGE_2 (0.5 μmol/L), and c-fos (Fig 4, *A*, *B*, *D*, and *E*) and MIP-1β (Fig 4, *C*) were analyzed. *$P < .05$, **$P < .01$, and ***$P < .001$.

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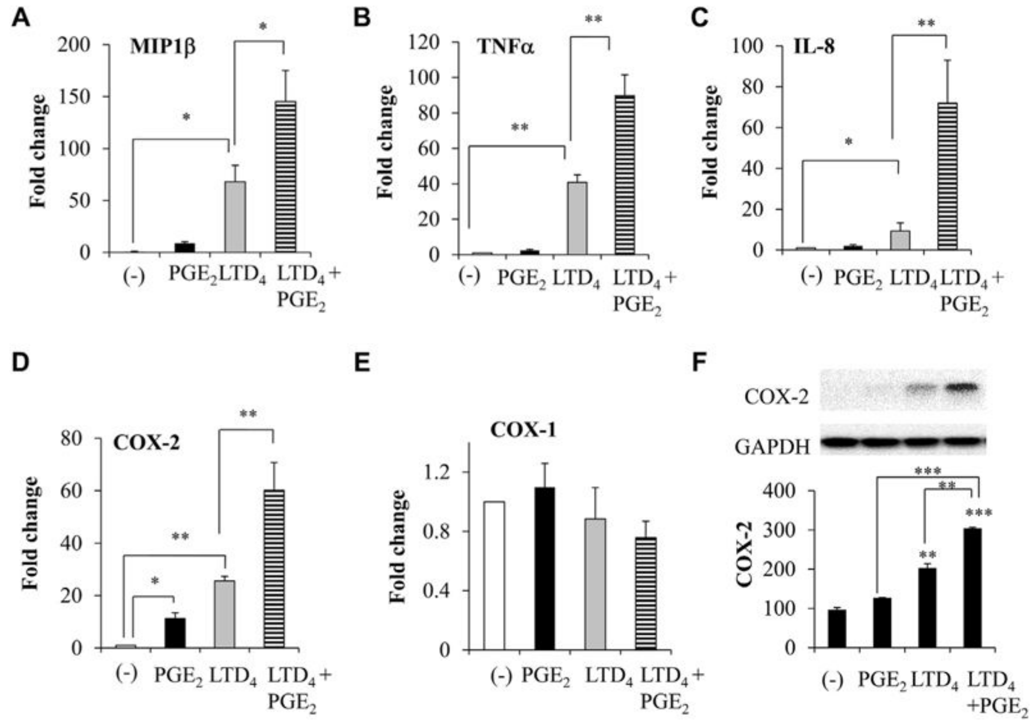

FIG 5.

Inflammatory gene induction and COX-2 protein by LTD_4 and PGE_2 in LAD2 cells. LAD2 cells were stimulated with 0.5 μmol/L LTD_4, PGE_2, or both; transcript levels of MIP-1β, TNF-α, IL-8, COX-2, and COX-1 were analyzed by means of real-time PCR at 2 hours of stimulation **(A–E)**; and COX-2 protein levels were determined **(F)** in cell lysates. $*P < .05$, $**P < .01$, and $***P < .001$.

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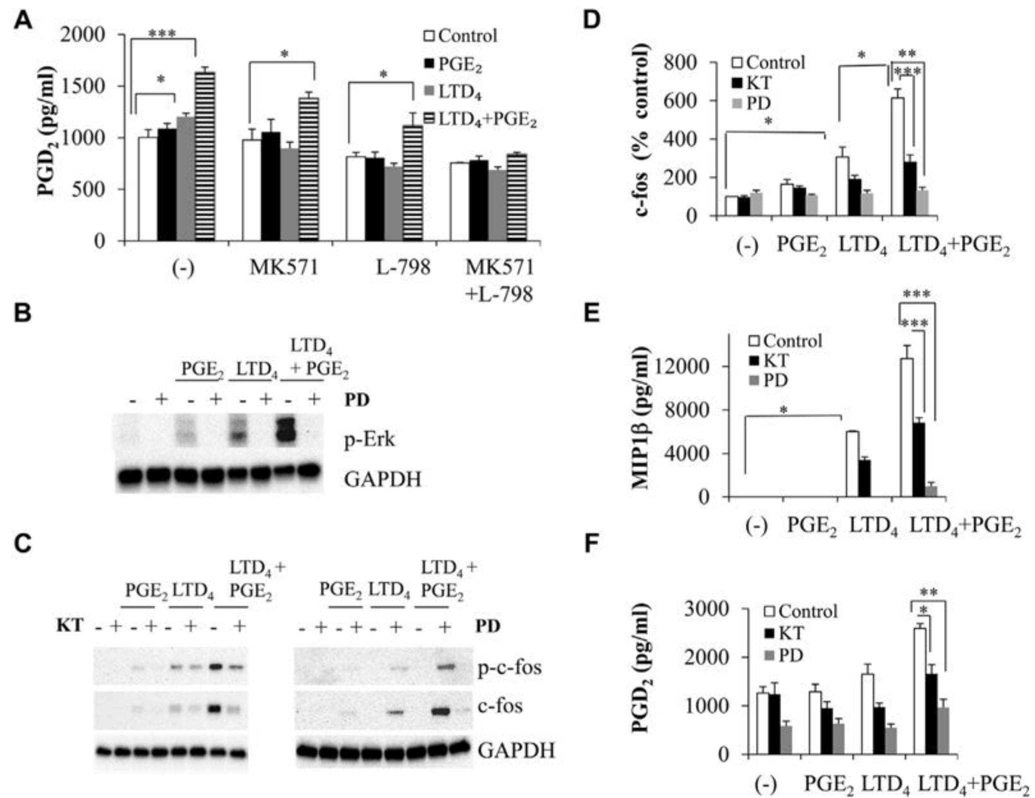

FIG 6.

LTD_4 and PGE_2 crosstalk causes a synergistic increase in PGD_2 secretion and Erk phosphorylation and is mediated through PKG and Erk. LAD2 cells were stimulated with 0.5 μmol/L LTD_4, 0.5 μmol/L PGE_2, or both in the presence or absence of MK571 (1 μmol/L), L-798 (100 nmol/L), KT5823 (*KT*; 5 μmol/L), or PD98059 (*PD*; 50 μmol/L) before treatment, and PGD_2 secretion (**A** and **F**), Erk phosphorylation **(B)**, c-fos (**C** and **D**), and MIP-1β generation **(E)** were analyzed. **P* < .05, ***P* < .01, and ****P* < .001.

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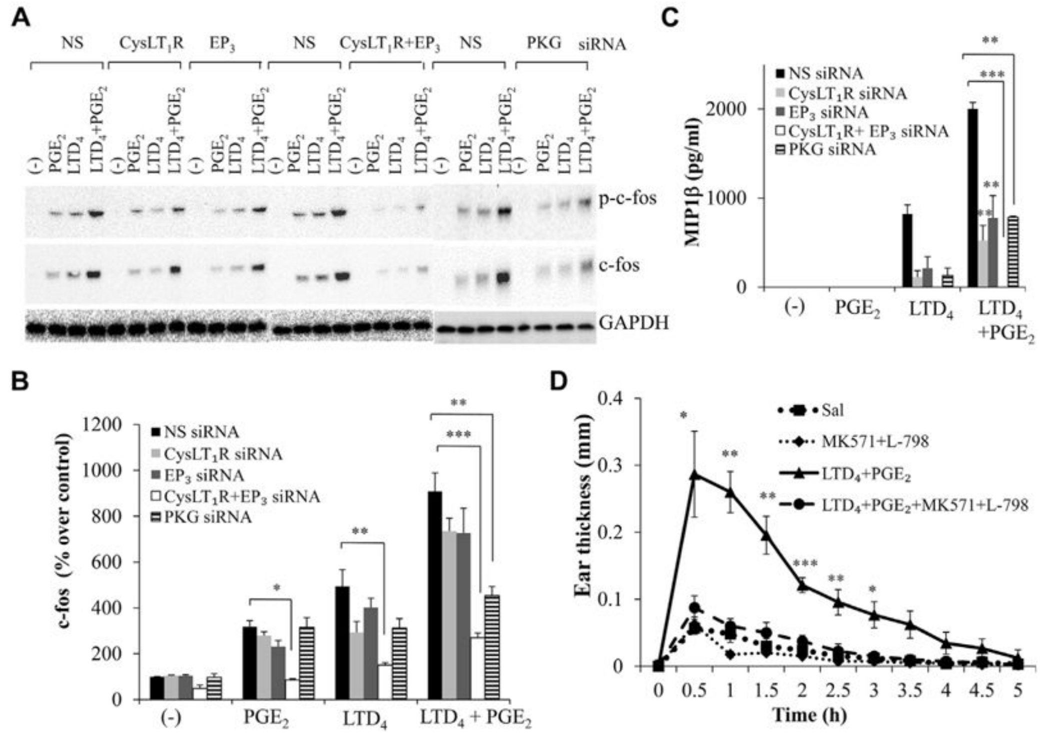

FIG 7.

Simultaneous inhibition of $CysLT_1R$ and EP_3 on LTD_4 plus PGE_2–mediated MC activation *in vitro* and ear edema *in vivo*. $CysLT_1R$, EP_3, or both and PKG were knocked down in LAD2 cells by corresponding siR-NAs (10 nmol/L), and c-fos (**A** and **B**) and MIP-1β (**C**) were analyzed with LTD_4 plus PGE_2 treatment. **D**, Ear thickness in BALB/c mice treated with 0.5 μmol/L LTD_4 plus PGE_2 ± MK571 (1 μmol/L) or L-798 (100 nmol/L). Results in Fig 7, *D*, are means ± SEMs from 4 to 6 mice per group per experiment and 3 experiments performed. $^*P < .05$, $^{**}P < .01$, and $^{***}P < .001$.