**P2Y6 Receptors Require an Intact Cysteinyl Leukotriene Synthetic and Signaling System to Induce Survival and Activation of Mast Cells**

Yongfeng Jiang, Laura Borrelli, Brian J. Bacsakai, Yoshihide Kanaoka and Joshua A. Boyce

*J Immunol* 2009; 182:1129-1137; doi: 10.4049/jimmunol.182.2.1129
http://www.jimmunol.org/content/182/2/1129

---

**References**
This article cites 58 articles, 31 of which you can access for free at:
http://www.jimmunol.org/content/182/2/1129.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
P2Y6 Receptors Require an Intact Cysteinyl Leukotriene Synthetic and Signaling System to Induce Survival and Activation of Mast Cells

Yongfeng Jiang,* Laura Borrelli,‡ Brian J. Bacskaı,§ Yoshihide Kanaoka,* and Joshua A. Boyce2*†

Cysteinyl leukotrienes (cys-LTs) induce inflammatory responses through type 1 (CysLT1R) and type 2 (CysLT2R) cys-LT receptors and activate mast cells in vitro. We previously demonstrated that cys-LTs cross-desensitized IL-4-primed primary human mast cells (hMCs) to stimulation with the nucleotide uridine diphosphate (UDP). We now report that hMCs, mouse bone marrow-derived mast cells (mBMMCs), and the human MC line LAD2 all express UDP-selective P2Y6 receptors that cooperate with CysLT1R to promote cell survival and chemokine generation by a pathway involving reciprocal ligand-mediated cross-talk. Leukotriene (LT) D4, the most potent CysLT1R ligand, and UDP both induced phosphorylation of ERK and prolonged the survival of cytokine-starved hMCs and mBMMCs. ERK activation and cytoprotection in response to either ligand were attenuated by treatment of the cells with a selective P2Y6 receptor antagonist (MRS2578), which did not interfere with signaling through recombinant CysLT1R. Surprisingly, both UDP and LTD4-mediated ERK activation and cytoprotection were absent in mBMMCs lacking CysLT1R and the biosynthetic enzyme LTC4 synthase, implying a requirement for a cys-LT-mediated autocrine loop. In IL-4-primed LAD2 cells, LTD4 induced the generation of MIP-1β, a response blocked by short hairpin RNA-mediated knockdown of CysLT1R or P2Y6 receptors, but not of CysLT2R. Thus, CysLT1R and P2Y6 receptors, which are coexpressed on many cell types of innate immunity, reciprocally amplify one another’s function in mast cells through endogenous ligands. The Journal of Immunology, 2009, 182: 1129–1137.

Leukotriene (LT)3 C4, LTD4, and LTE4a, collectively known as the cysteinyl LTs (cys-LTs), are arachidonic acid-derived inflammatory lipid mediators generated by mast cells (MCs), eosinophils, basophils, and alveolar macrophages (reviewed in Ref. 1). They potentially contract smooth muscle in humans (2) and regulate vascular responses to passive anaphylaxis and experimental peritonitis in mice (3). cys-LTs are established mediators of asthma (4). Two G protein-coupled receptors (GPCRs) for cys-LTs, the type 1 (CysLT1R) and type 2 (CysLT2R) receptors, mediate the effects of cys-LTs in vivo (5). CysLT1R binds cys-LTs with a rank order of LTD4 > LTC4 > LTE4 (5), whereas CysLT2R binds LTC4 = LTD4 > LTE4 (6). CysLT1R is expressed by airway and vascular smooth muscle, as well as by leukocytes such as neutrophils and eosinophils, macrophages (5, 7) and MCs (8). CysLT1R is expressed by endothelial and epithelial cells, heart, brain, adrenal medulla, certain leukocytes, macrophages, and mast cells (MCs) (6). In accord with their incompletely overlapping distributions, each receptor for cys-LTs has distinct functions. For example, the vascular smooth muscle contractile effects of cys-LTs depend either on CysLT1R solely or on both receptors, contingent on the tissue location (9). Whereas CysLT1R is obligate for pulmonary fibrosis induced by bleomycin, CysLT1R is protective in this circumstance (9). On MCs, CysLT1R and CysLT2R heterodimerize and the latter receptor acts as a negative regulator of the former’s actions (10).

Among the >400 GPCRs encoded by mammalian genomes, the closest homologs of the cys-LT receptors are the purinergic (P2Y) receptors for extracellular nucleotides. Eight such GPCRs (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14) have confirmed nucleotide specificity (reviewed in Ref. 11). Some P2Y receptors are expressed in the CNS and others are expressed by vascular structures, epithelial cells, and hematopoietic cells. Cellular damage due to vascular injury and shear stress causes the release of large amounts of nucleotides, which can also be released in a regulated fashion by activation of secretory cell types such as MCs (12) or platelets. Thus, during tissue injury or inflammatory responses, extracellular concentrations of nucleotides can reach micromolar concentrations, with even higher local concentrations at cell-cell junctions (11). In these contexts, P2Y receptors can function as “danger sensors,” activating pain fibers (13), and regulating platelet aggregation (14, 15). P2Y receptors can also induce
migration and phagocytic function of microglia (16, 17) and cause the migration of monocytes (18) and activation of macrophages (19), MCs (20), and myeloid dendritic cells (21). Although most P2Y receptors preferentially recognize adenine nucleotides (ATP or ADP), some recognize uracil nucleotides. The human P2Y4 receptor binds UTP and the P2Y14 receptor recognizes the uridine diphosphate (UDP) metabolite UDP-glucose (21). A recently deorphanized GPCR, GPR17, is expressed in the CNS and confers calcium signals in response to uracil-containing nucleotides and to LTD4 when expressed by transfection into an astrocyte cell line (22). The P2Y6 receptor is the only known UDP-specific GPCR and is strongly expressed by vascular smooth muscle (23) and the spleen (24). Many cells of myelomonocytic origin (osteoclasts (25), microglia (17), macrophages (19)) express P2Y6 receptors. Lung epithelial cells constitutively express P2Y6 receptors (26), while both mouse and human intestinal epithelium inducibly express P2Y6 receptors with inflammation (27). The expression of P2Y6 receptors is required for IL-8 production by a monocytic cell line stimulated with bacterial LPS (19) and by human intestinal epithelial cells stimulated with neutrophil-derived antimicrobial peptides (27). Thus, among the P2Y receptor class, P2Y6 receptors may be specialized to amplify the responses of the innate immune system to inflammatory stimuli that induce release of endogenous uracil nucleotides. The distributions of P2Y6 receptors and CysLT1R overlap, and many cell types (e.g., smooth muscle, leukocytes, microglia (17, 28)) express both receptors.

MCs are tissue-resident effector cells that can potentially induce innate immune responses and are central to the pathophysiology of both allergic and nonallergic inflammatory diseases. Primary human MCs derived in vitro from cord blood (hMCs) express both CysLT1R and CysLT2R (8, 29) and respond to exogenous cys-LTs with proliferating (30) and generating cytokines (31), including large quantities of the chemokine MIP-1β by a signaling mechanism augmented by cell priming by IL-4. LTD4-mediated signaling in MCs requires CysLT1R-mediated phosphorylation of ERK, reflecting transactivation of the Kit receptor tyrosine kinase (30). Previously, we had demonstrated that UDP, like LTD4, caused calcium flux, ERK phosphorylation, and cytokine generation by hMCs, each of which was attenuated by pretreatment of the cells with MK571, an antagonist that blocks the CysLT1R (5) and several P2Y2 receptors (32), but not CysLT2R (6). We hypothesized that the parallels between cys-LT- and UDP-induced signaling events in MCs reflect cross-talk between the CysLT1R and one or more UDP-responsive receptors. We now report that CysLT1R and the P2Y6 receptor require one another’s presence on MCs for maximal ligand-initiated cytokine generation, ERK activation, and cytoprotection. Pharmacologic blockade of P2Y6 receptors in LAD2 cells, a well-differentiated human MC sarcoma line (33), diminishes their ERK activation in response to LTD4. Likewise, UDP-dependent ERK activation of mouse bone marrow-derived MCs (mBMMCs) requires the presence of CysLT1R and endogenous cys-LT production. Under conditions of starvation from cytokines, both UDP and LTD4 sustain the survival of MCs, a function requiring CysLT1R, extracellular nucleotides, LTC4 synthase (LTC4S), and the intact functions of P2Y6 receptors. LTD4-mediated MIP-1β production by LAD2 cells is strikingly attenuated by knockdown or blockade of P2Y6 receptors, by depletion of extracellular nucleotides, and by inhibition of LTC4S synthesis. Collectively, these observations suggest that P2Y6 receptors are components of CysLT1R-dependent inflammatory signaling in MCs and that the two receptors cross-talk through their respective endogenous ligands to enhance cell activation and survival.

Materials and Methods

Reagents

LTD4 and MK571 were purchased from Cayman Chemical. Polyclonal anti-Ab Abs were raised against conserved sequences of human and mouse CysLT1R (RB34, against extracellular domain 3), CysLT1R (RB19, against extracellular domain 2), and the P2Y6 receptor (RB165, against the second intracellular loop of the human P2Y6 receptor CQRYLGICH PLAPWHKREG by Orbigen). An additional Ab against the C terminus of the human CysLT1R (Cayman Chemical) was used for the fluorescence lifetime imaging microscopy (FLIM) assays. Monoclonal anti-β2 and polyclonal anti-α1 integrin Abs were purchased from BD Pharmingen. Alexa Fluor 488-conjugated goat anti-rabbit IgG and Cy3-conjugated donkey anti-mouse IgG were purchased from Invitrogen. Alexa Fluor 488 and Cy3 (Amersham Biosciences) were used for direct labeling according to the manufacturer’s protocols. Recombinant human and mouse cytokines were all purchased from R&D Systems. The P2Y2 receptor antagonist suramin and the P2Y6 receptor-selective antagonist MRS2578 (34) were purchased from Sigma-Aldrich.

Derivation and culture of MCs

The use of cord blood for this study was covered under a protocol for the use of discarded human materials approved by the Partners Healthcare Human Research Committee. hMCs were derived from cord blood mononuclear cells as previously described (35). mBMMCs were derived from bone marrow (29) and studied at 4–6 wk when virtually all stained with toluidine blue. LAD2 cells (provided by Dr. A. Kirshenbaum, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Bethesda, MD) were maintained in SpenPro34 medium containing stem cell factor (SCF; 100 ng/ml) (33). LAD2 cells were used for the experiments involving calcium flux and cytokine generation due to their robust responses to cys-LTs in these assays (36), and hMCs were used as a comparison group for studies of P2Y6 receptor expression. mBMMCs from gene-targeted mice and wild-type controls were used for the studies of ERK activation and cytoprotection to determine the requirements for CysLT1R, CysLT2R, and LTC4S in this process.

Assessment of apoptosis

Aptosis assays were performed by the cytofluorographic detection of annexin V binding according to the supplier’s instructions (BD Biosciences). The cells were maintained for 48–72 h in the presence or absence of SCF (10 ng/ml) or various concentrations of LTD4 or UDP.

Cell activation

LAD2 cells were maintained for 72 h in the presence of recombinant human IL-4 to amplify their production of MIP-1β (30). For cross-linkage of the high-affinity Fc receptor for IgE (FceRI), cells were incubated overnight in the presence of a purified human myeloma IgE (Calbiochem). The cells were washed and resuspended in fresh StemPro medium containing SCF (100 ng/ml) at a density of 5 × 107/ml. Triplicate samples of cells were stimulated with exogenous LTD4 (100 or 500 nM), UDP (1 μM), or with a cross-linking rabbit anti-human IgE polyclonal IgG Ab in the wells of a 96-well flat-bottom plate. The supernatants were harvested at 6 h, a time point at which MIP-1β production was maximal. Calcium flux was measured as described previously (8).

Mice

Cysltr1−/− mice (C57Bl6/Cystlr1−/−), Cysltr2−/− mice (C57Bl6/Cystltr2−/−), and Ltcs4−/− mice (C129S7-Ltcs4−/−H11002), each backcrossed for at least 10 generations onto a BALB/c (background), were derived as described previously (3, 37). All animal studies were approved by the Animal Care and Use Committee of the Dana-Farber Cancer Institute.

SDS-PAGE immunoblotting

Cytokine-starved mBMMCs were stimulated for 15 min with various doses of LTD4, SCF (100 ng/ml), or medium, lysed, and processed for Western blotting as described previously (29). Blots were probed with anti-Ab Abs recognizing the uridine diphosphate (UDP) metabolite UDP-glucose (21). A recently deorphanized GPCR, GPR17, is expressed in the CNS and confers calcium signals in response to uracil-containing nucleotides and to LTD4 when expressed by transfection into an astrocyte cell line (22). The P2Y6 receptor is the only known UDP-specific GPCR and is strongly expressed by vascular smooth muscle (23) and the spleen (24). Many cells of myelomonocytic origin (osteoclasts (25), microglia (17), macrophages (19)) express P2Y6 receptors. Lung epithelial cells constitutively express P2Y6 receptors (26), while both mouse and human intestinal epithelium inducibly express P2Y6 receptors with inflammation (27). The expression of P2Y6 receptors is required for IL-8 production by a monocytic cell line stimulated with bacterial LPS (19) and by human intestinal epithelial cells stimulated with neutrophil-derived antimicrobial peptides (27). Thus, among the P2Y receptor class, P2Y6 receptors may be specialized to amplify the responses of the innate immune system to inflammatory stimuli that induce release of endogenous uracil nucleotides. The distributions of P2Y6 receptors and CysLT1R overlap, and many cell types (e.g., smooth muscle, leukocytes, microglia (17, 28)) express both receptors.

MCs are tissue-resident effector cells that can potentially induce innate immune responses and are central to the pathophysiology of both allergic and nonallergic inflammatory diseases. Primary human MCs derived in vitro from cord blood (hMCs) express both CysLT1R and CysLT2R (8, 29) and respond to exogenous cys-LTs by proliferating (30) and generating cytokines (31), including large quantities of the chemokine MIP-1β by a signaling mechanism augmented by cell priming by IL-4. LTD4-mediated signaling in MCs requires CysLT1R-mediated phosphorylation of ERK, reflecting transactivation of the Kit receptor tyrosine kinase (30). Previously, we had demonstrated that UDP, like LTD4, caused calcium flux, ERK phosphorylation, and cytokine generation by hMCs, each of which was attenuated by pretreatment of the cells with MK571, an antagonist that blocks the CysLT1R (5) and several P2Y2 receptors (32), but not CysLT2R (6). We hypothesized that the parallels between cys-LT- and UDP-induced signaling events in MCs reflect cross-talk between the CysLT1R and one or more UDP-responsive receptors. We now report that CysLT1R and the P2Y6 receptor require one another’s presence on MCs for maximal ligand-initiated cytokine generation, ERK activation, and cytoprotection. Pharmacologic blockade of P2Y6 receptors in LAD2 cells, a well-differentiated human MC sarcoma line (33), diminishes their ERK activation in response to LTD4. Likewise, UDP-dependent ERK activation of mouse bone marrow-derived MCs (mBMMCs) requires the presence of CysLT1R and endogenous cys-LT production. Under conditions of starvation from cytokines, both UDP and LTD4 sustain the survival of MCs, a function requiring CysLT1R, extracellular nucleotides, LTC4 synthase (LTC4S), and the intact functions of P2Y6 receptors. LTD4-mediated MIP-1β production by LAD2 cells is strikingly attenuated by knockdown or blockade of P2Y6 receptors, by depletion of extracellular nucleotides, and by inhibition of LTC4S synthesis. Collectively, these observations suggest that P2Y6 receptors are components of CysLT1R-dependent inflammatory signaling in MCs and that the two receptors cross-talk through their respective endogenous ligands to enhance cell activation and survival.
Flow cytometry

Surface CysLT1R and CysLT2R were detected in unpermeabilized cells using polyclonal Abs RB34 and RB19, respectively, directed against extracellular loops of the respective GPCRs (32). P2Y6 receptor protein was detected on permeabilized cells with polyclonal Ab RB34 (as the donor fluorophore). Positive controls were cells infected of 10, based on preliminary experiments in which FACS analysis virus was derived as described elsewhere (10). Viral stocks were added frame with a lentiviral vector (pLKo1; Open Biosystems), and infectious GCAAATATGAAGGCTGCTTTTT. Each hairpin sequence was cloned in TGTCTTAT TCTCGAGAATAAGACATAATCCTGCAGGTTTTT; and GTACATAAGTCACGCTTTTT; CysLT 2R, CCGGCCTGCAGGATTA TGTCTTAT TCTCGAGAATAAGACATAATCCTGCAGGTTTTT; and P2Y6 receptor, CCGGGCAGCTTATCCATTCCGTGACAAATATGAAGGCTGCTTTTT. Each hairpin sequence was cloned in frame with a lentiviral vector (pLKO1; Open Biosystems), and infectious virus was derived as described elsewhere (10). Viral stocks were added directly to the medium at a quantity sufficient to achieve a multiplicity of infection of 10, based on preliminary experiments in which FACS analysis was used to assess the effect of the transfection on the expression of the target receptors.

Immunostains and confocal imaging

LAD2 cells were fixed in a suspension with 2% paraformaldehyde in PBS on ice for 10 min. The cells were washed once with wash buffer (PBS containing 0.1% sodium azide) and resuspended in blocking buffer (PBS containing 5% horse serum), immobilized on round 12-mm cover slips by cytocentrifugation, and postfixed with methanol at −20°C for 10 min. The slides were washed twice with wash buffer and then blocked with blocking buffer, with shaking at room temperature for 30 min. The cells were stained with primary Abs that were labeled directly with Alexa Fluor 488 or Cy3 according to the manufacturer’s specifications. The cells were counterstained with DRAQ5 nuclear stain (1/1000; Biostatus). Anti-CysLT1R (RB34) and polyclonal anti-P2Y6 receptor Abs were used at 5 μg/ml and species-matched control IgG was used at the same concentration. Subcellular localization of the receptors was assessed using a Nikon TE2000-U inverted microscope with a Nikon C1 plus laser-scanning confocal system as detailed elsewhere (10).

FLIM analysis

The cells were fixed and stained for FLIM analysis as described above for confocal imaging, except for the omission of the nuclear stain. Cells were double stained with anti-C-terminal CysLT1R Ab directly labeled with Cy3 (as the acceptor fluorophore) and anti-P2Y6 receptor Ab labeled with Alexa Fluor 488 (as the donor fluorophore). Positive controls were cells stained with primary rabbit anti-human α5 integrin Ab and mouse anti-human β1 integrin Ab, followed by counterstaining with secondary anti-rabbit-Alexa Fluor 488 (donor) and anti-mouse-Cy3 (acceptor) conjugates at a concentration of 1 μg/ml each. Additional experiments were performed with directly labeled primary Abs (data not shown). Alexa Fluor 488 and Cy3 were chosen because the excitation spectrum of Cy3 overlaps with the emission spectrum of Alexa Fluor 488, making them a good pair for dem- onstrating fluorescence resonance energy transfer (FRET). Primary Ab was omitted from some samples as a control. Images were taken using 488- and 543-nm single-photon excitation to excite Alexa Fluor 488 and Cy3, re- spectively (Zeiss LSM510/NLO). Emissions were separated in multitrack mode. FLIM was performed using a femtosecond-pulsed Ti:Sapphire laser (Chameleon; Coherent), with a multichannel plate photon counting detector (Hamamatsu). A time-correlated single-photon counting board and software (SPC830; Becker and Hickl) was used for acquisition, and images were analyzed using SPC Image software (version 2.6.1.2711; Becker & Hickl) with mono- and bi-exponential lifetime curve fits (38, 39). At least three fields were analyzed for each condition in each experiment. Lifetimes were recorded as whole-cell measurements. FRET is indicated by shortening of the donor fluorophore’s lifetime and will occur if the two fluorophores are within 10 nm of each other. The lifetime of the donor fluorophore (Alexa Fluor 488) was measured in the absence of the acceptor fluorophores (negative control) in each experiment.

Statistical methods

Data are expressed as mean ± SEM from at least three experiments, except where otherwise indicated. Differences between treatment groups were de- termined with the Student t test. For the FRET studies, differences in life- times were assessed by ANOVA, with a Bonferroni analysis to correct for multiple comparisons.

Results

Human and mouse MCs express P2Y6 receptor protein

To determine whether MCs expressed P2Y6 receptors, we devel- oped an anti-epitope polyclonal Ab (RB165) against a sequence corresponding to the second intracellular loop of the mouse and human P2Y6 receptor. After purification, the Ab was used to stain Chinese hamster ovary (CHO) cells stably expressing human P2Y6 receptors (8) and the parent cell line as positive and negative controls, respectively. The same Ab was used in SDS-PAGE immu- noblots to detect P2Y6 receptor protein in lysates. The Ab detected a strong 34-kDa band in lysates of CHO cells transfected with human P2Y6 cDNA, corresponding to the published size of the P2Y6 receptor protein in intestinal epithelial cells (26) (Fig. 1A). The band was weakly detectable in some samples of the parent CHO cell line, suggesting low-level endogenous expression. Permeabilized transfectedants displayed a robust signal for P2Y6 receptor protein on FACS compared with the untransfected parent line (Fig. 1A). Primary hMCs, LAD2 cells, and mBMMCs all expressed P2Y6 receptor protein as determined by FACS analysis with RB165 (Fig. 1B). Confocal imaging of LAD2 cells (Fig. 1C) and mBMMCs (data not shown) revealed that P2Y6 receptor protein localized mainly to plasma membrane (Fig. 1C, arrows), particularly within cytoplasmic protrusions (microprotrices).
ERK activation in response to LTD₄ (500 nM) was diminished by MRS2578 (1 μM; Fig. 2, C and E), whereas phosphorylation induced by UDP (1000 nM) was completely blocked. Neither LTD₄ (500 nM) nor UDP (1000 nM) could induce ERK activation in mBMMCs from CysLT₁R-null or from LTC₄S-null mice (Fig. 2, D and E), whereas both ligands elicited responses from CysLT₂R-null mBMMCs that were similar to those observed in the wild-type cells (Fig. 2, C–E).

UDP and cys-LTs support survival of MCs through CysLT₁R-P2Y6 receptor cooperation and endogenous cys-LTs

CysLT₁R-mediated ERK phosphorylation promotes proliferation of MCs (30). ERK signaling is also essential for the survival of MCs in vivo. To determine whether LTD₄ and UDP could enhance the survival of MCs and whether these effects involved cooperation between P2Y6 receptors, CysLT₁R, and endogenously produced cys-LTs, mBMMCs were withdrawn from IL-3 and maintained in medium supplemented with SCF (10 ng/ml) as a positive control, UDP (0.01, 0.1, or 1 μM), or LTD₄ (100 or 500 nM). Entry into apoptosis was monitored by annexin V staining 48 h later. At the highest doses tested, both LTD₄ and UDP substantially attenuated apoptosis of wild-type mBMMCs (Fig. 3, A and B). LTD₄- and UDP-mediated cytoprotection were abolished in mBMMCs lacking CysLT₁R or LTC₄S (Fig. 3, A and B). CysLT₂R-null mBMMCs were not different from wild-type cells in their cytoprotective responses to LTD₄ or UDP. In wild-type

FIGURE 1. Development of an anti-P2Y6 receptor Ab and expression of P2Y6 receptor protein by MCs. A rabbit Ab (RB165) was developed against a conserved peptide within the human and mouse P2Y6 receptor second intracellular loop. A, SDS-PAGE immunoblot (top) showing recognition of a 34-kDa band in lysates of CHO cells stably expressing the human P2Y6 receptor. Flow cytometry (bottom) of the permeabilized CHO cells using the same Ab. Staining of the transfectant (CHO-P2Y6) is shown in the unshaded histogram. Stain of the same cells with isotype control IgG is superimposable with the P2Y6 receptor Ab stain of the parent cell line (CHO, shaded histogram). B, Flow cytometry of permeabilized primary hMCs, LAD2 cells, and mouse mBMMCs showing staining by RB165. The unshaded histogram is the stain with RB165. The isotype controls are shaded. C, Confocal imaging of LAD2 cells with Cy3-labeled RB165. Note prominent plasma membrane staining (arrows). Results in A–C are from experiments repeated at least three times.

FIGURE 2. Involvement of endogenous uracil nucleotides in LTD₄/CysLT₁R-dependent signaling events in MCs. A, Effect of the CysLT₁R antagonist MK571 (1 μM) and the P2Y6 receptor antagonist MRS2578 (at the indicated doses) on LTD₄ (500 nM)-mediated calcium flux in LAD2 cells. DMSO was used as the buffer control for the MRS2578. B, Lack of effect of MRS2578 (10 μM) on LTD₄-induced calcium flux in CHO cells stably expressing human CysLT₁R. C, Phospho-ERK blots showing effect of 15 min of stimulation of wild-type (WT) mBMMCs with the indicated agonists in the absence or presence of MRS2578 (1 μM; top). D, The effect of the same stimuli as in C on mBMMCs from the indicated genotypes. E, Quantitative densitometry showing the effects of MRS2578 (1 μM) and the null alleles on LTD₄- and UDP-induced ERK activation at the highest ligand concentrations (500 and 1000 nM, respectively). Results in A–D are representative of three experiments for each assay. E is the mean ± SEM from three experiments, including those displayed in C and D, each using cells from different cohorts of mice.
mBMMCs, both UDP- and LTD₄-mediated cytoprotection were sensitive to blockade of P2Y6 receptors by MRS2578 (1 μM; Fig. 3, C and D).

**LTD₄-induced MIP-1β generation requires extracellular nucleotides and P2Y6 receptors**

We had previously reported that UDP-mediated signaling and cytokine generation by hMCs was sensitive to blockade by MK571 (8, 30). Because chemokine generation by MCs in response to LTD₄ requires both calcium- and ERK-dependent transcriptional mechanisms (30) and because interference with P2Y6 receptors blunted ERK signaling in response to LTD₄ (Fig. 2), we sought to clarify whether P2Y6 receptors regulated CysLT₁R-dependent MIP-1β production by MCs and whether this response involved endogenous nucleotides. LAD2 cells were primed with IL-4 for 72 h to augment their generation of cytokines (30) and were treated

**FIGURE 3. LTD₄- and UDP-induced cytoprotection.** Cytokine-starved mBMMCs grown from mice of the indicated genotypes were maintained for 2 days in medium alone, SCF (10 ng/ml), LTD₄ (500 nM), or UDP (1 μM). A, Flow cytometry showing apoptotic cells (expressed as a percentage of total cells) quantified by cytofluorographic detection of annexin V binding. The percentages of cells exhibiting positive staining are noted for each histogram. The experiment depicted was repeated four times with similar results. B, Mean data from four experiments (including the one in A) showing the effect of SCF (10 ng/ml) or the indicated concentrations of UDP or LTD₄ on the survival of mBMMCs from mice with the indicated genotypes. Results are mean ± SEM. Values of p are indicated. C, Effect of the P2Y6 receptor-selective antagonist MRS2578 (1 μM) on survival of wild-type (WT) mBMMCs treated with the indicated conditions. Representative histograms are shown from one experiment, which is included in the mean data from three experiments performed using cells from three different animals (D).

**FIGURE 4.** Involvement of P2Y6 receptors in CysLT₁R-dependent chemokine generation. A, Cytofluorographic analyses of LAD2 cells subjected to transfection with the indicated shRNAs to achieve knockdown (KD) of CysLT₁R and P2Y6 receptors. CysLT₁R was stained in unpermeabilized cells using the polyclonal Ab RB34, raised against the third extracellular loop of this receptor. P2Y6 receptors are recognized by RB165 on permeabilized LAD2 cells. Data are representative of three experiments. B, Effect of the CysLT₁R, CysLT₂R, and P2Y6 knockdowns on MIP-1β generation by IL-4-primed LAD2 cells in response to the indicated stimuli. Results are the mean ± SEM from five different experiments. * p < 0.05 compared with vector control. C, Effect of nucleotide and cys-LT antagonists at the concentrations indicated in the text on LTD₄ (500 nM)- or UDP (1000 nM)-induced MIP-1β generation. Results are the mean ± SEM from four different experiments. * p < 0.05 compared with vehicle control.
with recombinant lentiviruses containing shRNAs directed toward the CysLT1R, CysLT2R, and P2Y6 receptor sequences, or with a control lentivirus without shRNA for the last 48 h of priming. FACS analyses confirmed the knockdown of the target proteins, without altering the nontarget receptors (as shown for CysLT1R, CysLT2R, and P2Y6 receptors, Fig. 4A). Stimulation of the empty vector-treated control cells with LTD₄-induced dose-dependent secretion of MIP-1β, exceeding the quantities generated in response to the positive control cells subjected to FcεRI cross-linkage. MIP-1β production in response to LTD₄ at 100 or 500 nM was abrogated by the knockdown of CysLT1R, but not knockdown of CysLT2R (Fig. 4B). Knockdown of P2Y6 receptors decreased production of MIP-1β in response to LTD₄ by >50% but did not interfere with FcεRI-mediated MIP-1β production (Fig. 4B). UDP tended to weakly induce MIP-1β production by itself at 1000 nM; this response was abrogated by knockdown of either CysLT1R or the P2Y6 receptor, but not by the CysLT2R knockdown.

To determine whether LTD₄-dependent MIP-1β production involved extracellular nucleotides or endogenous cys-LTs, IL-4-primed LAD2 cells were stimulated with LTD₄ in the presence or absence of suramin (100 μM), MRS2578 (1 μM), or apyrase (10 μM), an ectonucleotidase that degrades extracellular nucleotides, as well as MK886 (1 μM), an antagonist of LT synthesis. Treatment of the primed LAD2 cells with each of these agents blocked MIP-1β generation in response to LTD₄ (500 nM), as did MK571 (at 1 μM; Fig. 4C). None of these inhibitors blocked FcεRI-mediated MIP-1β generation.

Lack of direct interaction between CysLT₁R and P2Y6 receptors

To determine whether P2Y6 receptors colocalized with CysLT₁R, we performed confocal imaging of LAD2 cells that were double stained with RB34 and RB165 Abs directly conjugated to Alexa Fluor 488 and Cy3, respectively. CysLT₁R protein localized to the plasma membrane (Fig. 5A, left panel), as well as to the nuclear envelope and punctuate intranuclear structures as described previously (32). Double staining revealed colocalization of CysLT₁R and P2Y6 receptors primarily at the plasma membrane and within microplciae (Fig. 5A, middle panel, arrows). Double staining of the LAD2 cells with a monoclonal anti-β₁ integrin Ab and a polyclonal anti-α₄ integrin Ab showed the anticipated prominent colocalization at the plasma membrane (Fig. 5A, right panel, arrows). Multiphoton-based FLIM analysis (which reveals a shortening of the lifetime of the fluorescence emitted by Alexa Fluor 488 if the Cy3-conjugated Ab is within 10 nm of the donor fluorophore (39, 40)) showed no energy transfer between CysLT₁R and P2Y6 receptors (Fig. 5B, left and middle panels). In contrast, double staining for both α₄ and β₁ integrins showed strong energy transfer (Fig. 5B, right panel). These results suggest that despite the cellular (or membrane) colocalization of CysLT₁R and P2Y6, there is likely no direct interaction between these receptors.

Discussion

The results of this study demonstrate a bilateral functional interplay among the P2Y6 receptor, CysLT₁R, and their respective endogenous ligands. Nucleotides are released in large quantities in response to tissue injury, hypoxia, and other environmental danger signals (10). cys-LTs, generated by cells of the innate immune system (MCs, eosinophils, basophils, and macrophages) are the most potent known mediators of smooth muscle tone and permeability. cys-LT actions at CysLT₁R contribute substantially to bronchoconstriction during asthma attacks (40). Our previous studies suggested cross-talk between cys-LT- and UDP-mediated signaling events in primary hMCs (8, 30). Although we had suspected that a dual-selective cys-LT/UDP receptor was responsible for these effects, several other mechanisms of cross-talk between nucleotide and cys-LT systems have been described subsequently. These include reciprocal induction of extracellular ligand release (27) and heterologous protein kinase C-dependent desensitization of P2Y receptors by LTD₄ (41). Moreover, the fact that CysLT₁R antagonists can also broadly block P2Y receptor signaling (32) as well as sphingosine-1-phosphate transport (42) clouds interpretation of previous studies using these inhibitors to invoke mechanism. We thus sought to clarify the relationship between the cys-LT receptor system and UDP using molecular approaches.

Although human GPR17 and P2Y4 receptors recognize UDP at high concentrations when expressed in heterologous cell systems (21, 43), P2Y6 receptors are the only high-affinity, UDP-selective GPCR described thus far (22, 23). To determine whether human
and mouse MCs express this receptor, we raised an Ab against a conserved sequence of the second intracellular loop of P2Y6 receptors to detect both the mouse and human proteins. We verified that MCs from both species expressed P2Y6 receptors using flow cytometry (Fig. 1B). The specificity of the staining with the Ab was confirmed by its ability to recognize recombinant P2Y6 receptors expressed by transfected CHO cells (Fig. 1A) and by the abrogation of the FACS signal on MCs subjected to the knockdown procedure (Fig. 4A). Confocal imaging revealed prominent P2Y6 receptor staining of LAD2 cells (Fig. 1C). Thus like other cells of the innate immune system, MCs express P2Y6 receptors. Given that this receptor functions in autocrine signal amplification in other effector cell types (25, 26), we sought such functions in MCs following LTD4 stimulation (44), which is the highest affinity ligand for CysLT1R.

Nucleotides released by MCs in a regulated fashion after activation can propagate FceRI-mediated calcium signaling from cell to cell (12). Since there is no accurate method to measure UDP in extracellular fluids, we performed calcium flux assays in the presence of the selective P2Y6 receptor antagonist MRS2578 and the CysLT1R antagonist MK571. The complete blockade of the LTD4-mediated calcium flux by MK571 is consistent with a CysLT1R-dependent event (Fig. 2A), but does not preclude the involvement of extracellular nucleotides since this reagent can also block P2Y receptors. MRS2578 at 1 μM (a dose sufficient to completely block signaling through the recombinant P2Y6 receptors (34) did not alter calcium flux induced by LTD4, but did decrease the response to LTD4 when the concentration was increased to 10 μM (Fig. 2A). MRS2578 does not exert nonspecific actions at other P2Y receptors at this high dose (34), and the same dose of MRS2578 failed to alter calcium fluxes of recombinant CysLT1R-R in CHO cells (Fig. 2B), suggesting that it does not behave as a partial CysLT1R antagonist. We cannot completely exclude another potential off-target effect of MRS2578 in these experiments, but the results support a potential role for endogenous uracil nucleotides in regulating calcium signaling through CysLT1R and CysLT2R (31). We confirmed that CysLT1R and CysLT2R (31) is consistent with GPCR oligomerization.

Although mBMMCs do not exhibit the robust cys-LT-induced calcium fluxes of hMCs or LAD2 cells, they do respond to LTD4 with potent phosphorylation of ERK that depends on CysLT1R-dependent transactivation of the c-Kit receptor tyrosine kinase (29). We previously demonstrated that UDP, like LTD4, also induces ERK activation in primary hMCs (30). We found that UDP caused MRS2578-sensitive ERK phosphorylation, indicating a functional response mediated by P2Y6 receptors (Fig. 2C). At a low dose (1 μM), MRS2578 decreased the LTD4-dependent ERK signal (Fig. 2, C and E). It is thus likely that endogenous UDP released by stimulation through CysLT1R amplifies the subsequent phosphorylation of ERK through P2Y6 receptors. As anticipated, mBMMCs from CysLT1R-null mice (but not from CysLT2R-null mice) showed no LTD4-mediated ERK activation. Unexpectedly, however, UDP also failed to induce ERK activation in the absence of CysLT1R and in the absence of LTC4S (Fig. 2, D and E). Although UDP and LTD4 do not induce the release of cys-LTs from MCs in concentrations sufficient to be detected in the medium (30), these findings suggest that both stimuli induce the formation of intracellular LTC4. Because LTC4S is integral to the nuclear envelope (44), while CysLT1R localizes to both the cell surface and nucleus (31), endogenous LTC4 could either serve a synaptic function at the cell surface or could activate intracellular receptors.

ERK signaling is important for MC survival and UDP can act through P2Y6 receptors to promote survival in some cell types (23). We investigated whether LTD4 or UDP would protect cytokine-starved MCs from apoptosis, whether this response also involved reciprocal cross-regulation among CysLT1R, P2Y6 receptors, and endogenous cys-LTs. As was the case for the experiments involving ERK activation, we used mBMMCs to take advantage of cells with deletions of the relevant genes. As anticipated, SCF protected cells from apoptosis irrespective of genotype. Cytoprotection conferred by either LTD4 or UDP required the presence of CysLT1R and LTC4S, but not of CysLT2R (Fig. 3, A and B) and was blocked by interference with UDP signaling through P2Y6 receptors using MRS2578 (Fig. 3, C and D). Thus, the cross-talk among CysLT1R, LTC4S, and P2Y6 at the level of ERK activation (Fig. 2) is paralleled by effects on protection from apoptosis. Our previous study demonstrated that both LTC4S and CysLT1R expression were also necessary for IL-4, an accessory mitogen for MCs, to induce proliferation (29). The profound MC deficiency in the airway of allergen-challenged mice lacking LTC4S (45) could thus reflect a requirement for endogenous cys-LTs to facilitate cell survival and/or proliferation in circumstances where local concentrations of SCF, the obligate MC growth factor, are limited.

MC-derived chemokines play a key role in recruiting blood-borne effector cells during immune responses (46). We had previously reported that cys-LTs and UDP could induce chemokine generation by primary hMCs through an IL-4-dependent pathway (30), the latter being essential to amplify ERK activation (47). Because mBMMCs do not generate abundant chemokines when stimulated with cys-LTs (data not shown), we used LAD2 cells for these experiments and manipulated the key receptors using shRNA knockdowns. IL-4-primed LAD2 cells were stimulated with LTD4 or UDP after transfection with the shRNAs. The abolition of MIP-1β production in response to LTD4 by the knockdown of CysLT1R (Fig. 4B), but not by the CysLT2R knockdown, confirms the essential requirement for CysLT1R in chemokine production that had been inferred from earlier pharmacologic studies (30). Strikingly, P2Y6 receptor knockdown substantially decreased the generation of MIP-1β in response to LTD4, but not to FceRI cross-linkage (Fig. 4B). The effect was not due to an off-target effect of the P2Y6 knockdown on CysLT1R expression levels or vice-versa (Fig. 4A). Combined with the marked inhibition of LTD4-dependent MIP-1β production by MRS2578, suramin (a nonselective P2Y receptor antagonist), and depletion of extracellular nucleotides by apyrase (Fig. 4C), these studies indicate that P2Y6 receptors respond to UDP at the plasma membrane after its CysLT1R-mediated entry into the cell (30). Moreover, the fact that MK886 also abrogated the response to LTD4 indicates that endogenous cys-LTs are required for chemokine generation induced by exogenous LTD4, as was the case for ERK activation (Fig. 2) and cytoprotection (Fig. 3).

Structurally homologous GPCRs form multimers that function as signaling units (48–52), as we had demonstrated previously for CysLT1R and CysLT2R (31). We confirmed that CysLT1R and P2Y6 colocalized at the plasma membranes of LAD2 cells (Fig. 5A), but Ab-based FLIM, a sensitive method for detecting protein-protein interactions in primary cells, showed no evidence for a direct interaction between CysLT1R and P2Y6 receptors (Fig. 5B). The colocalization of CysLT1R, P2Y6 receptors, perhaps within lipid microdomains of the plasma membrane, may facilitate some of the synergy of their respective signaling events, particularly if LTD4 elicits UDP release in close proximity to CysLT1R at the plasma membrane. Since the FLIM assay requires that the Abs be directed against epitopes on the respective GPCRs that are in close proximity to detect oligomerization, we cannot completely exclude the existence of CysLT1R-P2Y6 receptor oligomers. However, the lack of a FRET signal in the same setting where CysLT1R and CysLT2R heterodimerizes (31) is consistent with GPCR oligomerization being tightly regulated and specific.
Our study indicates that CysLT₁R and P2Y6 receptors orchestrate a coordinated, complementary series of signaling events in MCs involving their respective endogenous ligands. Given its subcellular distribution, it is likely that the P2Y6 receptor responds primarily to extracellular UDP at the cell surface released in response to CysLT₁R-dependent MC activation. The requirement for endogenous cysteinyl leukotrienes and CysLT₁R for cytoprotection and cytokine generation could reflect synaptic signaling functions at the cell surface or possibly at intracellular sites. The interplay between these receptors could extend to smooth muscle, intestinal epithelial cells, macrophages, and other hematopoietic effectors that express both (53–55). The relevance of this interplay in vivo to determine the role for P2Y6 receptors in fibrosis, remodeling, and CysLT₂ receptor expression remains to be determined. It is important to note that uracil nucleotides and cysteinyl leukotrienes are both implicated in proliferation and/or cytoprotection of epithelial cells and smooth muscle (56, 57). Given the established role of cysteinyl leukotrienes in asthma, it is also noteworthy that bronchoalveolar lavage fluids from allergen-challenged asthmatic individuals contain high levels of extracellular nucleotides and that experimental allergen-induced bronchial inflammation in mice is profoundly inhibited by suramin treatment (58). It will be essential to determine the role for P2Y6 receptors in fibrosis, remodeling, cardiovascular disease, and other pathologic processes in which MCs and cysteinyl leukotrienes are implicated as effectors to reveal their potential as therapeutic targets.

Acknowledgments
We acknowledge Chunli Feng for outstanding technical support.

Disclosures
The authors have no financial conflict of interest.

References