

Cysteinyl Leukotrienes and Uridine Diphosphate Induce Cytokine Generation by Human Mast Cells Through an Interleukin 4–regulated Pathway that Is Inhibited by Leukotriene Receptor Antagonists

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Abstract

We previously reported that interleukin (IL)-4 upregulates the expression of leukotriene C₄ synthase (LTC₄S) by human cord blood–derived mast cells (hMCs), augments their high-affinity Fc receptor for IgE (FcεRI)-dependent generation of eicosanoids and cytokines, and induces a calcium flux in response to cysteinyl leukotrienes (cys-LTs) and uridine diphosphate (UDP) that is blocked by cys-LT receptor antagonists. We speculated that this IL-4–dependent, receptor-mediated response to the cys-LTs and UDP might induce cytokine generation by hMCs without concomitant exocytosis. Unlike hMCs maintained in cytoprotective stem cell factor (SCF) alone, hMCs primed for 5 d with IL-4 responded to UDP (1 μM), LTC₄ (100 nM), and LTD₄ (100 nM) by producing IL-5, tumor necrosis factor (TNF)-α, and especially large quantities of macrophage inflammatory protein (MIP)-1β de novo at 6 h, preceded by the induced expression of the corresponding mRNAs. Cys-LT- and UDP-mediated cytokine production by the primed hMCs occurred without histamine release or PGD₂ generation and was inhibited by the CysLT1 receptor antagonist MK571. Additionally, pretreatment of hMCs with MK571 or with the cys-LT biosynthetic inhibitor MK886 decreased IL-5 and TNF-α production in response to IgE receptor cross-linkage, implying a positive feedback by endogenously produced cys-LTs. Cys-LTs and UDP thus orchestrate a novel, IL-4–regulated, non-IgE-dependent hMC activation for cytokine gene induction that could be initiated by microbes, cellular injury, or neurogenic or inflammatory signals; and this pathobiologic event would not be recognized in tissue studies where hMC activation is classically defined by exocytosis.

Key words: mast cells • cytokines • leukotrienes • uridine diphosphate • receptors

Introduction

Mast cells (MCs)* are bone marrow–derived immune effector cells that are prominent at the interfaces between the immune system and the external environment, such as the skin and the gastrointestinal and respiratory tracts. Rodent

and human MCs activated in vitro by cross-linkage of their high-affinity Fc receptor for immunoglobulin E (FcεRI) release preformed histamine and proteases from secretory granules; generate the arachidonic acid–derived eicosanoid mediators PGD₂ and cysteinyl leukotrienes (cys-LTs; for a review, see reference 1); and subsequently transcribe, translate, and secrete several proinflammatory cytokines and chemokines (2, 3). Eicosanoid generation is initiated by a calcium-dependent cytosolic phospholipase A₂ (cPLA₂) that liberates arachidonic acid from membrane phospholipids (4). For cys-LT generation, 5-lipoxygenase (5-LO) acts in concert with the 5-LO-activating protein (FLAP) (5) to convert arachidonic acid sequentially to the unstable intermediates 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and then leukotriene (LT)A₄ (6). In MCs, eosinophils, and

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*Abbreviations used in this paper: Cys-LT, cysteinyl leukotriene; CysLTR_{IL-4}, IL-4–regulated receptor-mediated response to Cys-LTs and UDP; ERK, extracellular regulated kinase; FcεRI, high affinity Fc receptors for IgE; FLAP, 5-lipoxygenase activating protein; hMCs, human mast cells; LT, leukotriene; LTC₄S, LTC₄ synthase; MC, mast cell; MIP, macrophage inflammatory protein; NF-AT, nuclear factor of activated T cells; SCF, stem cell factor; UDP, uridine diphosphate.

basophils, LTA_4 is conjugated to reduced glutathione to form LTC_4 by LTC_4 synthase (LTC_4S) (7–9), an integral nuclear membrane protein with homology to FLAP. LTC_4 is released by a distinct cellular export mechanism (10) and metabolized sequentially to LTD_4 by either extracellular γ -glutamyl transferase or γ -glutamyl leukotrienase (11, 12), and then by a dipeptidase to LTE_4 (13). LTC_4 , LTD_4 , and LTE_4 then act through at least two 7 transmembrane-spanning G protein-coupled receptors, termed the CysLT1 and CysLT2 receptors (14, 15). The bronchoconstriction (16) and alterations in venular permeability (17) mediated by the cys-LTs reflect their effects at smooth muscle- and microvasculature-associated receptors. Cys-LT receptors are also expressed by hematopoietic cells, such as eosinophils, basophils, monocytes, and CD34-bearing progenitor cells (14, 15, 18, 19). They have been implicated in leukocyte recruitment on the basis of responses to cys-LTs in vitro (19) and in vivo (20). However, whether cys-LT receptors on hematopoietic immune effector cells might serve additional proinflammatory functions is not known.

The culture of cord blood mononuclear cells for 6–9 wk with recombinant stem cell factor (SCF), IL-6, and IL-10 results in the development of pure, mature human MCs (hMCs) (21). When these cells were primed for 5 d with recombinant IL-4 (10 ng/ml) in the continued presence of SCF, their IgE-dependent exocytosis and PGD_2 generation increased by four- and twofold, respectively, compared with replicates maintained in SCF alone (22). However, IL-4 augmented the cys-LT-generating capability of hMCs by more than 25-fold, due to a profound induction of LTC_4S transcript, protein, and function (22). The unprimed hMCs expressed both CysLT1 receptor mRNA and protein and responded to either LTC_4 or LTD_4 with a sustained calcium flux that was completely blocked by the selective CysLT1 receptor antagonist MK571 (23). In contrast, BAY-u9773, which desensitizes the CysLT2 receptor and blocks the CysLT1 receptor (24), elicited a small calcium flux and partly blocked calcium flux in response to LTC_4 but not LTD_4 . Priming with IL-4 markedly enhanced the sensitivity of hMCs to exogenous cys-LTs, with the EC_{50} for MK571-sensitive calcium flux in response to LTC_4 being decreased by 3.8 logs and that to LTD_4 by 1.3 logs. Furthermore, the IL-4-induced sensitivity of hMCs to LTC_4 was accompanied by a parallel enhancement of their sensitivity for calcium flux to a nucleotide agonist, uridine diphosphate (UDP), which was also blocked by MK571. LTC_4 and UDP cross-desensitized IL-4-primed hMCs to their respective calcium fluxes, whereas UDP did not desensitize hMCs to LTD_4 . These IL-4-induced changes in the sensitivity of hMCs to LTC_4 and UDP occurred without obvious changes in the levels of CysLT1 receptor protein or transcript, and without inducing the expression of the CysLT2 receptor (23). The augmented receptor-mediated response of hMCs primed with IL-4 to cys-LTs and UDP, termed CysLTR_{IL-4}, could reflect the induction of a previously unrecognized receptor gene, or the posttranslational modification of the conventional CysLT1 receptor.

The observations that IL-4 priming of hMCs increased both the expression of LTC_4S (22) and the receptor-mediated responses of these cells to cys-LTs prompted us to seek a feedback effect. Because IL-4 also primes hMCs for augmented Fc ϵ RI-mediated cytokine generation (2, 25), we speculated that CysLTR_{IL-4} might mediate ligand-induced cytokine gene expression. In this study, we demonstrate that the ligands for CysLTR_{IL-4} (cys-LTs and UDP) induce de novo expression and secretion of the proinflammatory cytokines IL-5, macrophage inflammatory protein (MIP)-1 β , and TNF- α by hMCs. Cytokine generation induced by cys-LTs and UDP is sensitive to inhibition by MK571, occurs without histamine release or PGD_2 generation, and involves de novo nuclear factor of activated T cells (NF-AT)-dependent transcription. The findings link hMCs and cys-LTs to a novel receptor-mediated pathway for cytokine gene expression that is modulated by IL-4, intersects ligands of the cys-LT and pyrimidinerbic classes, and functions independently of exocytosis. This pathway could be involved in MC-mediated amplification of innate as well as adaptive immune responses.

Materials and Methods

Culture, Priming, and Stimulation of hMCs. hMCs were derived from human umbilical cord blood as described (21). Briefly, cord blood was obtained from human placentas after routine caesarian sections in accordance with established institutional guidelines. Whole heparin-treated cord blood was sedimented with 4.5% dextran solution to remove erythrocytes. The buffy coats were layered onto 1.77 g/L Ficoll-Hypaque (Amersham Pharmacia Biotech), and mononuclear cell interfaces were obtained after centrifugation. Residual erythrocytes were removed by hypotonic lysis. The remaining mononuclear cells were suspended in RPMI 1640 (GIBCO BRL) containing 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 μ g/ml gentamycin (all from Sigma-Aldrich), and 0.2 μ M 2-me (GIBCO BRL). Cells were seeded at a concentration of 10^6 cells/ml and were cultured in the presence of 100 ng/ml SCF (Amgen), 50 ng/ml IL-6 (R&D Systems), and 10 ng/ml IL-10 (Endogen). The nonadherent cells were transferred every week for up to 9 wk into culture medium containing fresh cytokines. Cytospin preparations were examined weekly from samples of 2×10^4 cells with a cytocentrifuge (Shandon) and were stained with toluidine blue to assess metachromasia. hMCs were harvested for study when >95% stained positively with toluidine blue (typically 6–9 wk).

For stimulation, cultures of hMCs were washed extensively and resuspended at a concentration of 10^6 /ml fresh medium containing SCF (100 ng/ml) alone or with recombinant IL-4 (10 ng/ml). After 4 d, human myeloma IgE (2 μ g/ml) was added. The next day, the cells were again washed and resuspended at a concentration of 0.5×10^6 /ml in fresh medium containing SCF (100 ng/ml). They were then distributed to the wells of 96-well flat-bottom microtiter assay plates in samples of 10^5 cells for stimulation in triplicate at 37°C with one of the following agonists: LTC_4 or LTD_4 (10^{-7} M; both from Cayman Chemical), UDP (10^{-6} M; Sigma-Aldrich), or a rabbit anti-human IgE Ab (1 μ g/ml; Calbiochem). The final concentrations of the agonists were chosen on the basis of the doses necessary to elicit maximal generation of each cytokine from IL-4-primed hMCs and were the

same concentrations that elicited maximal calcium fluxes in our previous study (23). Medium alone was added to some wells as a negative control. In the initial experiments, serine borate (50 μM) was included in the samples treated with LTC_4 to inhibit its conversion to LTD_4 . This step was subsequently omitted because the cytokine quantities detected in the LTC_4 -stimulated samples were the same with or without serine borate and because HPLC of LTC_4 -stimulated hMC supernatants contained no detectable LTD_4 , indicating that no conversion had occurred. Some cell samples were preincubated for 1 min before ligand addition with the following inhibitors: MK571 (1 or 10 μM), a competitive antagonist of the CysLT1 receptor (14); MK886 (1 μM), an inhibitor of FLAP (26) and LTC_4S (27) (both from Cayman); the selective MEK inhibitor UO126 (5 $\mu\text{g/ml}$, Promega) (28); and the calcineurin inhibitor FK506 (29) (10 ng/ml; Fujisawa Health Care). For histamine release and eicosanoid production, the reactions were terminated at 30 min. Both supernatants and pellets were collected for measurement of histamine to allow calculation of percent release (2). For cytokine generation, the reactions were terminated at 6 h, the time at which the highest levels of each cytokine were detectable. All samples were frozen at -20°C until analysis. The quantities of cytokines were determined for each sample with commercial ELISA kits (Endogen) with the manufacturer's protocol. PGD_2 , histamine (Cayman), and cys-LTs (Amersham Pharmacia Biotech) were each measured by ELISA. The minimal quantities of cytokines, released histamine, and PGD_2 detected in the sham-stimulated samples were subtracted from the other samples in each experiment, and the data were expressed as the net production in response to each agonist based on the mean of the replicate experiments indicated in the text.

Steady-State RNA Analysis. Preliminary experiments revealed that each agonist elicited maximal steady-state levels of each cytokine at 2 h, and this was therefore chosen as the harvest point in each subsequent experiment. Total RNA was extracted from 10^7 cells with TRI-reagent according to the manufacturer's protocol (Molecular Research Center). After extraction with chloroform followed by overnight precipitation in isopropanol, total RNA was washed with 70% ethanol, and purity was assessed by spectrophotometry (Beckman DU 640; Beckman Coulter). Then, 10–15 μg of total RNA was loaded into a 1.2% agarose gel with $1\times$ MOPS (3-[N-morpholino] propan-sulfonic acid) buffer containing 20% formaldehyde. After electrophoresis, RNA was transferred to nylon membranes (Micron Separations) by capillary action overnight. RNA was fixed to the membrane by baking at 80°C for 1 h. The blot was prehybridized in $5\times$ standard sodium phosphate with EDTA (SSPE; GIBCO BRL) containing $2\times$ Denhardt's solution, 0.25% SDS, 50% formamide, and 100 $\mu\text{g/ml}$ denatured salmon sperm DNA (GIBCO BRL) overnight at 43°C . The blot was then probed with previously described probes for human IL-5 and TNF- α (2). A MIP-1 β probe was developed by PCR from hMC RNA that had been reverse-transcribed with a commercial kit (CLONTECH Laboratories, Inc.). Briefly, 10 pmol each of a sense strand (5'-CACACGAATCAAATGTGTTATCC-3') and an antisense strand (5'-AAGCTTCTGAGTTCTGCAGCCT-3') primer were added to a 50- μl reaction mixture containing 10 μl of diluted reverse-transcribed RNA, $1\times$ PCR buffer containing 1.5 mM MgCl_2 , 200 μM dCTP, dTTP, dGTP, and dATP, (PerkinElmer), and 2.5 U of Taq polymerase (PerkinElmer). The appropriately sized band was visualized on an ethidium bromide-stained 1.5% agarose gel after 30 cycles of amplification in an automated thermal cycler (PerkinElmer) with the following param-

eters: 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The identity of the probe was subsequently confirmed by automated sequencing (Dana Farber Cancer Institute Molecular Biology Core Facility). The blots were also probed with a human 18S RNA probe (CLONTECH Laboratories, Inc.). Each probe was randomly labeled with [^{32}P]dCTP with RediPrime II (Amersham Pharmacia Biotech). Blots were washed at high stringency with $0.2\times$ SSPE at 55°C , and hybridization signals were detected by autoradiography with Kodak XAR film (Eastman Kodak Co.). Because of a low to negligible signal on RNA blots, reverse transcription (RT)-PCR was used to detect steady-state TNF- α mRNA, with 35 cycles of amplification under the conditions described above with specific commercial primers (CLONTECH Laboratories, Inc.).

SDS-PAGE Immunoblot Analysis. Samples of 2×10^5 hMCs were stimulated for various intervals with LTD_4 , LTC_4 (10^{-7} M each), or UDP (10^{-6} M) in the presence or absence of UO126 (5 $\mu\text{g/ml}$). The reactions were stopped on ice at the appropriate times and the cells were lysed in a buffer containing 1% SDS, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 5 $\mu\text{g/ml}$ leupeptin, and 1 $\mu\text{g/ml}$ pepstatin in 10 mM Tris (pH 8.0). The samples were loaded with an equal volume of Tris-glycine-SDS loading buffer (Novex) onto precast 12% Tris-glycine-SDS gels, and the proteins were separated by electrophoresis at 30 mA and 150 mV. After overnight transfer to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories), the blots were blocked for 1 h in $1\times$ Tris-buffered saline with 0.5% Tween-20 (Bio-Rad Laboratories; TTBS) containing 3% nonfat dry milk and 0.25% normal goat serum, and incubated in the same buffer containing 1:2,000 dilutions of anti-ActiveTM extracellular regulated kinase (ERK), c-Jun NH₂-terminal kinase (JNK), and p38 mitogen-activated protein (MAP) kinase antibodies (Promega) for 2 h at room temperature. After extensive washing in TTBS, the blots were incubated again for 1 h in blocking buffer with a 1:5,000 dilution of a goat anti-rabbit horseradish peroxidase-conjugated secondary Ab (Bio-Rad Laboratories). The blots were washed again in TTBS and bands were detected using enhanced chemiluminescence (ECL). The same blots were stripped and probed again with rabbit polyclonal antibodies that detect total ERK, p38, and JNK at dilutions of 1:2,000 (Cell Signaling Technologies). Maximal phosphorylation of ERK was observed at 5 min, and this was chosen as the harvest time for subsequent experiments.

Statistics. The net data from triplicate samples were calculated for each experiment and were expressed as means \pm SEM. Differences between groups were compared with the paired Student's *t* test, and $P \leq 0.05$ was considered significant.

Results

Cytokine (IL-5, MIP1- β , TNF- α) Generation by hMCs With and Without Priming by IL-4. hMCs that were primed for 5 d with IL-4 in the presence of SCF or were maintained in SCF alone were stimulated with a range of concentrations of cys-LTs (10^{-7} – 10^{-9} M) or UDP (10^{-6} – 10^{-9} M) for 6 h. Unprimed hMCs did not produce either IL-5 or TNF- α when treated with LTD_4 or LTC_4 at doses of 10^{-9} – 10^{-7} M, or in response to UDP at doses up to 10^{-6} M ($n = 3$ for each cytokine). In contrast, hMCs primed with IL-4 generated IL-5 in response to the highest tested doses of LTC_4 , LTD_4 , and UDP (29 ± 7 , 38 ± 9 , and 11 ± 2 pg/ 10^6 hMCs, respectively; Fig. 1 A, $n = 5$ for

each agonist). The same three ligands also induced the generation of TNF- α (16 ± 10 , 22 ± 9 , and 31 ± 10 pg/ 10^6 hMCs, $n = 3$ for each agonist) by the IL-4-primed hMCs. In the unprimed hMCs, LTC₄ and LTD₄ each induced the generation of MIP-1 β (622 ± 286 pg and 508 ± 350 pg/ 10^6 hMCs, respectively, $n = 5$) at the highest doses tested, while UDP induced MIP-1 β production at doses of 10^{-7} M (670 pg/ 10^6 hMCs, not shown), and 10^{-6} M ($2,836 \pm 990$ pg/ 10^6 hMCs; Fig. 1 A, $n = 5$). MIP-1 β production by IL-4-primed hMCs was induced by all three agonists at doses as low as 10^{-9} M and was substantially enhanced in response to the highest agonist concentrations used ($2,428 \pm 670$, $3,018 \pm 848$, and $4,572 \pm 1,660$ pg/ 10^6 hMCs in response to LTC₄, LTD₄, and UDP, respectively, $P = 0.02$, 0.11 , and 0.005 compared with unprimed conditions, $n = 4$ for each agonist; Fig. 1 A).

2 h after stimulation, LTC₄, LTD₄, and UDP each induced increases in the steady-state levels of mRNA encoding

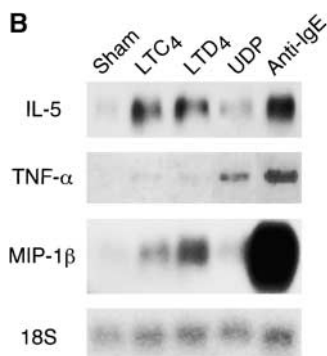
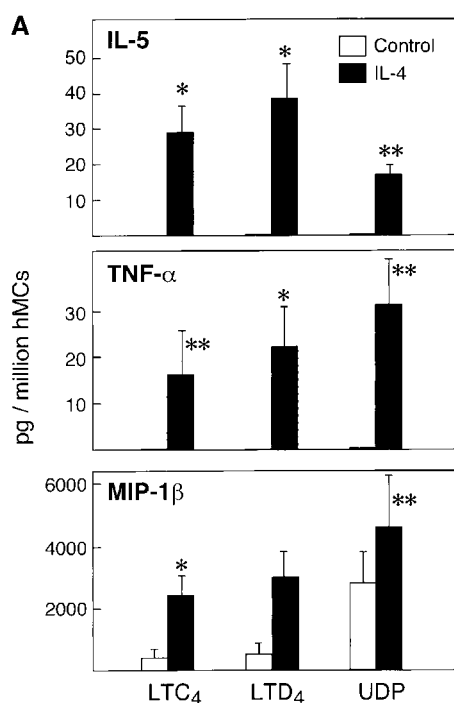


Figure 1. (A) Effect of IL-4 priming on cys-LT- and UDP-mediated cytokine generation by hMCs. P values reflect increases in ligand-induced product due to IL-4 priming (black bars) relative to the unprimed replicates (white bars). Results are based on $n = 3$ for TNF- α and $n = 5$ for both MIP-1 β and IL-5. * indicates $P \leq 0.05$ and ** indicates $P < 0.01$. (B) Steady-state levels of mRNA encoding IL-5 and MIP-1 β (by RNA blot) and TNF- α (by RT-PCR). The displayed signals are from a single experiment representative of four (for IL-5 and MIP-1 β) and three (for TNF- α) performed.

ing TNF- α , IL-5, and MIP-1 β compared with the levels detected in the sham controls. The IL-5 and MIP-1 β hybridization signals induced by LTC₄ and LTD₄ were generally equivalent in intensity ($n = 4$, as shown for one experiment; Fig. 1 B), and usually exceeded the UDP-induced signals. In contrast, UDP-induced TNF- α mRNA signals were consistently stronger than those elicited by cys-LTs as detected by RT-PCR ($n = 3$, as shown for one experiment; Fig. 3 B). The signals induced by each agonist were lower than those generated in response to IgE receptor cross-linkage ($n = 3$, as shown for one donor; Fig. 1 B).

Effect of MK571 on Cytokine Generation by Primed hMCs. To define the class of the receptors mediating the induction of cytokine expression in response to cys-LTs and UDP, the IL-4-primed hMCs were treated for 1 min with MK571 before ligand was added. 10-fold molar excesses of MK571 ($1 \mu\text{M}$) blocked MIP-1 β generation in response to 10^{-7} M LTC₄ and LTD₄ by $\sim 90\%$ (Fig. 2; $P = 0.07$ and 0.03 , respectively, $n = 6$). The same concentration of MK571 also partly and significantly blocked UDP-induced MIP-1 β production (31% inhibition, $P = 0.05$; Fig. 2, $n = 6$), and blocked UDP-induced MIP-1 β generation to a greater extent at $10 \mu\text{M}$ (75% inhibition; Fig. 2,

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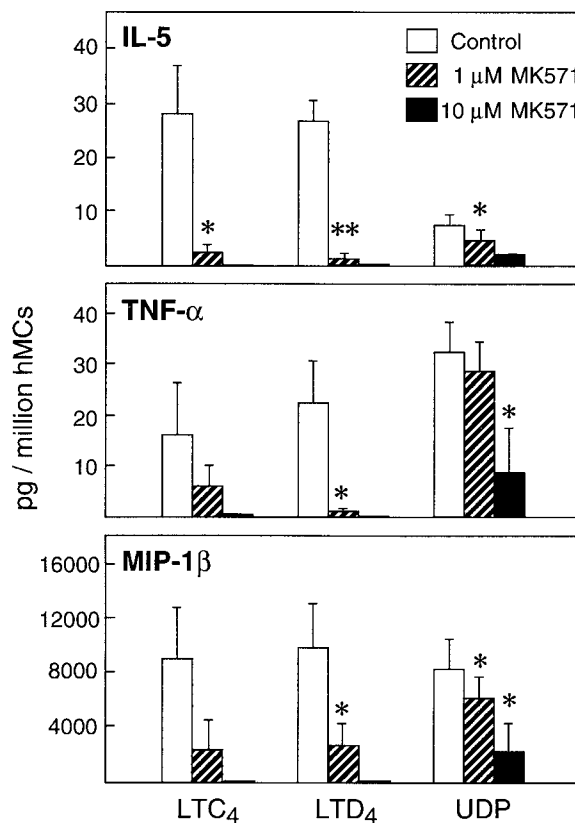


Figure 2. Effect of MK571 pretreatment of IL-4-primed hMCs on cytokine generation in response to cys-LTs and UDP. Data reflect the means \pm SEM for 6–12 experiments, except for the higher-dose MK571-treated samples, which reflect $n = 3$ for TNF- α and MIP-1 β , and $n = 1$ for IL-5. * indicates $P \leq 0.05$, while ** indicates $P < 0.01$.

$n = 2$). MK571 at $1 \mu\text{M}$ profoundly blocked production of both IL-5 and TNF- α in response to LTC₄ ($86 \pm 7\%$ and $83 \pm 17\%$ inhibition; $n = 6$), and to LTD₄ ($94 \pm 3\%$ and $96 \pm 4\%$ inhibition; $n = 6$). This dose of MK571 partially but significantly blocked UDP-induced IL-5 generation ($37 \pm 17\%$ inhibition, $n = 12$, $P = 0.04$). However, a higher ($10 \mu\text{M}$) dose of MK571 was required to diminish TNF- α generation in response to $1 \mu\text{M}$ UDP ($72 \pm 28\%$ inhibition, $P = 0.03$, $n = 3$; Fig. 2).

Effect of Cys-LT Receptor Antagonism and Cys-LT Synthesis Inhibition on Fc ϵ RI-mediated Cytokine Generation. IL-4-primed hMCs generated substantial quantities of IL-5 ($84 \pm 18 \text{ pg}/10^6$ hMCs, $n = 13$), TNF- α ($604 \pm 173 \text{ pg}/10^6$ hMCs, $n = 8$; Fig. 3) and MIP-1 β ($28,476 \pm 8,600 \text{ pg}/10^6$ hMCs, $n = 4$, not shown) after passive sensitization and challenge. Pretreatment with MK571 ($1 \mu\text{M}$) to block access to the CysLT1 and CysLTR_{IL-4} receptors partially and significantly attenuated the generation of IL-5 ($29 \pm 5\%$ inhibition, $P = 0.008$, $n = 13$; Fig. 3), and of TNF- α ($33 \pm 6\%$ inhibition, $P = 0.03$, $n = 8$; Fig. 3) to a comparable extent. In five experiments, hMCs pretreated with the FLAP/LTC₄S inhibitor MK886 ($1 \mu\text{M}$), were inhibited by $31 \pm 5\%$ and $26 \pm 6\%$, respectively, for IL-5 and TNF- α generation ($P = 0.05$ and 0.005 , respectively; Fig. 3); this finding was similar to that for MK571-treated hMCs. By comparison, interference by MK571 and MK886 with the substantial IgE-dependent generation of MIP-1 β did not approach significance (data not shown). Neither agent affected IgE-dependent histamine release (data not shown). As anticipated, MK886 nearly completely blocked cys-LT generation by IL-4-primed hMCs (from 9 ± 3 to $0.024 \pm 0.004 \text{ pg}/10^6$ hMCs), whereas MK571 had no effect ($n = 2$, data not shown).

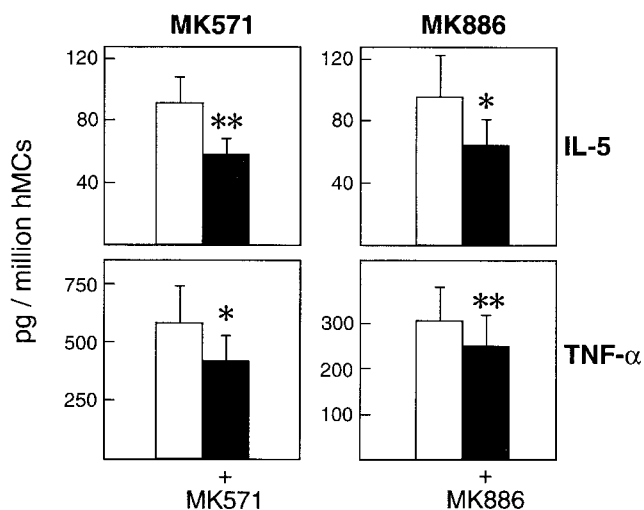


Figure 3. Effect of receptor blocker (MK571) and FLAP inhibitor (MK886) on IL-5 and TNF- α generation by IL-4-primed and sensitized hMCs stimulated with anti-IgE without (white bars) and with (black bars) inhibitors. Results for MK571 (left) reflect mean \pm SEM for 13 (IL-5) and 8 (TNF- α) experiments. Results for MK886 (right) reflect five of those experiments. * indicates $P \leq 0.05$, while ** indicates $P < 0.01$.

Effect of Stimulation of hMCs with Cys-LTs or UDP on Exocytosis and PGD₂ Generation. IL-4-primed hMCs stimulated with maximal concentrations of cys-LTs (10^{-7} M) or UDP (10^{-6} M) were studied 30 min after activation for their metachromatic staining with toluidine blue, their release of histamine, their generation of PGD₂, and, in the case of UDP, their production of cys-LTs. Replicate hMCs were passively sensitized and stimulated with an anti-IgE Ab or with a sham control. Toluidine blue staining of hMCs at both 30 min (Fig. 4, top) and 6 h (not shown) after stimulation revealed loss of granules in the anti-IgE-stimulated groups, but not in the groups stimulated with UDP or cys-LTs ($n = 3$, representative experiment shown; Fig. 4, top panels). Anti-IgE caused histamine release ($46 \pm 3\%$, $n = 3$; Fig. 4), cys-LT generation ($9 \pm 3 \text{ ng}/10^6$ hMCs, mean \pm 1/2 range, $n = 2$, data not shown), and PGD₂ production ($12 \pm 6 \text{ ng}/10^6$ hMCs, mean \pm 1/2 range, $n = 2$; Fig. 4) by IL-4-primed hMCs. In contrast, LTC₄, LTD₄, and UDP did not induce exocytosis of histamine ($n = 3$; Fig. 4), PGD₂ production ($n = 2$; Fig. 4), or, in the case of UDP, cys-LT production ($n = 2$, data not shown). Sham-treated hMCs retained their metachromatic granules, and did not release histamine, produce PGD₂, or generate cys-LTs ($n = 3$, not shown).

Involvement of NF-AT and ERK Signaling Pathways in Cys-LT- and UDP-Mediated Cytokine Generation. Stimulation of hMCs for 5 min with 10^{-7} M concentrations of LTC₄ and LTD₄ or with 10^{-6} M concentrations of UDP resulted in phosphorylation of ERK (Fig. 5 A). Phosphorylation of p38 MAP kinase was constitutive and not increased during the 5 min stimulation by any ligand, while phosphorylation of JNK was not detected (not shown). For each agonist, the phosphorylation of ERK was reduced to levels below baseline by a 5-min pretreatment with the selective mitogen-activated protein ERK kinase inhibitor U0126 ($5 \mu\text{g}/\text{ml}$; Fig. 5 A). Pretreatment of IL-4-primed hMCs with either UO126 or the calcineurin inhibitor, FK506 ($10 \text{ ng}/\text{ml}$), almost completely blocked their generation of both IL-5 and MIP-1 β in response to cys-LTs and UDP (Fig. 5 B). TNF- α production was also blocked by both inhibitors, although not completely in the cases of LTD₄ (77 ± 17 and $69 \pm 20\%$ inhibition in response to UO126 and FK506, respectively) and UDP (84 ± 10 and $56 \pm 26\%$ inhibition, respectively; Fig. 5 B).

Discussion

We describe a novel function for cys-LTs through their receptors, namely, the induction of proinflammatory cytokine gene expression and protein secretion by hMCs that does not involve exocytosis. As with calcium flux, the induction of cytokines is blocked by a selective, competitive CysLT1 receptor antagonist, MK571. The maximal induction of this function requires priming of the hMCs by IL-4, which on the basis of our prior studies does not induce significant changes in the expression of the known cys-LT receptors (23); thus we use Cys-LTR_{IL-4} to desig-

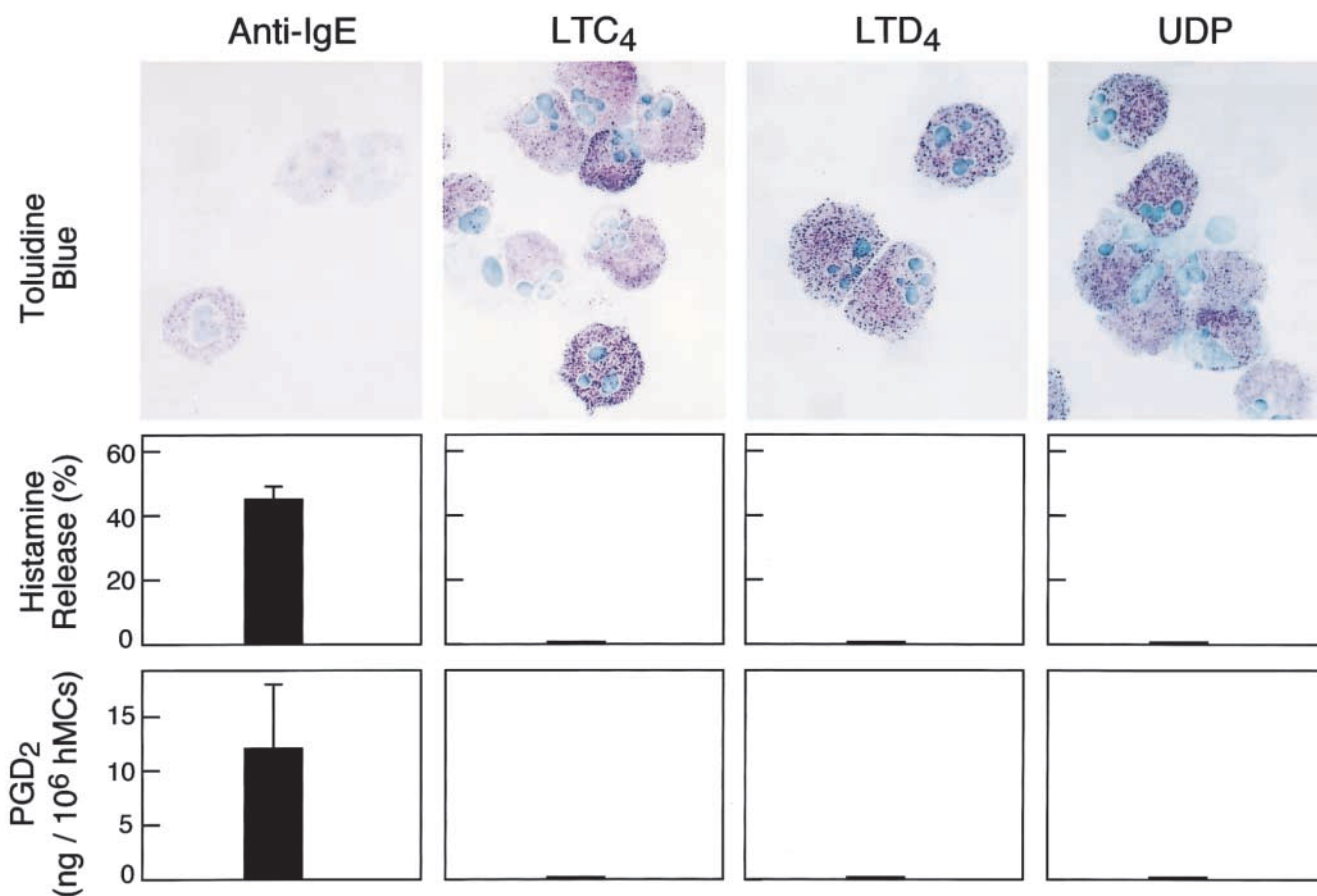


Figure 4. Comparison of histamine release and PGD₂ generation from IL-4-primed hMCs activated by anti-IgE, cys-LTs, or UDP. Toluidine blue staining for granules of IL-4-primed, passively sensitized hMCs stimulated with the indicated agonists for 30 min (top), percent release of histamine (middle), and PGD₂ generation in response to the same stimuli (bottom). The depicted toluidine blue stains are from a single experiment representative of three performed. Histamine release is the mean \pm SEM for three experiments, while PGD₂ generation is the mean \pm 1/2 range for two experiments.

nate the recognition/effector step. A key implication of these findings is that cys-LTs generated by eosinophils, macrophages, and hMCs could induce hMC-derived proinflammatory cytokines such as TNF- α without evidence of degranulation. This mechanism could operate in the setting of allergic and asthmatic inflammation, where IL-4 is prominently expressed by T cells. In innate immunity, natural killer T cells could provide IL-4 (30), and the cys-LTs could be derived by bacterial products acting on cells directly through β -glucan receptors (31, 32) or indirectly through complement activation products on their cellular receptors (33). Additionally, UDP provided by microbes, injured cells, or neurogenic signals (34) could initiate a similar activation cascade through receptors shared with cys-LTs on hMCs.

The previous recognition that cys-LT receptors are expressed by hematopoietic effector cells (18) implies that cys-LTs have functions distinct from their known, rapid-onset bronchoactive and vasoactive effects. Our recent observation that hMCs also express the CysLT1 receptor and the CysLTR_{IL-4} led us to hypothesize that cys-LTs might act through hMCs to initiate cytokine production, accounting for a more sustained, proinflammatory effect. We

therefore stimulated both IL-4-primed and unprimed hMCs with LTC₄, LTD₄, and UDP at the dosing ranges that had elicited maximal calcium responses previously (23). The sustained calcium fluxes elicited by these agonists in hMCs are typical of those that couple to cytokine production in T cells (35). MIP-1 β was included in our analysis, because it is a major lymphocyte chemoattractant that is generated exclusively by MCs in a mouse model of contact sensitization (36) and is generated abundantly by the leukemic MC line, HMC-1, activated through the transfected fMLP receptor (37). IL-5, the eosinophilopoietic cytokine, was included because its IgE-dependent expression by cord blood-derived hMCs requires priming with IL-4 (2). TNF- α was measured because of its importance in MC-dependent host resistance to bacteria (38) and its role in human diseases such as rheumatoid arthritis (39). Of these cytokines, only MIP-1 β was detected in supernatants from the stimulated, unprimed hMCs, and only at the highest cys-LT concentrations tested. As was the case for calcium flux in our previous studies, IL-4 priming sharply lowered the doses of cys-LTs needed to elicit dose-dependent MIP-1 β generation from 10⁻⁷ to 10⁻⁹ M; IL-4 priming was required for the detection of IL-5 and TNF- α

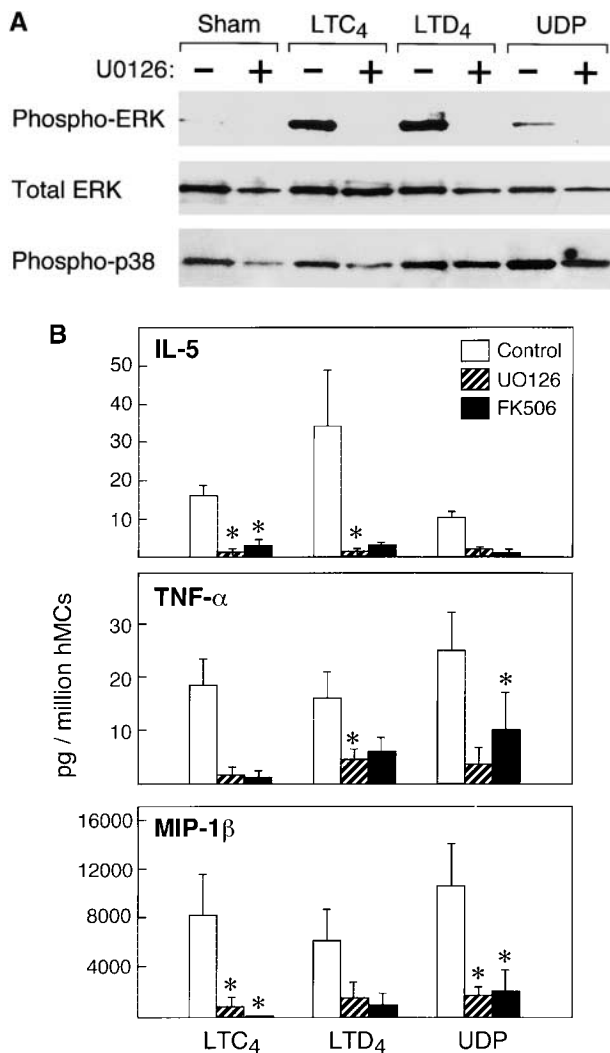


Figure 5. Involvement of the ERK and calcineurin/NF-AT pathways in cys-LT- and UDP-induced cytokine generation by IL-4-primed hMCs. (A) Anti-phospho-ERK, anti-total ERK, and anti-phospho-p38 immunoblots performed on lysates of hMCs stimulated for 5 min with LTC₄, LTD₄, or UDP in the presence (+) or absence (-) of the MEK inhibitor, UO126. Replicate hMCs treated with a sham control (SCF alone) are included. Results are from a single experiment representative of four performed. (B) Generation of IL-5, TNF-α, and MIP-1β by IL-4-primed hMCs in the presence or absence of UO126 or FK506. Results are the mean ± SEM of 3–4 experiments. * indicates $P \leq 0.05$.

production, which occurred only at the highest agonist concentrations tested (Fig. 1 A). The fact that the ligand-initiated cytokine generation depended on de novo gene induction was supported by two lines of evidence; first, each agonist induced steady-state IL-5, TNF-α, and MIP-β mRNA expression 2 h after stimulation (Fig. 1 B). Second, cytokine generation in response to each agonist was blocked by the calcineurin-dependent transcription inhibitor FK506 (Fig. 5 B). MK571, the competitive CysLT1 receptor antagonist, blocked the cys-LT-mediated production of each cytokine virtually completely (Fig. 2). Thus, IL-4 priming strongly enhances cys-LT-induced cytokine production, with LTC₄ and LTD₄ being equipotent

when signaling through the CysLTR_{IL-4} pathway, as was observed for calcium flux (23).

The fact that IL-4 priming of hMCs profoundly induces the expression of LTC₄S and IgE-dependent cys-LT generation (22) suggested the possibility that endogenously generated cys-LTs could modulate FcεRI-dependent hMC activation responses through the CysLTR_{IL-4} pathway. Pretreatment with MK571 to block access to the CysLTR_{IL-4} and CysLT1 receptors, or with MK886 to inhibit the production of endogenous cys-LTs did not alter histamine release in response to FcεRI cross-linkage. As expected, only MK886 blocked cys-LT generation. However, both MK571 and MK886 attenuated IL-5 production and TNF-α in response to IgE receptor cross-linkage by ~30%. (Fig. 3). IgE-dependent MIP-1β generation, which is 40–300-fold greater than that of IL-5 or TNF-α, was unaffected by either inhibitor (data not shown). Thus, endogenously produced cys-LTs act through the CysLTR_{IL-4} pathway to amplify cytokine generation in response to FcεRI cross-linkage. Furthermore, this amplification mechanism is especially important for IL-5 and TNF-α, the two cytokines most dependent on IL-4 priming for their expression (Fig. 1). The fact that hMCs activated by IgE receptor cross-linkage released quantities of cys-LTs (9 ng, or 14 pmol/10⁶ hMCs) well below the nanomolar ranges used for stimulation suggests that endogenously generated cys-LTs work as ligands in a “synapse” with their receptors on hMCs. In this context, it is noteworthy that LTC₄ is exported by a specific carrier-dependent mechanism (10), and that the CysLTR_{IL-4} pathway is marked by the preferential augmentation of LTC₄ responses over those to LTD₄ (23). The participation of endogenous cys-LTs in the regulation of TNF-α generation was demonstrated in a mouse model of SCF-induced bronchial inflammation and hyperreactivity, where MK886 pretreatment attenuated subsequent pulmonary expression of TNF-α (40).

We used a higher concentration of UDP (10⁻⁶ M) than cys-LTs (10⁻⁷ M) because this concentration had been required to achieve a maximal calcium flux and because this concentration was necessary to consistently recognize the IL-4-priming effect in a setting where unprimed hMCs produced nanogram quantities of MIP-1β in response to UDP (Fig. 1 A). Although treatment with 1 μM MK571 did not completely block UDP-induced production of any of the three cytokines by IL-4-primed hMCs, the percent inhibition for MIP-1β was significant and was further enhanced when MK571 was used at a 10-fold molar excess (Fig. 2). The higher concentration of MK571 attenuated the UDP-mediated production of MIP-1β, TNF-α, and IL-5, although in the latter case the experiment was not repeated sufficiently to attain statistical significance. The apparent limitations of MK571 as a UDP antagonist, compared with its full efficacy in blocking cys-LT responses, could reflect either the differences in the agonist concentrations used or overlapping but nonidentical respective binding sites for cys-LTs and UDP on the CysLT1 and CysLTR_{IL-4} receptors. The IL-4- and MK571-insensitive components of UDP-mediated MIP-1β generation, and

the differences between the cys-LT- and UDP-stimulated hMCs in relative levels of elicited steady-state MIP-1 β mRNA and protein secretion, could reflect activation of uridine binding P2Y receptors. Neither the UTP-binding P2Y4 receptor, which is expressed by hMCs, nor the UDP-specific P2Y6 receptor respond to cys-LTs, nor are they blocked by MK571 when cloned and expressed in transfected cell lines (23).

In comparison to the extensive literature on Fc ϵ RI-mediated signal transduction, little is known concerning the pathways used by G protein-coupled receptors for cytokine generation in MCs. MIP-1 β production by the HMC-1 cell line in response to stimulation through the transfected fMLP receptor involves convergent signaling through calcineurin-dependent NF-AT transcription factors and ERK (37). Both cys-LTs and UDP caused phosphorylation of ERK that was inhibited by UO126 pretreatment (Fig. 5 A), with the cys-LTs eliciting especially strong phosphorylation. Both FK506 and UO126 blocked the production of IL-5 and MIP-1 β in response to each agonist virtually completely, and substantially interfered with TNF- α generation (Fig. 5 B). Residual TNF- α production could reflect additional signaling pathways and transcription factors, such as nuclear factor κ B, which is involved in the transcriptional control of IgE-dependent TNF- α generation in the transformed rat MC line, RBL-2H3 (41).

IL-4 priming of hMCs *in vitro* amplifies their subsequent exocytosis of histamine and PGD₂ generation in response to IgE receptor cross-linkage, in parallel with up-regulation of Fc ϵ RI expression (42). As anticipated, primed, passively sensitized hMCs released histamine and generated PGD₂ in response to cross-linkage of Fc ϵ RI. However, neither histamine exocytosis nor PGD₂ production occurred in response to maximally effective doses of cys-LTs or UDP (Fig. 4). The lack of histamine release was associated with retention of toluidine blue-positive granules, which contrasted sharply with the marked degranulation observed in the hMCs stimulated by cross-linkage of Fc ϵ RI (Fig. 4). Mouse MCs stimulated with bacterial LPS through toll-like receptor 4 (TLR4) also generate several proinflammatory cytokines, including TNF- α , through new transcription and translation, with no exocytosis (43). UDP and the P2Y6 receptor were recently implicated in an autocrine feedback mechanism amplifying IL-8 generation by a LPS-stimulated human monocytic cell line (44). The diversity of calcium-dependent signaling cascades in MCs may include overlapping pathways elicited by the cys-LT receptors, P2Y receptors, and TLR4 to induce cytokine/chemokine production without concomitant exocytosis.

Our study demonstrates cytokine gene induction by hMCs through CysLT receptor pathway(s) and an autocrine/paracrine amplification mechanism via these receptors for IgE-dependent cytokine generation. In either case, hMCs stimulated through cys-LT receptors could promote neutrophil recruitment (through TNF- α), lymphocyte chemotaxis (through MIP-1 β), and eosinophilia (through IL-5). These findings may help to explain the observation that allergen-induced late phase pulmonary responses *in*

vivo are attenuated by pretreatment with CysLT1 receptor antagonists (45) and may relate to the observation that bronchial inflammation and eosinophilia are markedly attenuated in biopsy specimens taken from patients with asthma who are being treated with CysLT1 receptor antagonists (46). Furthermore, the steroid-sparing action of an inhaled soluble IL-4 receptor (47) could in part reflect inhibition of IL-4 priming for IgE receptor, LTC₄S, and CysL-TR_{IL-4} expression by hMCs.

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