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Cysteinyl leukotrienes acting via granule membrane-expressed receptors elicit secretion from within cell-free human eosinophil granules

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Abstract

Background—Cysteinyl leukotrienes are recognized to act via receptors (CysLTRs) expressed on cell surface plasma membranes. Agents that block $CysLT_1R$, are therapeutics for allergic disorders. Eosinophils contain multiple preformed proteins stored within their intracellular granules. Cell-free eosinophil granules are present extracellularly as intact membrane-bound organelles in sites associated with eosinophil infiltration, including asthma, rhinitis and urticaria, but have unknown functional capabilities.

Objective—We evaluated the expression of CysLTRs on eosinophil granule membranes and their functional roles in eliciting protein secretion from within eosinophil granules.

Methods—We studied secretory responses of human eosinophil granules isolated by subcellular fractionation. Granules were stimulated with cysteinyl leukotrienes and eosinophil cationic protein and cytokines were measured in the supernatants. Receptor expression on granule membranes and eosinophils was evaluated by flow cytometry and western blot.

Results—We report that receptors for cysteinyl leukotrienes, $CysLT_1R$, $CysLT_2R$ and the purinergic P2Y12 receptor (P2Y12R), are expressed on eosinophil granule membranes. Leukotriene (LT) C_4 and extracellularly generated LTD_4 and LTE_4 stimulated isolated eosinophil granules to secrete eosinophil cationic protein. MRS 2395, a P2Y12R antagonist, inhibited cysteinyl leukotrienes-induced eosinophil cationic protein release. Montelukast, likely not solely as an inhibitor of $CysLT_1R$, inhibited eosinophil cationic protein release elicited by LTC_4 and LTD_4 as well as by LTE_4.

Conclusion—These studies identify previously unrecognized sites of localization, the membranes of intracellular eosinophil granule organelles, and function for cysteinyl leukotriene-responsive receptors that mediate cysteinyl leukotriene-stimulated secretion from within eosinophil granules, including those present extracellularly.

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Clinical implications—Cysteinyl leukotrienes elicit cell-free eosinophil granule secretion suggesting new roles, amenable to therapeutic interventions, for these lipid mediators in eosinophil-associated diseases.

Keywords

granules; cysteinyl leukotriene; eosinophil; allergy; asthma; montelukast

INTRODUCTION

Cysteinyl leukotrienes (cys-LTs) constitute an important class of potent, pro-inflammatory mediators that are synthesized from membrane-derived arachidonic acid via the 5-lipoxygenase pathway leading to the formation of LTA_4 that is converted into LTC_4 by the action of LTC_4 synthase.[1] Intracellular LTC_4 is actively transported extracellularly, where it is enzymatically converted sequentially to LTD_4 and then to LTE_4.[1] Cys-LTs are cell-membrane impermeant and are recognized to mediate their actions by engaging two heptahelical G protein-coupled receptors (GPCRs), designated $CysLT_1R$ and $CysLT_2R$, that are expressed on cell surface plasma membranes.[1,2] The rank order of affinities of cys-LTs for human $CysLT_1R$ and $CysLT_2R$, based on transfected cells, is $LTD_4 \gg LTC_4 = LTE_4$ and $LTC_4 = LTD_4 > LTE_4$, respectively.[3,4]

Eosinophils, prominent leukocytes in allergic inflammation and anthelminthic responses,[5] are characterized by an abundance of intracellular granules that contain preformed proteins including distinct cationic proteins, such as eosinophil cationic protein (ECP), and a wide range of preformed cytokines, chemokines and growth factors.[6,7] Human eosinophils are major sources of cys-LTs and express both $CysLT_1R$ and $CysLT_2R$.[8] Cys-LTs and their receptors have critical roles in allergic diseases and represent important therapeutic targets for the control of asthma and other pathophysiological conditions.[9,10] Medications, including those recognized to inhibit ligand-binding to $CysLT_1R$, such as montelukast, are used in the management of asthma and related allergic diseases.[1]

In addition to its conventional plasma membrane expression, $CysLT_1R$ has been immunolocalized to nuclei in colorectal adenocarcinoma cells[11] and in a human mast cell line, [12] although the functions of nuclear $CysLT_1Rs$ have not been defined. We have recognized that some cytokine and chemokine receptors are richly present on eosinophil granules;[13] and we have recently demonstrated that eosinophil granules, upon extrusion from eosinophils, respond to a stimulating cytokine, interferon-γ, and a chemokine, eotaxin-1 (CCL11), via cognate granule membrane-expressed receptors, to activate intragranular signaling pathways that elicit granule protein secretion.[14] Intact membrane-bound eosinophil granules have long been recognized to be present extracellularly in tissues and secretions in many human eosinophil-enriched disorders, including asthma, rhinitis, urticaria, atopic dermatitis, eosinophilic esophagitis and helminth infections.15–21 The capacity of cell-free human eosinophil granules to act via receptor-mediated responses to polypeptide agonists and secrete granule-derived cytokines and cationic proteins has indicated that cell-free eosinophil granules may be functionally significant.14 In the present study we have evaluated whether receptors for cys-LTs, in addition to their conventional plasma and nuclear membrane localizations, are expressed and functional on the surface membranes of cell-free human eosinophil granules. We investigated the efficacy of intracellular LTC_4 and extracellular LTD_4 and LTE_4 as potential agonists of eosinophil granule secretion and the capacity of montelukast to inhibit cys-LT-elicited eosinophil granule secretion.

METHODS

Eosinophil purification and subcellular fractionation

Eosinophils were purified from the blood of healthy and atopic donors as previously described.[14,22] Experiments were approved by the Beth Israel Deaconess Medical Center Committee on Clinical Investigation, and informed consent was obtained from all subjects. Subcellular fractionation and eosinophil granule isolation was performed as described.[14,22] Briefly, eosinophils were disrupted by nitrogen cavitation (600 psi, 10 min) and post-nuclear supernatants were ultracentrifuged (100,000 g, 1 h at 4°C) in linear isotonic Optiprep (Axis-Shield, Oslo, Norway) gradients (0–45%). Purity of isolated granules free of plasma membranes or other contaminating structures was rigorously ascertained as previously documented.[14,22]

Stimulation of isolated eosinophil granules

Subcellular fractions containing isolated granules were mixed with RPMI + 0.1% ovalbumin (without phenol red) (Sigma, St. Louis, MO, USA) followed by centrifugation (2,500 g, 10 min). Granule pellets were resuspended in 250 μl of the same medium. Treatments with montelukast (0.1 and 1 μM, Merck) and MRS 2395 (2,2-dimethyl-propionic acid 3-(2-chloro-6-methylaminopurin-9-yl)-2-(2,2-dimethyl-propionyloxymethyl)-propyl ester) (1 and 10 μM, Sigma), a selective P2Y12R antagonist, were performed for 15 min prior to stimulation with LTC_4, LTD_4, or LTE_4 for 30 min at 37°C. Thereafter, following centrifugation at 4°C (2,500 g, 10 min), granule supernatants were collected and stored at −80°C. Drugs were diluted in DMSO at a final concentration <0.01%, which had no effect on granule secretion.

Assays of granule-secreted proteins

ECP levels in eosinophil granule supernatants were analyzed by a quantitative ECP ELISA kit (Medical & Biological Labs, Nakaku Nagoya, Japan) according to the manufacturer's instructions. Stimulated ECP secretion represents ECP levels from stimulated samples minus ECP levels from unstimulated samples. Cytokines, IL-4, IL-6, IFN-γ, IL-10, Il-12 (p70), IL-13 and TNF-α, were quantified using multiplex assays (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Flow cytometry of isolated granules and eosinophils

Isolated granules or eosinophils were incubated for 1 h with primary antibody (Ab) or primary Ab premixed with blocking peptide. Then the granules were washed and incubated with the respective FITC-conjugated secondary Abs for 15 min on ice in the absence of granule fixation. After staining, granules were fixed in buffer containing 2% paraformaldehyde without methanol (Electron Microscopy Sciences, Fort Washington, PA, USA) for 5 min. Control or non-immune Abs were included for all. Analyses were performed on a FACScan with CELLQUEST software (BD Biosciences, San Jose, CA, USA).

Mouse anti-human P2Y12R polyclonal Ab (1:100) was purchased from Abnova Corporation (Taipei, Taiwan). Goat polyclonal Ab against a peptide mapping the N-terminus domain of $CysLT_1R$ (N-20) (5 μg/ml) and the blocking peptide (20 μg/ml) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal Abs against a peptide mapping the C-terminus of the $CysLT_1R$ (5 μg/ml) and the N-terminus of the $CysLT_2R$ (5 μg/ml) and the blocking peptide (20 μg/ml) were purchased from Cayman Chemical (Ann Arbor, MI, USA). FITC-conjugated F(ab′)2 goat anti-mouse, donkey anti-goat and goat anti-rabbit IgGs were used as secondary antibodies (1:100). Mouse, goat and rabbit normal IgGs were used as control Abs (Jackson Immuno Research Inc. West Grove, PA, USA).

Western blotting

Granules and eosinophils were lysed in LDS sample reducing buffer (Nupage, Invitrogen, Carlsbad, CA, USA) and boiled for 5 min. Samples were loaded on 10% Bis-Tris gels (Invitrogen) and run using 3-(n-morpholino) propanesulfonic acid MOPS running buffer. Gels were transferred to nitrocellulose membranes (Thermo Fisher Scientific, San Jose, CA, USA), blocked with 5% milk for at least 1 h and probed with rabbit anti-P2Y12R polyclonal Ab (1:400, Alomone Labs, Jerusalem, Israel) or the Ab premixed with the blocking peptide overnight. Anti-rabbit Ab conjugated to HRP (1:15000, Jackson Immuno Research) was used as secondary Ab. Membranes were developed with West Femto chemiluminescence kits (Thermo Fisher Scientific).

Statistical analysis

Secreted ECP levels, means of duplicates ± SD, are ECP levels from stimulated granules minus ECP levels from unstimulated granules. Data are shown for one experiment representative of three. Quantities of unstimulated and stimulated ECP secreted varied amongst replicate experiments, but the patterns of release and statistical differences were consistent in each of the replicate experiments. Results were analyzed by one-way ANOVA, followed by the Newman-Keuls test. P values < 0.05 were considered significant (two tailed test).

RESULTS

Extracellular eosinophil granules express on their membranes amino-terminal, ligand-binding domains for cys-LTs receptors

To evaluate whether secretory responses of eosinophil granules, as intracellularly resident or extracellularly released organelles, might be mediated by intracrine or paracrine acting cys-LTs, we first analyzed by flow cytometry the expression of $CysLT_1R$ and $CysLT_2R$ proteins on the surface membranes of isolated human eosinophil granules. Without membrane permeabilization, granules displayed immunoreactivity for both $CysLT_1R$ (Fig 1, *A*) and $CysLT_2R$ (Fig 1, *B*) using polyclonal Abs raised against epitopes present specifically in the nominally "extracellular," ligand-binding regions of each CysLTR. Specificities of each polyclonal Ab for $CysLT_1R$ (Fig 1, *A*) and $CysLT_2R$ (Fig 1, *B*) were corroborated by complete neutralization of immunostaining through preincubation of the anti-$CysLT_1R$ and anti-$CysLT_2R$ polyclonal Abs with their respective specific blocking peptide immunogens. In contrast, eosinophil granules exhibited no staining with a polyclonal Ab raised to an "intracellular" carboxyl-terminal domain of $CysLT_1R$ (Fig 1, *C*).

Extracellular eosinophil granules secrete eosinophil cationic protein (ECP) in response to cysteinyl leukotrienes

Given our recent finding that a cytokine and a chemokine could elicit receptor-mediated secretion by cell-free eosinophil granules,[14] we evaluated whether cys-LTs might elicit functional secretory responses by extracellular human eosinophil granules that express CysLTRs. Isolated eosinophil granules were stimulated with LTC_4, LTD_4 and LTE_4; and all three cys-LTs effectively stimulated secretion of ECP from within eosinophil granules (Fig 2). Notably, dose-responses to the three cys-LTs differed. LTC_4 (Fig 2, *A*) and LTE_4 (Fig 2, *C*) elicited ECP secretion only in lower concentrations (including well below 30 nM), fully compatible with physiologic signaling responses *in vivo*. Inhibited ECP secretion at higher LTC_4 and LTE_4 levels of 300 and 3000 nM is consistent with the high-dose inhibition characteristic of GPCRs. Likewise, LTD_4 (Fig 2, *B*) induced granule ECP secretion at significant levels at very low physiologic concentrations (0.3 and 0.03 nM) and not at higher intermediate (3 and 30 nM) concentrations. In contrast, LTD_4 also elicited granule ECP secretion at higher concentrations (300 and 3000 nM). This dose-response suggests

engagements of two receptors for LTD_4 – the first responds to low LTD_4 levels and then exhibits higher dose inhibition and a second receptor putatively mediates secretion elicited by much higher concentrations of LTD_4. Whereas a cytokine (interferon-γ) and a chemokine (eotaxin-1, CCL11) stimulated selective, differential secretion of specific cytokines, as well as ECP, from eosinophil granules,14 we evaluated whether cys-LTs elicited cytokine secretion from within eosinophil granules. All three cys-LTs failed to induce cytokine secretion of known eosinophil secretable cytokines,23 as measured in granule supernatants using cytokine multiplex assays for IL-4, IL-6, IFN-γ, IL-10, IL-12 (p70), IL-13 and TNF-α. Within the limits of cytokine detection, these findings suggest that cys-LT stimulation of eosinophil granules may provide a means for selective mobilization and secretion of the granule cationic protein, ECP, without concomitant mobilization of eosinophil granule-stored cytokines.

A notable finding above was the capacity of low concentrations of LTE_4 to elicit granule ECP secretion (Fig 2, *C*). As a major extracellularly generated cys-LT, the capacity of LTE_4 to stimulate eosinophil granule secretory responses could be pertinent to the known, albeit often over-looked, presence of free membrane-bound eosinophil granules in human diseases, including allergic asthma and rhinitis, dermatitis, helminth infections, eosinophilic esophagitis, and urticaria.[14] LTE_4 is a weak stimulus for both human $CysLT_1R$ and $CysLT_2R$.[3,4] Moreover, LTE_4, in contrast to LTD_4, has elicited airways responses in humans not likely based on $CysLT_1R$ and $CysLT_2R$.[24–26]

Cysteinyl leukotriene-induced eosinophil cationic protein (ECP) release is inhibited by MRS 2395 via eosinophil granule-expressed P2Y12 receptors

An additional human LTE_4 receptor, the purinergic P2Y12R, was identified by *in silico* and *in vitro* methods.[27] To assess if ECP secretion induced by cys-LTs on eosinophil granules might be mediated by P2Y12R, isolated granules were pretreated with MRS 2395, a P2Y12R antagonist. LTC_4 (Fig 3, *A*), LTD_4 (Fig 3, *B* and *C*), and LTE_4 (Fig 3D) -induced ECP secretion was dose-dependently inhibited by MRS 2395. Nonaka *et al* in their *in silico* screening for P2Y12R ligands identified both LTE_4 and LTD_4 (LTC_4 not tested) as potential endogenous ligands for this receptor.[27] In our assays, a P2Y12R antagonist effectively inhibited ECP secretion from eosinophil granules induced by all three cys-LTs.

Human eosinophils express several mRNAs that encode P2X and P2Y receptor subtypes: P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y14, P2X1, P2X4 and P2X7.[28] However, the expression of the subtype P2Y12R had not been recognized for human eosinophils. To ascertain that the inhibitory effects of MRS 2395 on granules were due to its inhibitory actions on P2Y12R, we investigated the expression of this receptor on eosinophils and isolated granules. By flow cytometry, under membrane non-permeabilizing conditions, eosinophils (Fig 3, *E*) and isolated, extracellular granules (Fig 3, *F*) exhibited immunostaining for P2Y12R. Moreover, expression of P2Y12R on isolated granules and eosinophils was confirmed by Western blots (Fig 3, *G*). The specificity of the polyclonal Ab was ascertained by complete neutralization through preincubation of the anti-P2Y12R polyclonal Ab with its respective specific blocking peptide immunogen.

Montelukast inhibits eosinophil cationic protein (ECP) secretion from cysteinyl leukotriene-stimulated eosinophil granules

To evaluate the therapeutic potential of montelukast, currently used clinically based on its actions as a $CysLT_1R$ antagonist, we assessed the capacities of montelukast to inhibit LTC_4-, LTD_4- and LTE_4-elicited ECP secretion from human eosinophil granules. Notably, montelukast inhibited eosinophil granule ECP secretion induced by LTC_4 (30 nM) (Fig 4, *A*), LTD_4 (0.3 and 300 nM) (Fig 4, *B* and *C*), and LTE_4 (30 nM) (Fig 4, *D*).

DISCUSSION

Our findings of functional receptors for cys-LTs on cell-free extracellular human eosinophil granule membranes, sensitive to inhibition by montelukast and a P2Y12R antagonist, identify novel mechanisms whereby cys-LTs may serve as intracrine and paracrine mediators of eosinophil granule-derived secretion. Cys-LTRs, heretofore, have been widely recognized to localize and function principally at cell plasma membranes.[1] To date, recognition of intracellular sites of CysLTRs has been limited to their immunolocalization, without defined functional roles, on nuclei of a human mast cell line[12] and colon adenocarcinoma cells.[11] We now demonstrate that receptors for cys-LTs are expressed and functional on the membranes of an intracellularly derived organelle, the granules of human eosinophils. With Abs specific to each receptor. the protein receptor components immunologically demonstrated to be present on human eosinophil granules include those for $CysLT_1R$, $CysLT_2R$ and the purinergic receptor P2Y12. Notably, each CysLTR protein was expressed with its ligand-binding domain on the outer membranes of human eosinophil granules.

As is true of other GPCRs, there is the potential for individual protein chains of Cys-LTRs to form hetero-dimers or hetero-oligomers, which might influence their pharmacology and function.[29] Previously in a human mast cell line, functionally significant heterodimeric combinations of $CysLT_1R$ and $CysLT_2R$ were demonstrated.[12] We document that three protein components of Cys-LT receptors, $CysLT_1R$, $CysLT_2R$ and the purinergic receptor P2Y12, are expressed on human eosinophil granules. Whether the three candidate CysLTR protein chains function or interact as homodimers, heterodimers, or oligomers in human eosinophil granules can not be ascertained in these “primary” cells in contrast to transfectable cell lines. Nevertheless, of potential clinical pertinence, whatever the interactions maybe amongst the Cys-LTR protein chains, cell-free human eosinophil granules, long recognized to be present as intact membrane bound structures in tissues and secretions associated with varied allergic (e.g., asthma, rhinitis, urticaria) and other eosinophil-associated diseases,[15–21] have the capacity to secrete ECP in response to low and even sub-nanomolar concentrations of the three cys-LTs, including the two extracellularly generated cys-LTs, LTD_4 and LTE_4.

Cys-LT-elicited ECP secretion was inhibited by MRS 2395, a selective P2Y12R antagonist. Clinically, this receptor is blocked by clopidogrel. Moreover, isolated granule secretory responses to each of the cys-LTs were blocked by montelukast. The 1 μM concentration of montelukast that uniformly inhibited LTC_4-, LTD_4- and LTE_4-elicited granule secretion of ECP is in accord with levels of montelukast achieved in patients receiving this agent.[30] Decreases in serum ECP levels have been demonstrated in asthmatic subjects treated with montelukast.[31] Montelukast is known as a potent selective $CysLT_1R$ antagonist effective as a therapeutic for asthma and other allergic conditions.[32] Inhibitory actions of montelukast beyond those based mainly on inhibition of $CysLT_1R$ have been reported.[33–35] Montelukast inhibits the recently de-orphanized GPR17[33] and several purinergic G protein-coupled receptors, suggesting that $CysLT_1R$ antagonists possibly interact functionally with signaling pathways of P2Y receptors.[34] Besides, on human eosinophils, montelukast was suggested as a regulator of eosinophil protease activity through a leukotriene-independent mechanism.[35] In part, because LTE_4 is a weak agonist for $CysLT_1R$, montelukast sensitive mechanisms that are operative in stimulating eosinophil granule ECP secretion are likely other than singularly by $CysLT_1R$ blockage.

Within eosinophils, synthesis of LTC_4 (but not of extracellularly formed LTD_4 or LTE_4) occurs at perinuclear membranes and cytoplasmic lipid bodies.[8,36–38] For granules, as intracellular organelles, the presence of functional membrane-expressed receptors might be indicative of intracrine roles for LTC_4 as regulators of granule protein mobilization, sorting and secretion. We previously recognized a role principally for LTC_4, and not LTD_4 or LTE_4, as an intracrine

mediator of CCR3 receptor-elicited, vesicular transport-mediated interleukin-4 secretion by human eosinophils.[39] Such release of IL-4 via vesicular transport from within eosinophils[13] likely involves more complex steps beyond intracellular granule secretion that may be regulated by actions of LTC_4. That intracellular LTC_4 can activate release of ECP from cys-LT responsive receptors expressed on eosinophil granule membranes helps in further delineating roles for LTC_4 as an intracrine mediator of eosinophil secretory responses. For granules, as extruded extracellular "organelles," the expression of functional CysLTRs on granule membranes capable of responding to the extracellularly formed cys-LTs, LTD_4 and LTE_4, underscores the secretion-competence of cell-free eosinophil granules. Together, the recognition that CysLTRs are localized on the outer membranes of human eosinophil granules and mediate cys-LT-elicited secretion from within these granules identifies new roles, amenable to therapeutic interventions, for cys-LTs as mediators in eosinophil-associated diseases.

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Abbreviations used

Ab	antibody
cys-LTs	cysteinyl leukotrienes
CysLTRs	cysteinyl leukotriene receptors
ECP	eosinophil cationic protein
GPCRs	G protein-coupled receptors
LTs	leukotrienes
P2Y12R	P2Y12 receptor

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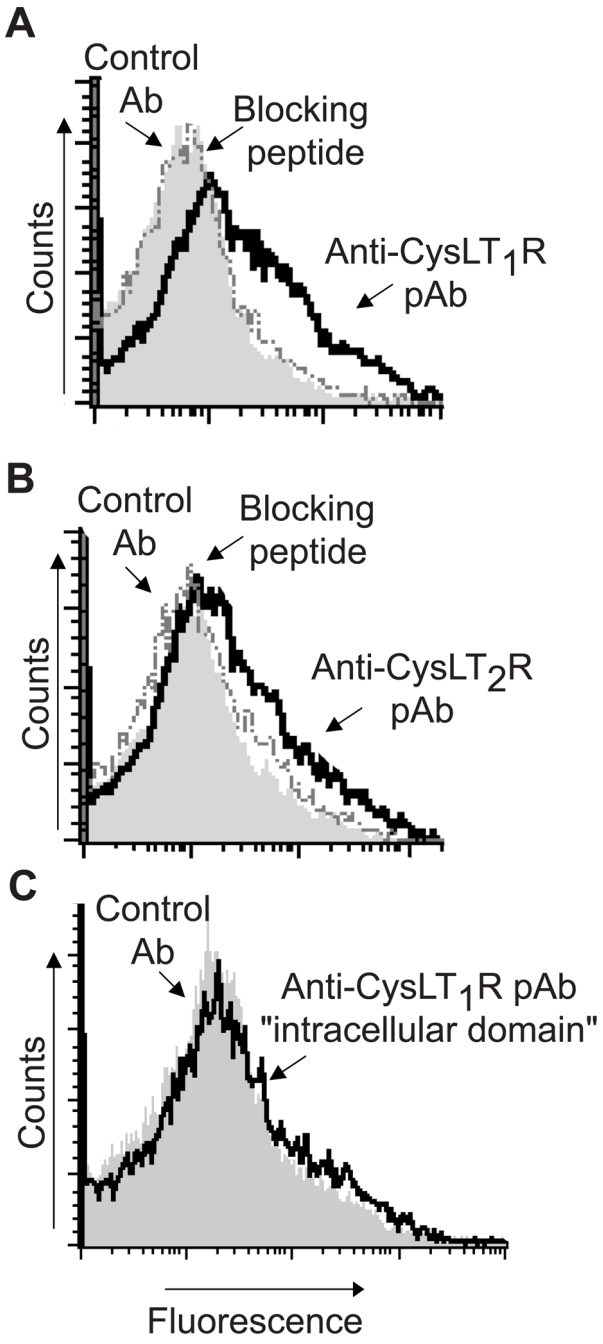

FIG 1.

Isolated granules expressed extracellular domains of the **A,** $CysLT_1$ receptor ($CysLT_1R$) and **B,** $CysLT_2$ receptor ($CysLT_2R$), but not **C,** the carboxy-terminal intracellular domain of $CysLT_1R$. Shaded histograms represent staining with control antibody (Ab). Solid and dashed lines represent staining with polyclonal specific antibodies (pAb) and anti-CysLTR pAbs neutralized by absorption with their respective immunogen peptide, respectively. Data are from one experiment, representative of three.

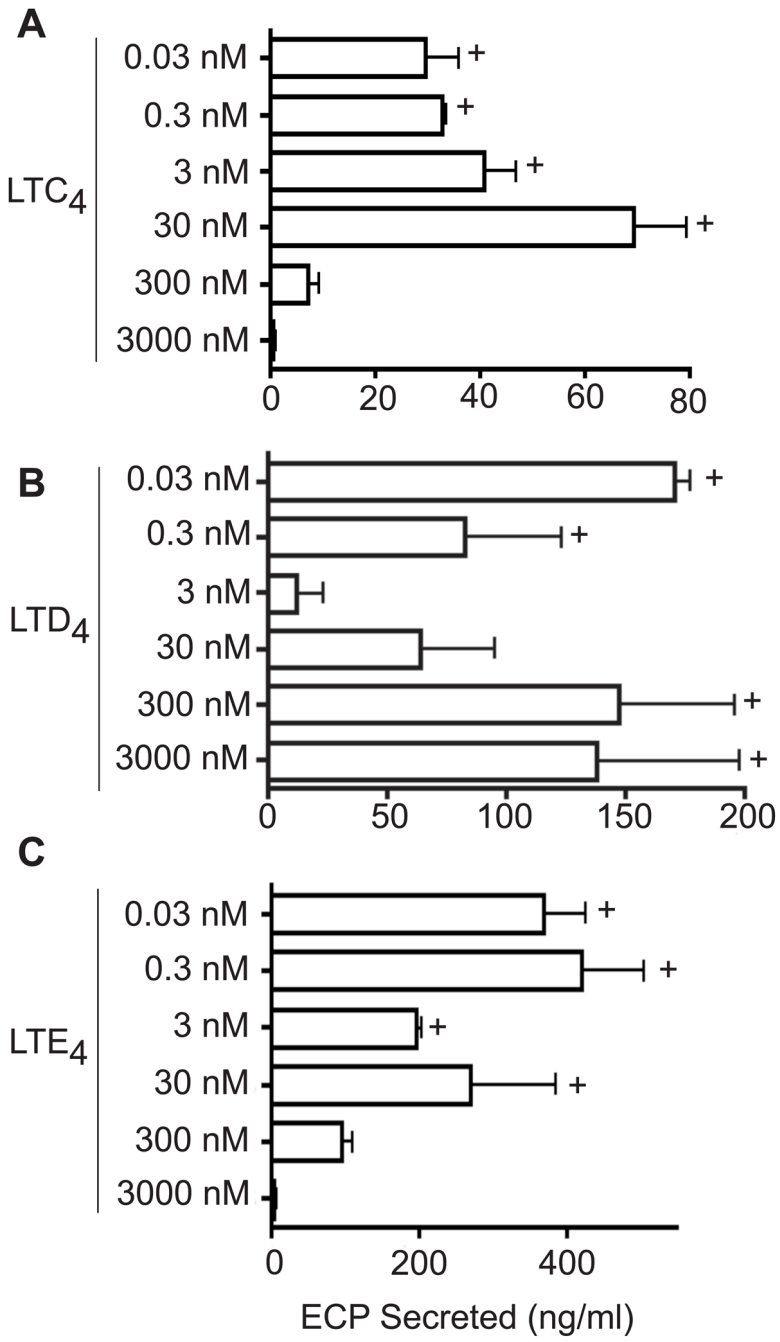

FIG 2.

Isolated granules were stimulated with different concentrations (0.03 – 3,000 nM) of **A,** LTC_4, **B,** LTD_4 and **C,** LTE_4 and eosinophil cationic protein (ECP) levels were measured in the supernatants. Data is shown for one experiment representative of three. + represents significantly increased ECP release ($P < 0.05$) compared with non-stimulated granules.

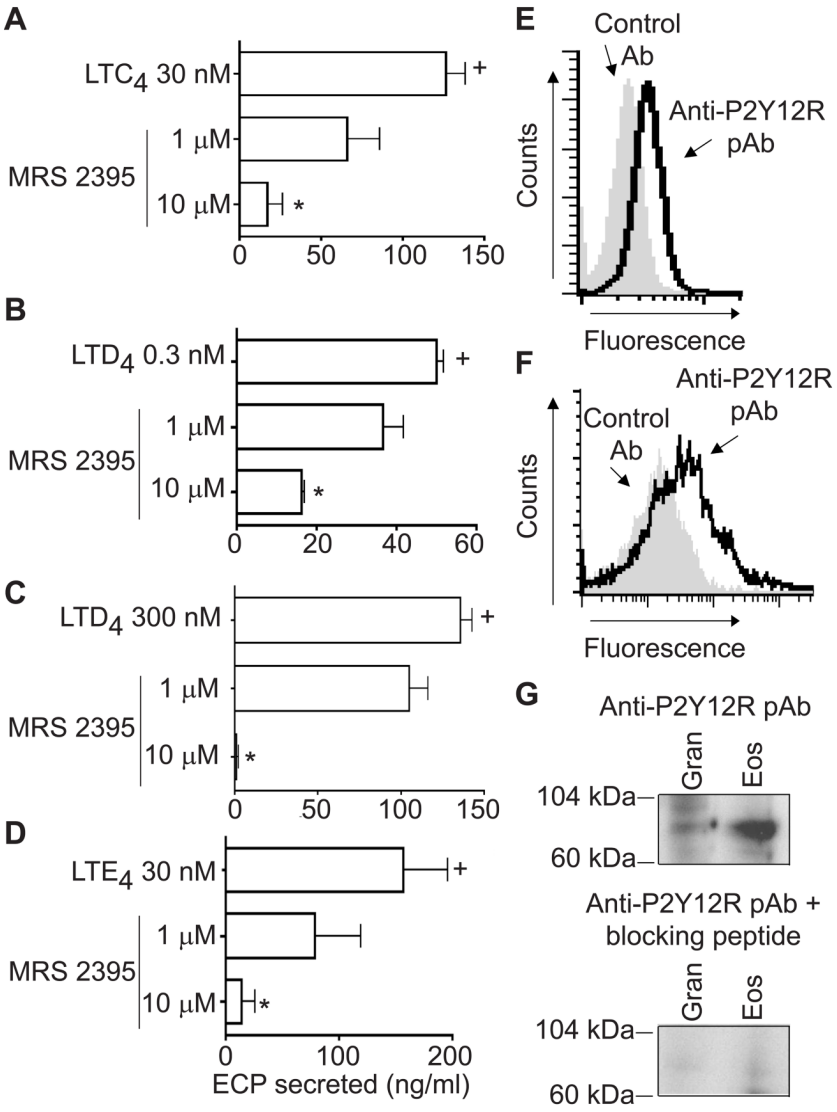

FIG 3.

MRS 2395, a selective P2Y12 receptor (P2Y12R) antagonist, dose-dependently inhibited the eosinophil cationic protein (ECP) release induced by **A,** LTC_4 30 nM, **B,** LTD_4 0.3 nM and **C,** 300 nM and **D,** LTE_4 30 nM. + and * represent $P < 0.05$ for ECP released compared with non-stimulated and leukotriene-stimulated granules, respectively. **E,** Eosinophil and **F,** isolated granule surface membranes expressed the P2Y12R. Shaded histograms and solid lines represent staining with control Ab and with an anti- P2Y12R specific polyclonal Ab, respectively. **G,** The expression of the P2Y12R was confirmed on eosinophil and isolated granules by Western blots. Specificity of immunodetection was confirmed by neutralization with the anti-P2Y12R pAb by absorption with its respective immunogen peptide. All data is shown for one experiment representative of three. Gran, granules; Eos, eosinophils.

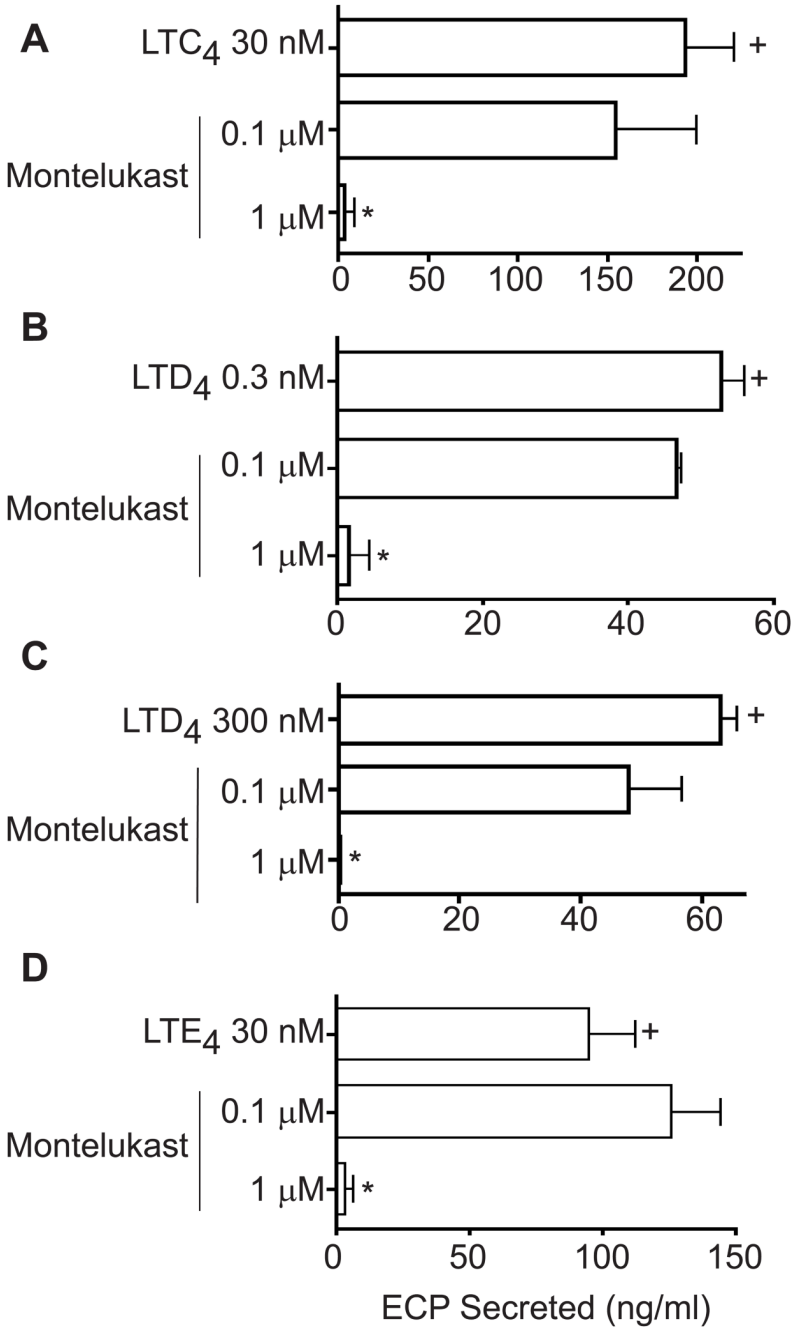

FIG 4.

Montelukast dose-dependently inhibited eosinophil cationic protein (ECP) secretion induced by **A**, LTC_4 30 nM, **B**, LTD_4 0.3 nM and **C**, 300 nM and **D**, LTE_4 30 nM. Data is shown for one experiment representative of three. + and * represent $P < 0.05$ for ECP released compared with non-stimulated granules and leukotriene-stimulated granules, respectively.