Mechanisms of allergic diseases

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The leukotriene E_4 puzzle: Finding the missing pieces and revealing the pathobiologic implications

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Activity Objectives

1. To better understand the biologic activities of leukotriene (LT) E_4.
2. To understand the interactions of the cysteinyl leukotrienes (cysLTs) with their receptors.
3. To appreciate the key biochemical aspects of the LT pathway.

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The intracellular parent of the cysteinyl leukotrienes (cysLTs), leukotriene (LT) C_4, is formed by conjugation of LTA_4 and reduced glutathione by LTC_4 synthase in mast cells, eosinophils, basophils, and macrophages. After extracellular export, LTC_4 is converted to LTD_4 and LTE_4 through sequential enzymatic removal of glutamic acid and then glycine. Only LTE_4 is sufficiently stable to be prominent in biologic fluids, such as urine or bronchoalveolar lavage fluid, of asthmatic individuals and at sites of inflammation in animal models. LTE_4 has received little attention because it binds poorly to the classical type 1 and 2 cysLT receptors and is much less active on normal airways than LTC_4 or LTD_4. However, early studies indicated that LTE_4 caused skin swelling in human subjects as potently as LTC_4 and LTD_4, that airways of asthmatic subjects (particularly those that were aspirin sensitive) were selectively hyperresponsive to LTE_4, and that a potential distinct LTE_4 receptor was present in guinea pig trachea. Recent studies have begun to uncover receptors selective for LTE_4: $P2Y_{12}$, an adenosine diphosphate receptor, and $CysLT_ER$, which was observed functionally in the skin of mice lacking the type 1 and 2 cysLT receptors. These findings prompt a renewed focus on LTE_4 receptors as therapeutic targets that are not currently addressed by available receptor antagonists. (J Allergy Clin Immunol 2009;124:406-14.)

Key words: *Leukotriene E_4, G protein–coupled receptor, bronchial asthma, inflammation, knockout mouse*

Of the 3 ***cysteinyl leukotrienes*** (cysLTs; leukotriene [LT] C_4, LTD_4, and LTE_4), only LTE_4 is sufficiently stable so as to be detectable in extracellular fluids. Although widely used as a biomarker of cysLT pathway activity in clinical studies, LTE_4 has received little attention in recent literature as a mediator of inflammation because of its poor activity at the classical cysLT receptors (type 1 ***cysteinyl leukotriene receptor*** [$CysLT_1R$] and type 2 cysteinyl leukotriene receptor [$CysLT_2R$]). However, several earlier studies clearly demonstrated that LTE_4 had biologic activity that differed from that of its precursors, predicting (correctly in retrospect) the existence of specific LTE_4-reactive receptors. This review will highlight LTE_4 from a historical

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Terms in boldface and italics are defined in the glossary on page 407.

Abbreviations used
cysLT: Cysteinyl leukotriene
$CysLT_1R$: Type 1 cysteinyl leukotriene receptor
$CysLT_2R$: Type 2 cysteinyl leukotriene receptor
ERK: Extracellular signal-regulated kinase
GPCR: G protein–coupled receptor
LT: Leukotriene
LTC_4S: Leukotriene C_4 synthase
MIP-1β: Macrophage inflammatory protein 1β
PG: Prostaglandin
PPAR-γ: Peroxisome proliferator-activated receptor γ
SRS-A: Slow-reacting substance of anaphylaxis
WT: Wild-type

perspective, from its discovery to the identification of its receptors and mechanisms of action.

DISCOVERY OF LTE_4

The slow-reacting substance of anaphylaxis (SRS-A), so named by Brocklehurst,[1] was identified as a substance generated by *in vitro* antigen/allergen challenge of perfused lungs of actively sensitized guinea pigs or human lung fragments of allergic patients requiring resection. Its potent constrictor activity on guinea pig or human bronchioles in the presence of an antihistamine provided compelling evidence for its potential role in asthma. The initial analyses into the physical characteristics and composition of SRS-A from the rat suggested possible sulfur content.[2] This led to the identification by Murphy et al[3] of LTC_4, the first component of SRS-A, by loading a mastocytoma cell line with [^{35}S]cysteine and identifying the radiolabeled component released in response to activation with calcium ionophore. LTC_4 was composed of a metabolite of ***arachidonic acid*** (eicosatetraenoic acid) with 3 conjugated double bonds and a peptide adduct through a sulfur bridge. The exact stereochemistry of the lipid and the amino acid sequence of the S-linked peptide of LTC_4 were obtained by comparing purified natural SRS-A with candidate synthetic cysLTs prepared by E. J. Corey.[4] These synthetic cysLTs showed bioactivity consistent with the functional definition of SRS-A offered by Brocklehurst,[1] who noted that a range of activities could contract the guinea pig ileum in the presence of an antihistamine.

Because we had anticipated that the activity of SRS-A could be attributed to a single product, we were surprised to find that partially purified SRS-A from the peritoneal cavity of rats

GLOSSARY

ARACHIDONIC ACID: Arachidonic acid is the precursor for both LTs and prostaglandins and is found on the nuclear membrane. Together, LTs and prostaglandins are called eicosanoids.

ASPIRIN-EXACERBATED RESPIRATORY DISEASE (AERD): AERD consists of a clinical constellation of nasal polyposis with eosinophilic sinusitis, asthma, and idiosyncratic sensitivity to nonsteroidal anti-inflammatory agents that inhibit COX-1. Treatment of patients with AERD includes LT inhibitors.

COX-2: COX-2 is induced by LPS, IL-1, and IL-2 to produce prostaglandin intermediates from arachidonic acid.

CYSTEINYL LEUKOTRIENE (cysLT): LTs, so named because of their generation from "leuko"cytes and conserved 3-double-bond "trienes," are generated from arachidonic acid by 5-lipoxygenase/5-lipoxygenase–activating protein. Conversion of LTA_4 to LTC_4 by means of addition of glutathione is the first step to generating cysLTs. LTA_4 hydrolase converts LTA_4 to LTB_4. The major sources of LTC_4S (and thus cysLTs) are eosinophils, basophils, and macrophages. Mast cells make both LTC_4S and, along with neutrophils and macrophages, LTA_4 hydrolase.

CYSTEINYL LEUKOTRIENE RECEPTOR (CysLTR): Both cysLT receptors and LTB_4 receptors are 7-transmembrane GPCRs. CysLT receptors can be upregulated by IL-4. LTB_4 uses 2 receptors, BLT1 and BLT2, which are expressed on most tissues and upregulated by IFN-γ and promote neutrophil chemotaxis when activated by LTB_4.

DYNAMIC COMPLIANCE: Airway compliance is a measure of volume change per unit of pressure. Lungs from patients with longstanding asthma have been reported to show decreased compliance, perhaps because of airway remodeling and associated fibrosis.

INDOMETHACIN: Indomethacin inhibits COX-1 and can trigger AERD.

IL-6: IL-6 primes for T_H2 effector cells, inhibits the suppressive functions of $CD4^+CD25^+$ regulatory T cells, and protects mast cells from apoptosis.

IL-10: Generally associated with dampening immune responses, IL-10 decreases mast cell functions, such as IgE-mediated activation and anaphylaxis, in murine models.

IL-13: IL-13 and IL-6 can be produced by mast cells in response to activating signals, such as IL-33.

"LUKASTS": Montelukast, pranlukast, and zafirlukast all block the $CysLT_1R$, whereas biosynthetic pathway inhibitors, such as zileuton, block the production of both cysLTs and LTB_4.

MACROPHAGE INFLAMMATORY PROTEIN 1β (MIP-1β): MIP-1 is involved in the chemotaxis and activation of monocytes. CysLTs can induce MIP-1β and MIP-1α production from monocytes through the $CysLT_1R$.

PERMEABILITY-ENHANCING ACTIVITY: Activated vascular endothelium allows leakage of dyes (eg, Evans blue and fluorescein isothiocyanate–albumin) into the extravascular space, making tissues appear colored (blue) or fluorescent (fluorescein isothiocyanate).

PERTUSSIS TOXIN: Pertussis toxin inhibits the function of GPCRs through adenosine diphosphate ribosylation of Gα.

PULMONARY RESISTANCE: Mechanical factors that limit alveolar access to air. Pulmonary resistance is calculated by using Ohm's and Poiseuille's laws, which factor in the pressure difference at the mouth and alveoli, the rate of airflow, viscosity, and the length and radius of the airways.

PROSTAGLANDIN D_2 (PGD_2): A mast cell eicosanoid made in large quantities after IgE-mediated mast cell activation. PGD_2 levels are increased after allergen challenge, and it functions as a bronchconstrictor and vasodilator and is associated with eosinophil influx.

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ (PPAR-γ): Transcription factors in the nuclear hormone receptor superfamily that binds retinoic acid. PPAR-γ1 and PPAR-γ2 are expressed in adipocytes and decrease proinflammatory cytokine production from macrophages, B and T cells, eosinophils, dendritic cells, and airway epithelium.

RESPIRATORY BURST: The neutrophil oxidative burst generates superoxide anions and reactive oxygen intermediates important for microbial killing through the reduced nicotinamide adenine dinucleotide phosphate oxidase complex. Mutations in this complex (gp91, $gp67^{phox}$, gp22, and $gp47^{phox}$) cause chronic granulomatous disease and recurrent infections with organisms such as *Staphylococcus aureus*, *Aspergillus* species, *Serratia* species, and *Burkholderia cepacia*.

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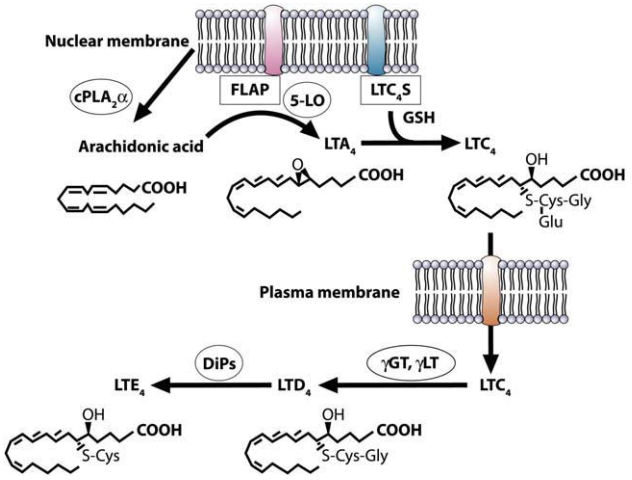

FIG 1. Biosynthesis and molecular structures of cysLTs. Cytosolic phospholipase $A_2\alpha$ *(cPLA$_2\alpha$)* catalyzes the liberation of arachidonic acid from nuclear membranes. 5-Lipoxygenase *(5-LO)* translocates to the nuclear envelope, requiring the integral membrane protein 5-LO–activating protein *(FLAP)* to convert arachidonic acid to the precursor LTA_4. LTA_4 is further conjugated to reduced glutathione *(GSH)* by LTC_4S, forming LTC_4, the first committed molecule of the cysLTs. After energy-dependent export, LTC_4 is converted by the extracellular enzymes γ-glutamyl transpeptidase *(γGT)* or γ-glutamyl leukotrienase *(γLT)* to LTD_4 and to LTE_4 by dipeptidases *(DiPs)*.

undergoing IgGa-dependent anaphylaxis was comprised of 3 products, all with 3 conjugated double bonds and each having contractile activity for the guinea pig ileum. By comparison with active and inactive standards with different peptide adducts, we recognized the 3 components of authentic SRS-A to elute with the retention times of the previously defined LTC_4[4,5] and 2 additional theoretic structures, LTD_4 and LTE_4.[6] These 2 additional structures differed from LTC_4 in that the former possessed the sulfur-linked glutathione tripeptide adduct composed of glutamic acid, glycine, and cysteine, whereas LTD_4 lacked the glutamic acid residue and LTE_4 lacked both the glutamic acid and glycine residues (Fig 1). Because LTC_4 is the only intracellular cysLT generated by LTC_4 synthase (LTC_4S),[7] it seemed likely that LTD_4 was formed extracellularly from LTC_4 by means of deletion of glutamic acid (by a γ-glutamyl transpeptidase or γ-glutamyl leukotrienase) after the export of the former compound to the extracellular space. Further removal of glycine from the remaining dipeptide adduct of LTD_4 by dipeptidases accounted for LTE_4 with a remaining cysteine adduct.[6,8] That LTC_4 underwent enzymatic modification in the extracellular space accounted for the fact that it was the only component of SRS-A to be detected in single-cell systems, whereas all 3 components were detected in biologic fluids. These early studies not only showed natural SRS-A to be composed of 3 cysLTs but also demonstrated that each cysLT had contractile activity for ileal smooth muscle *in vivo* (Fig 2) and ***permeability-enhancing activity*** by means of intradermal injection into the guinea pig prepared with Evans blue dye.[6]

Members of the pharmaceutical industry then used contractile assays to characterize the putative "receptors" for cysLTs and to identify potential antagonists. This approach permitted the development of the prototypes of the orally available $CysLT_1R$-selective antagonists ***("lukasts")*** more than a decade before any receptor was defined in molecular terms. Human $CysLT_1R$ and $CysLT_2R$ were cloned by Evans and colleagues.[9,10] $CysLT_1R$ exhibited a marked preference for binding of LTD_4 over LTC_4 and was the only receptor that was competitively blocked by the lukasts. $CysLT_2R$ had equal affinity for LTD_4 and LTC_4 and bound LTD_4 at 10-fold lower affinity than did $CysLT_1R$. It was surprising that LTE_4 did not register as an appreciable binding ligand for these "classical" receptors expressed individually in cloned cells. The poor affinity of LTE_4 for these cloned receptors prompted some to suggest that LTE_4 was a relatively impotent extracellular metabolite and perhaps discouraged others from seeking a third receptor. In contrast, we believed that the LTE_4 agonist activity that had been demonstrated in pharmacologic studies in guinea pigs and human subjects was impressive and that the greater stability of LTE_4 relative to the other cysLTs might favor a distinct pathobiologic role. The sections that follow will consider some of the early findings for LTE_4, favoring the existence of a distinct receptor and revealing its relative biologic stability. We will also consider the noteworthy potency of LTE_4 as a contractile agonist in guinea pig airways and in the human microvasculature, as well as a proinflammatory function based on studies using aerosolization challenge in human subjects with asthma and in allergen-sensitized mice. These studies have been key to the recognition of 2 functional receptors activated by LTE_4 by 2 different laboratories with different experimental approaches.

EARLY PHARMACOLOGY OF LTE_4 IN ANIMALS

The potency of LTE_4 for contraction of guinea pig tracheal spirals *in vitro* was 10-fold greater than that of either LTC_4 or LTD_4, whereas for guinea pig parenchymal strips, the potency of LTD_4 was 6-fold that of LTE_4 and 20-fold that of LTC_4. Furthermore, the concentration effect for LTD_4 and LTE_4 on parenchymal strips observed by Drazen et al[12] was biphasic, with the initial low concentration effect (studied only for LTD_4) being competitively antagonized by FPL55712. In contrast, LTC_4 was the least potent ligand and produced only a linear response.[11,12] When these ligands were administered intravenously to the intact anesthetized or unanesthetized guinea pig, LTD_4 and LTC_4 elicited a small increase in ***pulmonary resistance*** compared with the magnitude of the decrease in ***dynamic compliance***, whereas LTE_4 decreased compliance together with a robust increase in resistance, indicating both peripheral and central airway effects.[12,13]

Another distinctive effect of LTE_4 was that it enhanced the contractile responses of the guinea pig tracheal smooth muscle to histamine, a property not shared with LTC_4 or LTD_4. The latter effect of LTE_4 could be prevented by treatment of the tracheal tissue with ***indomethacin***, indicating a key role for a COX product.[14] Together, these *in vitro* and *in vivo* functional findings suggested the presence of 3 receptors for cysLTs: a high-affinity receptor for LTD_4, a lower-affinity receptor for LTC_4, and a separate receptor for LTE_4, with the latter potentially capable of eliciting the secondary production of a prostanoid (Table I).[12]

The observed ratio of potency for the 3 cysLTs in different tissues could reflect not only the profile of receptor expression in the target tissue but also the rate of conversion of 1 cysLT to another of greater or lesser activity. This is readily demonstrated when LTC_4 and LTD_4 are separately applied to the guinea pig ileum at concentrations sufficient to result in their maximum isotonic responses. Tritiated LTC_4 has a 60-second latent period before initiating a linear contractile response to 80% of maximum over 2 minutes, which is followed by a further contraction associated with slow metabolism to the more potent tritiated LTD_4.

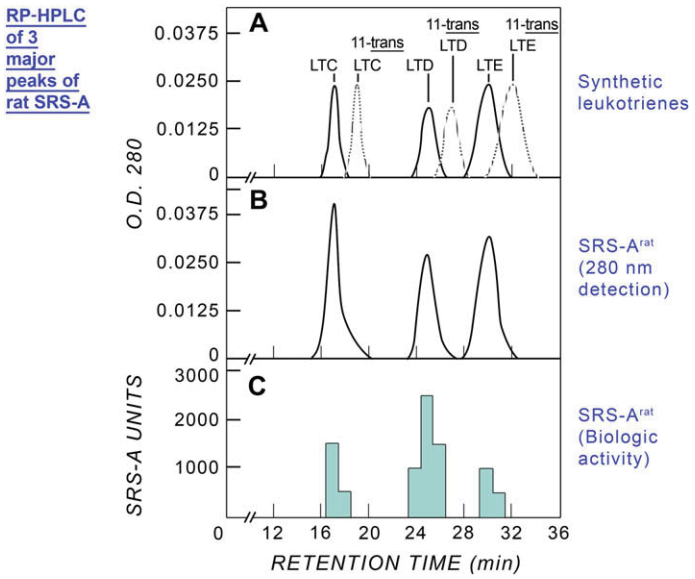

FIG 2. Resolution by means of RP-HPLC of 3 major peaks of SRS-A produced in the peritoneal cavities of rats. **A,** retention times of synthetic cysLTs. **B,** Retention times of the resolved natural components of SRS-A. **C,** Arbitrary units of biologic activity of these natural components for contraction of the guinea pig ileum. Reproduced from Lewis et al[6] with permission from Elsevier, Inc.

TABLE I. Effects of various LTs on airways

	LT		
	C	D	E
*In vitro**			
Parenchymal strip	1/300	1/6000	1/1000
Tracheal spiral	1/30	1/30	1/300
In vivo†			
C_{dyn}	3+	4+	3+
R_L	1+	1+	3+

Reproduced from Drazen et al[12] with kind permission of Springer Science and Business Media.

C_{dyn}, Dynamic compliance; R_L, lung resistance.

**In vitro* activity is recorded as the ratio of the molar concentration of LTs required to achieve a half-maximal response to the concentration of histamine required to achieve an equivalent response.

†*In vivo* activity is recorded as the response to infusion of 3 μg/kg LT (*1+*, minimal response; *4+*, maximal response).

There is negligible conversion of tritiated LTC_4 to tritiated LTD_4/LTE_4 during the linear phase. Furthermore, the inclusion of serine borate to block bioconversion of LTC_4 to LTD_4 by membrane γ-glutamyl transpeptidase does not change the response of the tissue to LTC_4, thus confirming that this response is mediated by a specific LTC_4 receptor.[15] In contrast, tritiated LTD_4 initiated an immediate linear contraction that reached maximum at 1 minute and then decreased sharply with linear conversion to tritiated LTE_4, which has only one quarter the potency of LTD_4 in this assay. When the mucosa containing the dipeptidase activity had been removed from the ileal muscle, the linear contractile response to tritiated LTD_4 was maintained, reflecting the loss of metabolism to tritiated LTE_4 by means of removal of the glycine. These findings nicely reflect the exquisite receptor specificity of the cysLT system that is conferred by modifications of the peptide adduct (Fig 3).

EARLY STUDIES OF LTE_4 METABOLISM

The products of the granulocyte ***respiratory burst***, which are abundant with inflammation, can alter the stability of each cysLT *in vitro* and *in vivo*. Phorbol 12-myristate 13-acetate–activated human neutrophils converted each cysLT to their subclass-specific S-diastereoisomeric sulfoxides, which retained their ability to be detected by cysLT–specific antibodies but lost greater than 95% of function. Each sulfoxide was further processed to identical diastereoisomers of 6-trans LTB_4, which were nonfunctional and no longer immunoreactive with the original antibodies. This neutrophil-mediated inactivation involved interaction of released myeloperoxidase, newly generated H_2O_2, and extracellular chloride ion to form hypochlorous acid. Dose-dependent attack on the sulfur bridge during hypochlorous acid formation showed that LTE_4 was substantially more resistant than the other cysLTs.[16,17] Systemic metabolism of the cysLTs begins after export of intracellular LTC_4 and its rapid extracellular physiologic sequential conversion through LTD_4 to LTE_4. Studies using intravascular administration of labeled LTE_4 or LTC_4 to human subjects indicate that approximately 5% is recovered in the urine and is composed of LTE_4 and N-acetyl LTE_4. At the level of tissue peroxisomes, omega oxidation at the C-terminus yields 20-COOH-LTE_4 and formation of the 20-CoA ester, which allows for sequential β oxidation with shortening of the carbon chain to 18-COOH-dinor-LTE_4 and beyond.[18-20]

EARLY PHARMACOLOGY OF LTE_4 IN HUMAN SUBJECTS

Although the early pharmacology of the 3 sequentially generated cysLTs identified LTE_4 as the most stable in physiologic and pathobiologic models, clinical attention shifted to LTD_4 and LTC_4, which on inhalation were up to 1000 times as potent as histamine.[21,22] LTE_4 was only 39 times as potent as histamine in reducing maximum expiratory flow at 30% of vital capacity

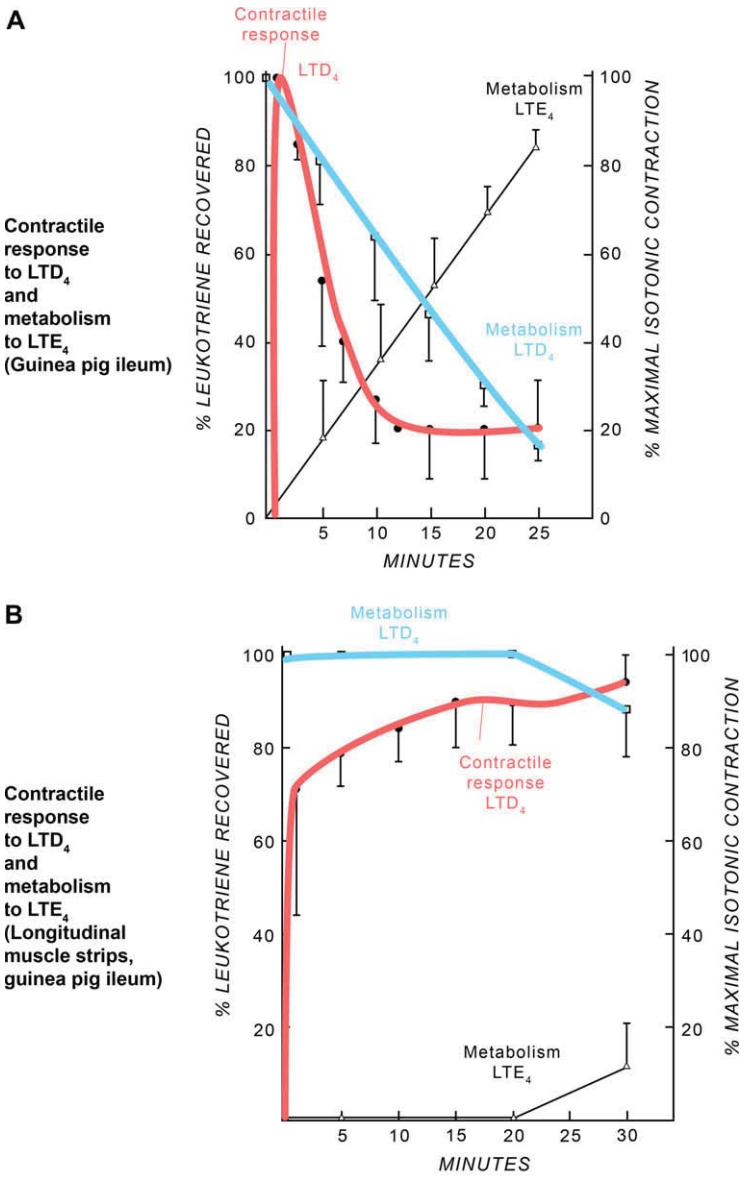

FIG 3. LTD_4-elicted contraction of guinea pig ileum and associated metabolism to LTE_4. **A,** Time course of contractile response to 3.6 ng of tritiated LTD_4 from guinea ileum expressed as a percentage of maximal response *(solid circles)* and of metabolism of tritiated LTD_4 *(open squares)* to tritiated LTE_4 *(open triangles)* expressed as a percentage of total labeled LTs recovered. **B,** Time course of contractile response to 3.6 ng of tritiated LTD_4 from longitudinal muscle strips of guinea pig ileum expressed as a percentage of maximal response *(solid circles)* and of metabolism of tritiated LTD_4 *(open squares)* to tritiated LTE_4 *(open triangles)* expressed as a percentage of total LTs recovered. Reproduced from Krilis et al[15] with permission from the American Society for Clinical Investigation.

in healthy human subjects.[21-23] Although each cysLT was a potent bronchoconstrictor in patients with bronchial asthma, there was little difference between asthmatic and healthy control subjects in sensitivity to cysLTs, which is in contrast to the hyperresponsiveness to histamine or methacholine that is characteristic of asthma.[24] An exception to that rule is in ***aspirin-exacerbated respiratory disease***, an asthma variant associated with marked overproduction of the cysLTs. In these subjects Christie et al[25] showed selective hyperresponsiveness to LTE_4, but not to LTC_4, relative to that seen in aspirin-tolerant asthmatic subjects.

Because we had recognized that LTE_4 induced permeability in guinea pig skin over the same dose range (5.0-50 ng) as for LTC_4 and LTD_4,[6] we compared their action at 1.0 nmol per site by means of intradermal injection in 3 human volunteers. Each cysLT elicited a wheal-and-flare response by 10 minutes, which peaked at 1 to 2 hours with a 10- to 20-mm wheal and a 20- to 25-mm flare. The wheal resolved by 4 hours (Fig 4), whereas the flare was still evident at 6 hours.[26] The 3 cysLTs produced equiactive responses in each subject. Biopsy specimens at 2 hours showed dermal edema, marked and uniform dilation of the microvasculature,

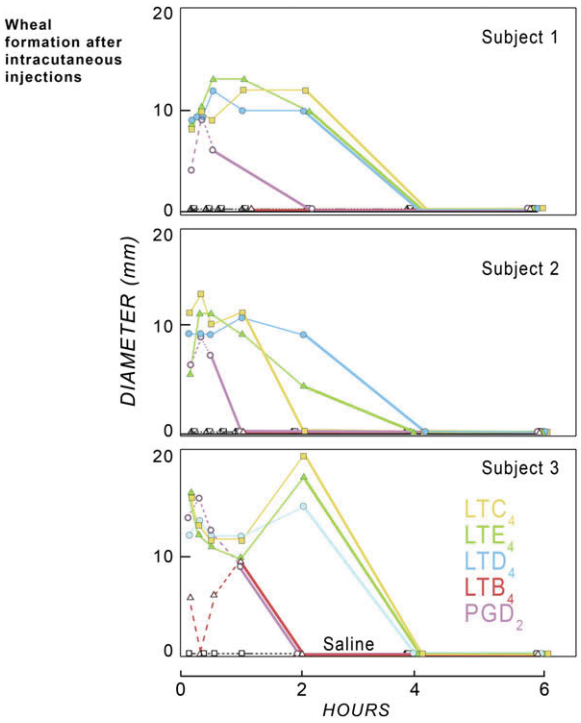

FIG 4. Wheal formation occurring with intracutaneous injections of various eicosanoids into 3 human subjects. The agonists were LTC_4 (*solid squares*; 1.0 nmol per site), LTD_4 (*solid circles*; 1.0 nmol per site), LTE_4 (*solid triangles*; 1.0 nmol per site), LTB_4 (*open triangles*; 1.6 nmol per site), PGD_2 (*open circles*; 3.0 nmol per site), and saline *(open squares)*. The greatest diameter of the wheal in millimeters is depicted versus time from 10 minutes to 6 hours. Reproduced from Soter et al[26] with permission from Nature Publishing Group.

and deep venules with activation of endothelial cells and some dilation of arterioles. That LTE_4 was apparently as potent a permeability factor as LTC_4 and LTD_4 in 2 species clearly indicated that it was not a disposal product. The advent of molecular biology and the development of gene-deleted mice later permitted the use of murine microvasculature to seek a functional receptor for LTE_4.

FUNCTIONAL AND PHARMACOLOGIC CHARACTERIZATION OF $CysLT_ER$, A CUTANEOUS RECEPTOR PREFERENTIAL FOR LTE_4

In addition to addressing the pharmacology of the cysLTs during the 1980s, we began to characterize LTC_4S, the integral protein of the outer nuclear membrane responsible for biosynthesis of LTC_4, by means of conjugation of glutathione to LTA_4.[7] After expression cloning of human LTC_4S and then homology cloning of murine LTC_4S,[27,28] we turned to targeted disruption of murine LTC_4S to explore for phenotypic characteristics that might depend on the functions of the cysLTs.[29] In a model of passive cutaneous anaphylaxis, there was more than 50% reduction in ear swelling (indicative of vascular leak) in LTC_4S-deficient mice *($Ltc4s^{-/-}$)* compared with wild-type (WT) mice after local sensitization of mast cells with specific IgE and systemic challenge with hapten-specific antigen. Thus the permeability-enhancing function of mast cell–derived cysLTs was at least as important as the preformed amines in this model. To analyze the contributions from individual cysLT receptors, we next generated strains deficient in $CysLT_1R$ *($Cyslt1r^{-/-}$)* and $CysLT_2R$ *($Cyslt2r^{-/-}$)*, respectively,[30,31] based on the prior cloning of these 7-transmembrane, human G protein–coupled receptors (GPCRs).[9,10] In response to an intraperitoneal injection of zymosan, a yeast cell-wall material that elicits cysLT generation from macrophages, both the *$Ltc4s^{-/-}$* and *$Cyslt1r^{-/-}$*, but not the *$Cyslt2r^{-/-}$*, strains showed approximately 50% reductions in vascular leak, implying a key role for cysLTs acting at $CysLT_1R$ for this innate immune response.

The responsiveness of the murine vasculature to the cysLTs suggested that the existence of a distinct LTE_4-reactive receptor could be proved by studying cysLT-dependent swelling responses in mice deficient in both receptors. This strain *($Cyslt1r/Cyslt2r^{-/-}$)* was created by intercrossing the *$Cyslt1r^{-/-}$* and *$Cyslt2r^{-/-}$* strains.[32] The resulting double-receptor deficiency of the *$Cyslt1r/Cyslt2r^{-/-}$* strain was confirmed by the absence of both receptor transcripts. We then examined the dose-dependent ear edema elicited by each cysLT in the respective *$Cyslt1r^{-/-}$*, *$Cyslt2r^{-/-}$*, and *$Cyslt1r/Cyslt2r^{-/-}$* strains.[32] The dose-dependent ear edema elicited by means of injection of LTD_4 and LTC_4 in the *$Cyslt1r/Cyslt2r^{-/-}$* strain was equivalent to that in the WT control animals, indicating the presence of a previously unrecognized receptor. The *$Cyslt1r/Cyslt2r^{-/-}$* mice were especially sensitive to LTE_4, exhibiting the same extent of ear swelling in response to an LTE_4 dose of 0.008 nmol as the response of the WT mice to 0.5 nmol (a 64-fold increase in sensitivity to LTE_4). Histologic analysis of biopsy specimens at 30 and 240 minutes showed an exaggerated magnitude and duration of ear edema without cellular infiltration in response to LTE_4 in the *$Cyslt1r/Cyslt2r^{-/-}$* strain. The LTE_4-mediated vascular leak in the *$Cyslt1r/Cyslt2r^{-/-}$* strain was markedly inhibited by pretreatment of the mice with ***pertussis toxin*** or a Rho kinase inhibitor, supporting that the mechanism involved a GPCR linked to Gαi proteins and Rho kinase.[32] Additionally, the response to LTE_4 was blocked by approximately 30% by means of treatment of the mice with indomethacin, which is reminiscent of the indomethacin sensitivity of the LTE_4 response of guinea pig tracheal rings.[14] The particular sensitivity of this novel receptor to LTE_4 prompts the designation of $CysLT_ER$ rather than a number until it is cloned (Fig 5).

The discovery of a $CysLT_ER$ prompted us to re-evaluate the findings in WT mice and single-receptor-deficient strains. The permeability response to 0.5 nmol LTC_4 or LTD_4 was 50% reduced in *$Cyslt1r^{-/-}$* mice and normal in magnitude but delayed in *$Cyslt2r^{-/-}$* mice, suggesting that $CysLT_1R$ is the major signaling receptor for LTC_4 and LTD_4, whereas $CysLT_2R$ is a negative regulator of $CysLT_1R$. LTE_4-elicited vascular leak was not attenuated in the *$Cyslt1r^{-/-}$* mice but delayed and sustained in the *$Cyslt2r^{-/-}$* strain, suggesting that $CysLT_ER$ is the dominant receptor for this ligand and that $CysLT_2R$ is again a negative regulator. That the enhanced sensitivity to LTE_4-induced ear edema observed in the *$Cyslt1r/Cyslt2r^{-/-}$* strain was not seen with either single-receptor-null strain, *$Cyslt1r^{-/-}$* or *$Cyslt2r^{-/-}$*, implies that both $CysLT_2R$ and $CysLT_1R$ negatively regulate $CysLT_ER$. Indeed, administration of the selective $CysLT_1R$ antagonist MK571 to *$Cyslt2r^{-/-}$* mice mimicked the phenotype of the *$Cyslt1r/Cyslt2r^{-/-}$* strain in terms of the markedly increased vascular leak of the ear to intradermal LTE_4. This, of course, meant that MK571 was not an inhibitor of $CysLT_ER$. Curiously, pretreatment with MK571 had opposing effects in WT mice and the *$Cyslt1r/Cyslt2r^{-/-}$* strain.[32] Specifically, MK571 suppressed swelling of the skin in WT mice challenged intradermally with 0.5 nmol of LTD_4, LTC_4, or LTE_4. In contrast, the same MK571 pretreatment and dose of ligand produced an enhanced

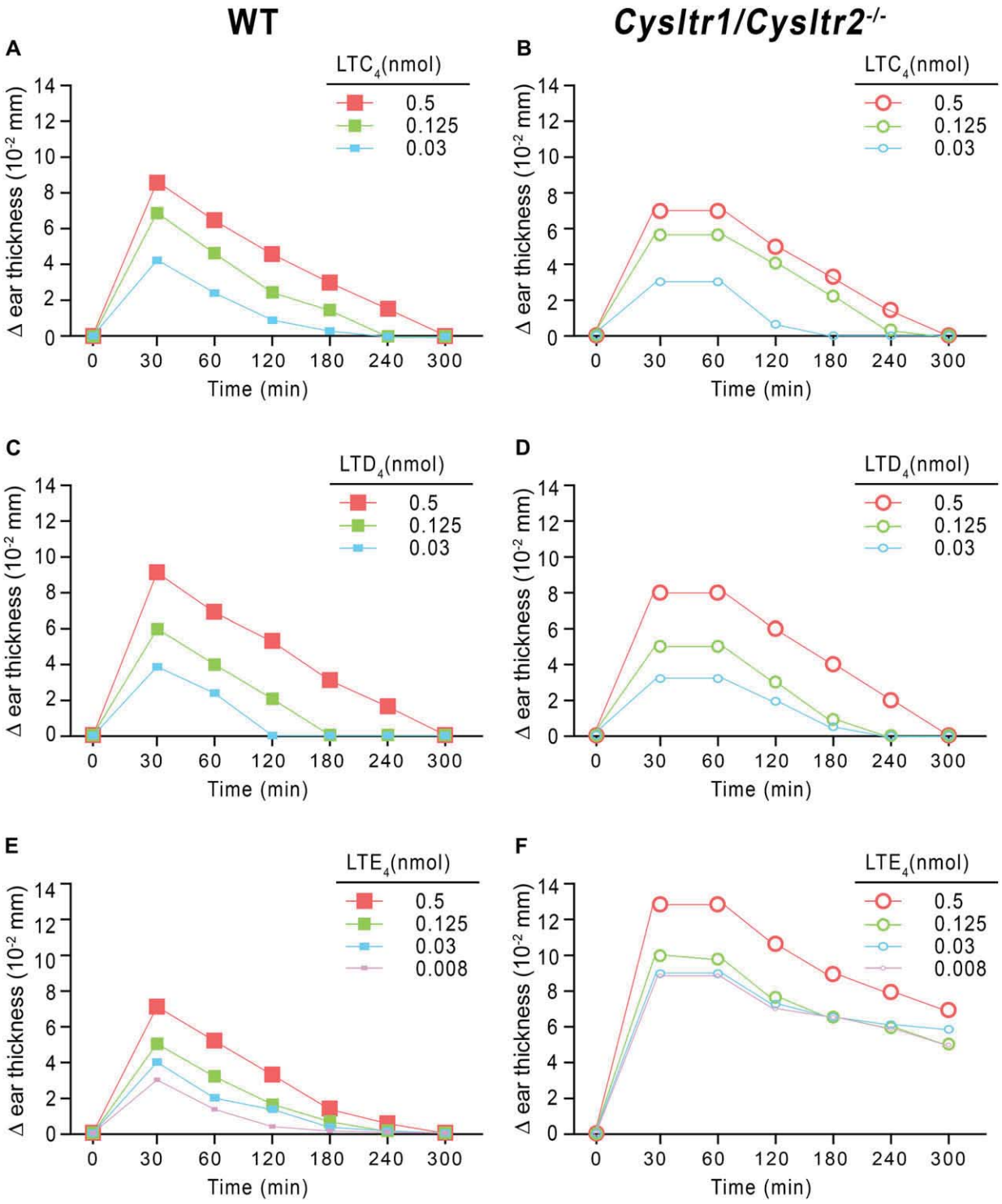

FIG 5. Dose dependence of LTC_4-, LTD_4-, and LTE_4-induced ear edema in WT and *Cysltr1/Cysltr2*$^{-/-}$ mice. WT (**A, C,** and **E**) and *Cysltr1/Cysltr2*$^{-/-}$ (**B, D,** and **F**) mice received intradermal injections of LTC_4 (Fig 5, *A* and *B*), LTD_4 (Fig 5, *C* and *D*), or LTE_4 (Fig 5, *E* and *F*) in the right ear and vehicle in the left ear (2 mice per group). Ear thickness was measured with calipers at the indicated times after the injection. *Error bars* indicate SDs. Reproduced from Maekawa et al[32] with permission from the *Proceedings of the National Academy of Sciences*.

response to cysLTs in *Cysltr1/Cysltr2*$^{-/-}$ mice. Thus MK571, which is a prototype of the lukast drugs, potentiates responses apparently mediated through $CysLT_ER$, in a setting in which neither $CysLT_1R$ nor $CysLT_2R$ is present to impart negative regulation. It is possible that MK571, which is now known to block certain transporter proteins and some purinergic (P2Y) receptors for nucleotides,[33,34] might block a yet-to-be-defined receptor with negative regulatory properties for $CysLT_ER$.

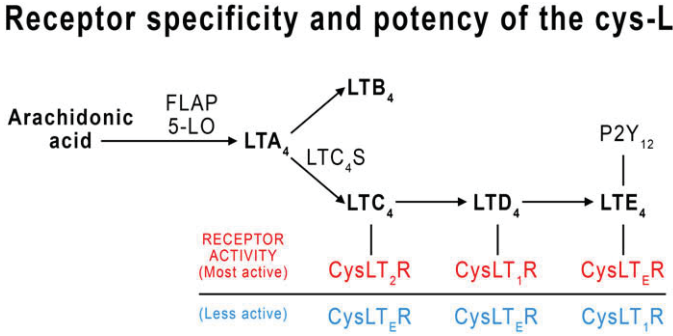

FIG 6. Schematic presentation of the diversity of the cysLT receptor system. *5-LO,* 5-Lipoxygenase; *FLAP,* 5-LO–activating protein.

DISCOVERY THAT THE $P2Y_{12}$ RECEPTOR MEDIATES MAST CELL ACTIVATION AND PULMONARY INFLAMMATION BY LTE_4

As is the case for many effector cells of bone marrow origin, mast cells express both $CysLT_1R$ and $CysLT_2R$.[35,36] LTC_4 and LTD_4 both induce calcium flux, cytokine and chemokine generation, phosphorylation of extracellular signal-regulated kinase (ERK), and proliferation of human mast cells *in vitro*.[35-37] These responses, like those of the cutaneous microvasculature, are regulated positively by $CysLT_1R$ but negatively regulated by $CysLT_2R$ based on experiments in which each receptor is selectively knocked down by using RNA interference in primary human mast cells.[38] During these studies, Jiang et al[38] made the unanticipated finding that LTE_4 exceeded the potency of LTC_4 and LTD_4 for increasing the numbers of human mast cells arising from cultures of cord blood–derived progenitor cells maintained in the presence of stem cell factor, ***IL-6***, and ***IL-10***.[37] Subsequently, Paruchuri et al[39] demonstrated that LTE_4 not only exceeded the potency of LTC_4 and LTD_4 as a mitogen for a human mast cell line, LAD2, but far exceeded its potency for causing the production of the inflammatory chemokine ***macrophage inflammatory protein-1***β (MIP-1β) and was also substantially more potent for causing the expression of inducible ***COX-2*** and promoting delayed ***prostaglandin D_2*** (PGD_2) generation. Curiously, the latter effects required the activation of ***peroxisome proliferator-activated receptor*** γ (PPAR-γ), a nuclear transcription factor that is activated by several dietary lipids and eicosanoids. However, the effect of LTE_4 on PPAR-γ is indirect because LTE_4 failed to activate a PPAR-γ–driven reporter in bovine endothelial cells.[39] Indeed, the effects of LTE_4 on PGD_2 generation and MIP-1β production were sensitive to MK571 and pertussis toxin, whereas LTE_4-mediated ERK activation was insensitive to MK571, and all LTE_4 responses were completely resistant to knockdowns of $CysLT_1R$ and $CysLT_2R$. It was thus clear that mast cells expressed at least 1 previously unrecognized LTE_4 receptor that was MK571 resistant (and perhaps another that was sensitive).

Based on sequence homology between $CysLT_1R$, $CysLT_2R$, and the P2Y receptor family, it seemed likely that a putative $CysLT_3R$ might be among the orphan P2Y-like GPCRs or even a known member. Human mast cells express several such receptors,[40] including the $P2Y_{12}$ receptor, a Gαi-linked receptor for adenosine diphosphate and the target of thienopyridine antithrombotic drugs. Because a computer modeling study had predicted that LTE_4 might be a surrogate ligand for this receptor,[41] we sought to determine whether recombinant $P2Y_{12}$ receptors reacted to LTE_4 and mediated the LTE_4-dependent signaling events recognized in mast cells. LTE_4 induced the activation of ERK in Chinese hamster ovary cells stably transfected with human $P2Y_{12}$ receptors exceeding the potency of LTD_4. This signaling event was sensitive to pertussis toxin but resistant to MK571 (unpublished data). Although $P2Y_{12}$ did not bind LTE_4 directly, knockdown of $P2Y_{12}$ receptors by RNA interference blocked LTE_4-mediated MIP-1β generation and PGD_2 production by LAD2 cells without significantly altering their responses to LTD_4. Because LTE_4 (but not LTD_4) was previously shown to induce bronchial eosinophilia when administered by means of inhalation to asthmatic human subjects,[42] we sought to determine whether pulmonary inflammation amplified by LTE_4 in mice depended on $P2Y_{12}$ receptors. Administration of LTE_4, but not LTD_4, to the airways of sensitized BALB/c mice potentiated eosinophilia, goblet cell metaplasia, and expression of ***IL-13*** in response to low-dose aerosolized ovalbumin. These effects were completely intact in the *Cyslt1r/Cyslt2r*$^{-/-}$ mice but were completely blocked by oral administration of the $P2Y_{12}$ receptor–selective antagonist clopidogrel. The effect of $P2Y_{12}$ receptor blockade was similar to the effect of platelet depletion with an antibody, suggesting that LTE_4 acted as an agonist for platelet activation in the pulmonary vasculature in this model. Importantly, clopidogrel had failed to block the response of the murine skin microvasculature to LTE_4, indicating that $P2Y_{12}$ receptors are separate and distinct from the $CysLT_ER$ in the skin.[32]

EPILOGUE

At this early stage, clinical considerations must be circumspect and limited based on these findings for receptors in naive mice in model systems or with targeted disruption of classical receptors. Nonetheless, the history of cysLT-mediated permeability effects in guinea pigs and human subjects suggests that this important aspect of the inflammatory process is as responsive to LTE_4 as to its precursors, LTC_4 and LTD_4, which are only transiently present during an inflammatory process. The finding that the classical $CysLT_1R$ and $CysLT_2R$ are negative regulators of $CysLT_ER$ function in mice was certainly unexpected but is supported by literature showing $CysLT_2R$ to be a negative regulator of $CysLT_1R$ for murine and human mast cell proliferation[38] and by the potentiation of LTE_4-mediated skin swelling of *Cyslt2r*$^{-/-}$ mice occurring in the presence of a $CysLT_1R$ antagonist.[32] In an inflammatory process it seems possible that the generation of

LTC_4/LTD_4 and occupancy of classical receptors followed by receptor internalization[43] could allow increased $CysLT_ER$ function. The identification of at least 2 LTE_4-reactive GPCRs provides potential mechanistic explanations for the potency of LTE_4 as an inducer of vascular permeability and potentiator of mucosal inflammation, which were identified by previous pharmacologic profiling studies in human and guinea pig tissues.[13-15,25,26,42] Moreover, the fact that $P2Y_{12}$ (and not $CysLT_ER$) is responsible for LTE_4-mediated activation and proliferation of mast cells, as well as amplification of allergic pulmonary inflammation, indicates that the receptors for LTE_4 evolved to serve functions that are anatomically and contextually distinct yet potentially complementary in inflammation (Fig 6).

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