Attenuated Zymosan-induced Peritoneal Vascular Permeability and IgE-dependent Passive Cutaneous Anaphylaxis in Mice Lacking Leukotriene C₄ Synthase*  

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Leukotriene C₄ synthase (LTC₄S), the terminal 5-lipoxygenase pathway enzyme that is responsible for the biosynthesis of cysteinyl leukotrienes, has been deleted by targeted gene disruption to define its tissue distribution and integrated pathway function in vitro and in vivo. The LTC₄S−/− mice developed normally and were fertile. LTC₄S activity, assessed by conjugation of leukotriene (LT) A₄ methyl ester with glutathione, was absent from tongue, spleen, and brain and ≥ 90% reduced in lung, stomach, and colon of the LTC₄S−/− mice. Bone marrow-derived mast cells (BMMC) from the LTC₄S−/− mice provided no LTC₄ in response to IgE-dependent activation. Exocytosis and the generation of prostaglandin D₂, LTB₄, and 5-hydroxyeicosatetraenoic acid by BMMC from LTC₄S−/− mice and LTC₄S+/+ mice were similar, whereas the degraded product of LTC₄, 6-trans-LTB₄, was doubled in BMMC from LTC₄S−/− mice because of lack of utilization. The zymosan-elicited intraperitoneal extravasation of plasma protein and the IgE-mediated passive cutaneous anaphylaxis in the ear were significantly diminished in the LTC₄S−/− mice. These observations indicate that LTC₄, but not microsomal or cytosolic glutathione S-transferases, is the major LTC₄-producing enzyme in tissues and that its integrated function includes mediation of increased vascular permeability in either innate or adaptive immune host inflammatory responses.

The cysteinyl leukotrienes (cysLTs),¹ leukotriene (LT) C₄, and its metabolites, LTC₄ and LTE₄, are potent mediators of tissue inflammation, particularly implicated in allergic and asthmatic diseases (1, 2). In humans, inhalation of cysLTs constricts bronchial smooth muscle and attracts eosinophils, and intradermal injection elicits an increase in vascular permeability (3–6). The contribution of the cysLTs to the pathophysiology of bronchial asthma is established by the therapeutic efficacy of inhibitors of their biosynthesis (7) and antagonists of their receptor-mediated action (8). The cellular generation of LTC₄ requires activation with Ca²⁺-dependent translocation of cytosolic phospholipase A₂ and 5-lipoxygenase (5-LO) to the perinuclear and endoplasmic reticular membranes. There, in the presence of 5-LO-activating protein (FLAP), the arachidonic acid released by cytosolic phospholipase A₂ is converted to 5-hydroperoxyeicosatetraenoic acid and then to LTA₄ (9–11). LTA₄ is processed either to the dihydroxy leukotriene, LTB₄, by LTA₄ hydrolase (12) or to LTC₄ through conjugation with reduced glutathione by LTC₄ synthase (LTC₄S) (13–15). After carrier-mediated export of LTC₄ (16), glutamic acid and glycine are sequentially cleaved by γ-glutamyl transpeptidase and dipeptidase to form LTF₄ and LTE₄, respectively (17, 18). Two cysLT receptors, termed CysLT₁ and CysLT₂ receptors, are presently known. Whereas the CysLT₁ receptor has a marked preference for signal activation by LTA₄, the CysLT₂ receptor has a similar recognition of LTC₄ and LTF₄ with a higher Kᵩ value relative to that of the CysLT₁ receptor (19, 20). Mast cells also metabolize the released arachidonic acid to prostaglandin (PG) D₂ by the successive action of PG endoperoxide synthase-1 or PG endoperoxide synthase-2 (21, 22) and hematopoietic PGD synthase (23, 24). Whereas a PGD₂ receptor, termed DP, is prominent on smooth muscle such as airways and microvasculature, a recently identified PGD₂ receptor, termed CRTH₂, is localized to hematopoietic cells such as T helper type 2 (Th2) cells, basophils, and eosinophils (25, 26).

LTC₄S is an 18-kDa integral membrane protein that shows glutathione S-transferase (GST) activity that is strictly specific for LTA₄ as a substrate (13–15). The human LTC₄S cDNA encodes a protein of 150 amino acids and belongs to a recently recognized superfamily of membrane-associated proteins in eicosanoid and glutathione metabolism that includes FLAP and microsomal GSTs (MGSTs) (27). LTC₄S shows 44% amino acid identity with MGST2 (28) and 31% identity with FLAP. MGST2 and MGST3 (29) conjugate glutathione not only to xenobiotics but also to LTA₄ to form LTC₄, and they are ubiquitously expressed even in cells lacking the capacity to provide LTC₄. LTC₄ can be formed through the transcellular metabolism of LTA₄ by cells that express LTC₄S, such as platelets (30), or MGST2, such as endothelial cells (31). With the exception of platelets, LTC₄S has been identified only in hematopoietic cells that also express 5-LO.

We sought to establish that LTC₄S was the dominant constitutive enzymatic source of LTC₄ in situ in the mouse by targeted gene disruption of the LTC₄S gene. The loss of function in the gene-disrupted mice relative to their controls was

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The abbreviations used are: cysLT(s), cysteinyl leukotriene(s); LT, leukotriene; 5-LO, 5-lipoxygenase; FLAP, 5-LO-activating protein; LTC₄S, LTC₄ synthase; PG, prostaglandin; GST, glutathione S-transferase; MGST(s), microsomal GST(s); BMMC, bone marrow-derived mast cells; 5-HETE, 5-hydroxyeicosatetraenoic acid; PBS, phosphate-buffered saline; ME, methyl ester; RP-HPLC, reverse phase high-performance liquid chromatography; β-HEX, β-hexosaminidase; kb, kilobase; DNP, dinitrophenyl; HBSA++, Hanks’ balanced salt solution containing bovine serum albumin.
then used to quantitate the roles of LTC₄S in models of innate and adaptive immune inflammation. Zymosan, a yeast cell wall polysaccharide, was used to elicit an intraperitoneal extravasation of plasma proteins, and hapten-specific, IgE-mediated passive cutaneous anaphylaxis provided a permeability increment at the skin. We also assessed for the catalytic function of LTC₄S in various tissues relative to the bifunctional GSTs by the decrement in LTC₄ biosynthesis in the LTC₄⁻/⁻ mice. Finally, we determined whether the absence of LTC₄S in bone marrow-derived mast cells (BMMC) subjected to IgE-dependent activation would lead to increased non-enzymatic hydrolysis of the substrate LTA₄ to 6-trans-LTB₄ diastereoisomers or to the shunting of LTA₄ to LTA₄ hydrolase to form more LTB₄.

**EXPERIMENTAL PROCEDURES**

**Generation of LTC₄S gene-disrupted mice.**—A 5.7-kb 129/Ola mouse genomic fragment containing the LTC₄S gene (32) was subcloned into a pcDNA3 vector (Invitrogen). After the endogenous neomycin resistance gene (neo) of the pcDNA3 and ~400 base pairs of the 5′ region of the 5.7-kb genomic fragment were removed, a neo gene cassette from pMC1Neo Poly(A) (Stratagene) was inserted to replace 289 nucleotides of intron 1, exon II to exon IV, and 32 nucleotides of intron 4 of the mouse LTC₄S gene. The herpes simplex virus thymidine kinase (TK) gene was inserted at the 3′-end of the gene. The resultant targeting vector was linearized and electroporated into the embryonic stem cell line, AB2.2 (Stratagene). The embryonic stem cells were selected with G418 (200 µg/ml; Life Technologies, Inc.) and ganciclovir (2 µm) and homologous recombination was confirmed by Southern blot analysis of EcoRI-digested genomic DNA from each embryonic stem cell clone with the ~400-base pair 5′ fragment as a probe (see Fig. 1A). The verified embryonic stem cell clones were microinjected into blastocysts from C57BL/6 and BALB/c mice, and chimeric mice were obtained. Chimeras were bred to C57BL/6 and BALB/c mice, and offspring were genotyped by Southern blot analysis of tail DNA as described above. Heterozygotes were backcrossed to a C57BL/6 or BALB/c genetic background, and heterozygotes in the N₂ or N₃ generation were intercrossed to obtain homozygotes. All the experiments were performed with LTC₄S⁻/⁻ mice derived from a BALB/c background except for the zymosan-induced peritonitis model in which mice from the C57BL/6 background were utilized. All procedures were approved by the Harvard Medical Area Standing Committee on Animals.

**Culture of BMMC and LTC₄S mRNA Analysis**—Bone marrow cells were collected from femurs and tibiae of mice and cultured for 4–6 weeks in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM l-glutamine, 0.1 mM nonessential amino acids, 100 units/ml penicillin, 100 µg/ml streptomycin, and 1% culture supernatant from Chinese hamster ovary cells expressing mouse interleukin-3 (33, 34). The culture medium for the BMMC was changed every week, and the cell density was adjusted to 3 × 10⁶/ml at every passage. After 4 weeks, more than 97% of the cells were BMMC as assessed by staining with Wright-Giemsa and toluidine blue.

Total RNA was isolated from BMMC with TRI-Reagent (Sigma). A 20-µg sample of the total RNA was resolved by electrophoresis on a formaldehyde-denatured gel and transferred to a nylon membrane (Gelman Sciences) with 20× SSC for 2 h. The membrane was baked at 80 °C for 2 h, prehybridized at 42 °C for 2 h in 5× SSC, 5× Denhardt’s solution, 50% formamide, 0.2% SDS, 100 µg/ml denatured salmon sperm DNA, and then hybridized at 42 °C for 16 h with a 3²P-labeled mouse LTC₄S cDNA fragment prepared with a Megaprime DNA labeling kit (Amersham Pharmacia Biotech). The blot was washed once in 0.5× SSC, 0.1% SDS at 60 °C for 30 min and twice in 0.2× SSC, 0.1% SDS at 60 °C for 30 min and was then exposed to an Eastman Kodak Co. AR film for 24 h at −80 °C with an intensifying screen. The probe was stripped, and the blot was hybridized with a 3²P-labeled mouse glyceraldehyde-3-phosphate dehydrogenase cDNA probe.

**Enzyme Assay**—Mice were euthanized with CO₂, and various tissues were isolated and homogenated with a Tissue-Tearor homogenizer (BioSpec Products) in five volumes of a buffer containing 50 mM HEPES, 0.1 mM EDTA, 1 mM β-mercaptoethanol, pH 7.9. BMMC were washed, suspended in PBS-buffered saline containing 2 mM L-glutamine as a standard. Enzyme activity was assayed by spectrophotometric analysis of the hydrolysis of LTB₄-methyl ester (ME) (Dr. J. Rokach, Florida Institute of Technology, Melbourne, FL), which had been dried in nitrogen and dissolved in methanol containing 3% triethylamine, to give a final concentration in the reaction of 20 µM. After incubation for 10 min at room temperature, the reactions were terminated by the addition of three volumes of methanol containing 200 ng of PGB₂. Samples were analyzed for LTC₄, ME by reverse phase-high performance liquid chromatography (RP-HPLC) (14). Protein concentration was determined by the Bradford method (35) with bovine γ-globulin as a standard. Enzyme activity was expressed as pmol of LTC₄-ME/mg/10 min.

**IgE-dependent Activation of BMMC—**BMMC were suspended, washed at a concentration of 1 × 10⁶ cells/ml in Hanks’ balanced salt solution containing 1 mM CaCl₂, 1 mM MgCl₂, and 0.1% bovine serum albumin (HBSA2), and sensitized with 2 µg/ml monoclonal anti-dinitrophenyl (DNP) IgE (Sigma) for 1 h at 4 °C. After being washed with HBSA2, the cells were resuspended at a concentration of 1 × 10⁶ cells/ml in HBSA² and stimulated with 10 µg/ml goat anti-mouse immunoglobulin (Jackson ImmunoResearch). After 15 min, the reaction was stopped by centrifugation at 120 × g for 5 min at 4 °C, and the supernatants were retained for assays of β-hexosaminidase (β-Hex) and eicosanoids. The cell pellets were suspended in HBSA² and disrupted by repeated freeze-thawing. β-Hex, a marker of mast cell degranulation, was quantitated by spectrophotometric analysis of the hydrolysis of p-nitrophenyl-β-D-acetamido-2-deoxyglucopyranoside (36). The percent release of β-Hex was calculated by the formula $\frac{[S/S + P]}{[S]} \times 100$, where $S$ and $P$ are the β-Hex contents of equal portions of supernatant and cell pellet, respectively. PGD₂ was measured by enzyme immunoassay according to the manufacturer’s instructions (Cayman Chemical). Leukotrienes and 5-hydroxyeicosatetraenoic acid (5-HETE), the decay product of 5-hydroperoxyeicosatetraenoic acid, were measured by RP-HPLC as described (37). Briefly, samples were applied to a C18 Ultrasphere RP column (Beckman Instruments) equilibrated with a solvent of methanol/acetonitrile/water/acetic acid (10:15:100:0.2, v/v/v). After injection of the sample, the column was eluted at a flow rate of 1 ml/min with a programmed concave gradient, 55% of the equilibrated solvent A and 45% of Solvent B (100% methanol) over 2.5 min. After 5 min, Solvent B was increased linearly to 75% over 15 min and was maintained at this level for an additional 15 min. The UV absorbance at 280 and 235 nm and the UV spectra were recorded simultaneously. The retention times for PGB₂, LTC₄, 6-trans-LTB₄, LTB₄, LTE₄, and 5-HETE were 21.1, 21.6, 23.2, 24.2, 25.0, and 30.7 min, respectively. LTC₄, 6-trans-LTB₄, LTB₄, LTE₄, and 5-HETE were quantitated by calculating the ratio of each

**FIG. 1. Generation of LTC₄S gene-disrupted mice.** A, genetic organization of the mouse LTC₄S gene (upper), structure of the targeting vector (middle), and organization of the putative recombinant LTC₄S allele (lower). The five exons are shown as boxes with the coding regions in black. Restriction enzyme sites include BamHII (B), EcoRI (E), HindIII (H), PstI (P), Pmnl (Pnm), and XmnI (X). The location of a 400-base pair fragment used for Southern blot analysis is shown as a thick line. B, Southern blot analysis of EcoRI-digested tail DNAs from LTC₄S⁻/⁻, LTC₄S⁻/-, and LTC₄S⁺/⁺ mice. The hybrid blot analysis of RNAs from BMMC of LTC₄S⁻/⁻ and LTC₄S⁻/- mice and hybridizations were performed with a 3²P-labeled mouse LTC₄S cDNA probe (upper) and then with a 3²P-labeled mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) dehydrogenase cDNA probe (lower). Molecular size markers are shown at left.
peak area to the peak area of the internal standard PGB2.

Zymosan A-induced Peritoneal Inflammation—Each mouse received an intravenous injection of 0.5% Evans blue dye (10 ml of dye solution/kg of body weight) in PBS immediately before the intraperitoneal injection of 1 ml of zymosan A suspension (1 mg/ml in PBS; Sigma). Mice were euthanized by CO2 at time points of 10, 30, 60, and 120 min and underwent peritoneal lavage with 4 ml of cold PBS. Cells were sedimented from the lavage fluid by centrifugation at 500 g for 5 min, and Evans blue dye extravasation was assessed by light spectrophotometry of the supernatants at 610 nm. In separate experiments to determine the levels of LTC4 and cysLTs in the lavage fluid, mice were injected with zymosan A suspension without intravenous injection of Evans blue dye. After 2 h, the peritoneal lavage fluid was collected and centrifuged at 500 g x g for 5 min. After the addition of ~10,000 dpm of [3H]LTB4 and 100 ng of PGB2 in ethanol (4 times the volume of the supernatant), the lavage fluid supernatant was incubated on ice for 30 min and centrifuged at 10,000 g x g for 10 min at 4 °C. The ethanolic supernatant was evaporated by vacuum centrifugation, dissolved in 200 μl of 50 mM HEPES buffer, pH 7.1, containing 50% methanol, and analyzed by RP-HPLC as described above. The UV absorbance at 280 nm and the UV spectra were recorded simultaneously. The fraction containing [3H]LTB4 was collected, evaporated by vacuum centrifugation, and dissolved in 200 μl of enzyme immunoassay buffer for detection of LTB4 (Cayman Chemical). LTC4, the only cysLT detected, was quantitated against the internal standard as described above.

Passive Cutaneous Anaphylaxis—LTC4 S−/− and LTC4 S+/− mice received intradermal injections of 25 μg of mouse monoclonal anti-DNP IgE in 25 μl of saline in the right ear and 25 μl of saline only in the left ear. After 20 h, mice were injected intravenously with 100 μg of DNP-human serum albumin in 100 μl of PBS. At 0, 15, 30, 45, 60, 120, and 240 min after the intravenous injection, ear thickness was measured with calipers (Dyer Company). The difference between the thickness of the right and left ears at each time point reflects the extravasation of plasma proteins because of the alteration in vascular permeability induced by the activation of the local mast cells.

Statistical Analysis—Results of the experiments were expressed as means ± S.E. Student’s t test was used for the statistical analysis of the results. P values < 0.05 were considered to be significant.

RESULTS AND DISCUSSION

Generation of LTC4 S−/− Mice—The strategy to disrupt the LTC4S gene is shown in Fig. 1A. The neo insertion interrupts the coding sequence in exons II, III, and IV, including Arg-51 in exon II and Tyr-93 in exon IV, respectively, which are the critical residues for LTC4S activity (38). Mouse embryonic stem cells derived from the 129/Sv strain were transfected with the linearized targeting vector and selected with G418 and ganciclovir. Of 228 G418- and ganciclovir-resistant colonies isolated, 12 clones were identified as targeted clones by Southern blot analysis. The verified embryonic stem cell microinjected blastocysts from C57BL/6 and BALB/c mice for subsequent transfer to pseudopregnant ICR female mice. 8 and 4 chimeric males with more than 50% chimerism were obtained from C57BL/6 and from BALB/c blastocysts, respectively, and bred to C57BL/6 or BALB/c female mice. Of 12 chimeric males, 10 were found to be fertile and provided only 2 male heterozygotes from a total of 478 offspring, one from a C57BL/6 mother and the other from a BALB/c mother, as genotyped by Southern blot analysis. These heterozygotes were backcrossed to the respective wild-type females to produce heterozygotes (N2 generation), and N2 or N3 heterozygotes were interbred to generate LTC4 S−/− homozygous mice. Southern blot analysis of EcoRI-digested DNA from the N2F1 progeny demonstrated a 4.5-kilobase (kb) band for the disrupted gene and a 5.8-kb band for the wild-type gene and revealed that the ratio of wild-type, heterozygote, and homozygote offspring was 1:2:1 as illustrated for one litter (Fig. 1B). The LTC4 S−/− mice developed normally without any apparent defects, and both genders were fertile. Thus, we concluded that the disruption of LTC4S gene did not cause embryonic lethality, developmental defects, or abnormalities in fertility or parturition in the mouse.

We used BMMC, which are known to generate LTC4 (39), to confirm that the mRNA for LTC4S is not expressed because of the homologous recombination of the targeting construct. Northern blot analysis with total RNAs from BMMC of LTC4 S−/− and LTC4 S+/− mice revealed that the mature LTC4S mRNA with a size of 0.8 kb present in BMMC from the LTC4 S−/− mice was not detected in BMMC from the LTC4 S−/− mice (Fig. 1C). A 1.4-kb band detected in BMMC from the LTC4 S−/− mice was considered to be an unspliced LTC4S mRNA. Because we used a LTC4S cDNA probe that included exon V, a 1.8-kb band detected in BMMC from the LTC4 S−/− mice was considered to be an unstable transcript that contained exon V and presumably the neo gene.

Functional Disruption of LTC4S in Mouse Tissues and BMMC Assayed in Vitro—We sought to establish that LTC4S function was absent in BMMC from the LTC4 S−/− mice. LTC4 biosynthesis was detected in extracts of BMMC from LTC4 S−/− mice, whereas BMMC from LTC4 S−/− and LTC4 S+/− mice showed LTC4 biosynthesis of 2.6 ± 0.78 pmol/min/106 cells (n = 3) and 15.1 ± 0.77 pmol/min/106 cells (n = 3), respectively.
Leukotriene C₄ Synthase Gene-disrupted Mice

TABLE I

| Genotype       | LTC₄  | 5-HETE | 6-trans-LTB₄ | LTB₄ | PGD₂ | β-HEX (%) |
|----------------|-------|--------|--------------|------|------|-----------|
| LTC₄S⁻/⁻       | <1.0  | 87.3 ± 8.0 | 108.7 ± 8.3³ | 29.5 ± 3.2 | 11.1 ± 1.7 | 33.3 ± 4.2 |
| LTC₄S⁺/⁺       | 100.2 ± 14.3 | 94.9 ± 9.9 | 54.3 ± 5.9 | 28.1 ± 4.5 | 8.27 ± 1.9 | 25.0 ± 4.6 |

*includes its diastereoisomer.

⁻⁻ p < 0.01 as compared to LTC₄S⁺/⁺.

respective. To examine the contribution of LTC₄S to the production of LTC₄ in various mouse tissues, we measured the LTC₄S activity by monitoring the conjugation of LTC₄ to glutathione in tissues from LTC₄S⁻/⁻, LTC₄S⁻/⁺, and LTC₄S⁺/⁺ mice (Fig. 2). In the LTC₄S⁻/⁻ mice, no GST activity specific for LTC₄ was detected in the tongue, spleen, brain, and only slight activity was detected in the lung (−3% of the activity of wild-type), stomach (−10% of the activity of wild-type), and colon (−3% of the activity of wild-type). These results indicate that targeted disruption of the LTC₄S gene effectively eliminated the conjugation of LTC₄ with glutathione in these tissues and establish LTC₄S as the dominant enzyme for this reaction in these tissues.

GST activity for LTC₄ in the testis of LTC₄S⁻/⁻ mice was essentially the same as that of the LTC₄S⁺/⁺ and LTC₄S⁺/⁺ mice. The LTC₄ activity measured by the testis likely reflects the activity of MGST2, MGST3, and/or Mu-class GSTs (40, 41). However, it appears that cysteine LTB₄ is not involved in reproduction, because both 5-LO- and FLAP-deficient mice, which lack the cellulosic enzymatic capacity to generate the required LTC₄ substrate, had normal fertility and parturition (42–44).

The activation of BMMC through their high affinity receptor for IgE (FcεRI) was used to demonstrate the absence of LTC₄S in an integrated response of the 5-LO/LTC₄S pathway and to delineate the impact of that absence on arachidonic acid metabolism by 5-LO, by PG endoperoxide synthase-I, and by the terminal pathway enzymes, LTC₄ hydrolase and hematopoietic PGD synthase. As shown in a representative RP-HPLC assay (Fig. 3), no LTC₄ was detected after activation of the BMMC from the LTC₄S⁻/⁻ mice; LTB₄ was relatively unaffected, and the 6-trans-LTB₄ diastereoisomers were increased in this analysis at 280 nm. The data for three independent experiments, including the quantitation of 5-HETE at 235 nm, are shown in Table I. The BMMC from the LTC₄S⁻/⁻ mice generated no LTC₄, or its metabolites, LTC₄ and LTE₄, LTC₄, a decay product of 5-hydroperoxyeicosatetraenoic acid, and LTB₄, LTC₄, at the same level as the BMMC from the LTC₄S⁺/⁺ mice but did exhibit a 2-fold increment in the elaboration of 6-trans-LTB₄, LTC₄ diastereoisomers, the nonenzymatic breakdown products of LTC₄, BMMC from LTC₄S⁻/⁻ and LTC₄S⁺/⁺ mice released comparable amounts of 5-HETE, a marker for exocytosis (p = 0.2533), and generated comparable amounts of PGD₂, another mast cell-derived eicosanoid (p = 0.3243). These results indicate that LTC₄, a common substrate for LTC₄ hydrolase and LTC₄S, is not converted to additional LTB₄ but rather undergoes non-enzymatic hydrolysis to 6-trans-LTB₄ diastereoisomers in BMMC from the LTC₄S⁻/⁻ mice and that the loss of LTC₄S does not affect the IgE-mediated exocytosis or major cyclooxygenase pathway in BMMC from the LTC₄S⁻/⁻ mice. We conclude that LTC₄S is the major LTC₄-producing enzyme in mouse mast cells and tissues and that the disruption of the LTC₄ gene does not affect release of mast cell granules or shunt substrate to other eicosanoid pathways of BMMC.

Functional Assessment of LTC₄S Disruption in Vivo—To elucidate the possible role of cysteine LTs in acute inflammation, we examined the in vivo responses of LTC₄S⁻/⁻ and LTC₄S⁺/⁺ mice to the intraperitoneal injection of zymosan A by measurement of plasma protein extravasation and of cysteine LTs and LTB₄ concentrations in the peritoneal lavage fluid. Protein extravasation in the LTC₄S⁻/⁻ mice was significantly reduced at 10, 30, and 60 min after zymosan injection as compared with the LTC₄S⁺/⁺ mice (Fig. 4A). RP-HPLC analyses of the lavage fluids collected 120 min after zymosan injection revealed a prominent peak of LTE₄ (80.7 ± 4.1 ng/ml; n = 4) as the only cysteine LT detected in the wild-type mice but no LTE₄ in the lavage fluid of the LTC₄S⁻/⁻ mice (Fig. 4, B and C). However, the LTB₄ level in the peritoneal lavage fluid at this time point was comparable for the LTC₄S⁻/⁻ (1.34 ± 0.53 ng/ml; n = 4) and LTC₄S⁺/⁺ mice (0.82 ± 0.10 ng/ml; n = 4) (p = 0.3722) (Fig. 4C).

Zymosan is a yeast cell wall polysaccharide that can directly stimulate monocytes to generate leukotrienes (45) or can act indirectly via activation of the alternative complement pathway to provide peptides (46) capable of eliciting leukotrienes (47). Together with the ability of peritoneal macrophages to generate LTC₄ in response to zymosan ex vivo (45), the findings that the zymosan-induced elaborations of LTC₄ in the peritoneal cavity are similar in time course and concentration in C5a-deficient and sufficient mice and also in mast cell-deficient and control mice (48) suggest that the monocyte/macrophages in the peritoneal cavity are directly activated with zymosan to produce leukotrienes. In the zymosan-elicted peritoneal inflammation model, LTC₄ was generated with a peak at 30 min and was converted to LTE₄ with a peak at 60 min, whereas vascular permeability, assessed by protein concentration, increased rapidly after injection and reached a peak at 120 min (45). In other studies, the level of LTB₄ peaked at 120–180 min (45), followed by the recruitment of neutrophils to a plateau 360–420 min after zymosan injection (48, 49). We focused on the vascular permeability component of the zymosan effect, which was significantly impaired from 10 to 60 min in the LTC₄S⁻/⁻ mice compared with their controls and noted an absence of LTE₄, the most stable of the cysteine LTs (50). That the cysteine LTs are major mediators of the increase in vascular permeability induced by zymosan is supported by the previous findings of a similar attenuation of this response in 5-LO-deficient and FLAP-deficient mice (44, 49) but not in LTC₄ hydrolase-deficient mice (49). Thus, we conclude that the initial phase of zymosan-elicted plasma protein extravasation in the peritoneal cavity is in large part because of the cysteine LTs and not because of LTB₄ or even other 5-LO metabolites.

To examine the contribution of cysteine LTs to a mast cell-mediated acute allergic reaction in the skin, we performed passive cutaneous anaphylaxis in the ears of the LTC₄S⁻/⁻ and LTC₄S⁺/⁺ mice (Fig. 5). The intravenous injection of DNP-human serum albumin elicited rapid ear swelling in both the LTC₄S⁻/⁻ and wild-type mice that peaked at 15 min, persisted to 45 min, and then declined with resolution at 240 min. However, ear swelling was significantly reduced from 15 to 60 min by ~50% in the LTC₄S⁻/⁻ mice. As BMMC from the LTC₄S⁻/⁻ mice had no capacity to generate LTC₄ in response to IgE-dependent activation while being fully competent in secretory
granule exocytosis and generation of LTB₄ and PGD₂, we conclude that cysLTs are major mediators of the ear swelling initiated in situ by antigen activation of IgE-sensitized mast cells.

Topical administration of mast cell-derived mediators such as LTC⁴, LTD⁴, histamine, and serotonin has been demonstrated to induce ear edema in the mouse as assessed after 30 min by Evans blue dye extravasation. Serotonin and LTC⁴ were about 100 and 10 times, respectively, more potent than histamine on a weight basis in eliciting increased vascular permeability in the mouse ear (51). The contribution of the cysLTs to the ear edema in a model of passive IgE-induced cutaneous anaphylaxis had been considered to be minimal, because CysLT₁ receptor antagonists did not block increased vascular permeability (52, 53). The 5-LO²/², FLAP²/², or LTA₄ hydrolyase²/² mice also failed to show attenuated ear edema after passive systemic sensitization with monoclonal anti-DNP IgE followed in 24 h by antigen challenge (44, 49). In as much as there was a fall in body temperature similar to that of the normal mice, these gene-disrupted mice did experience systemic anaphylaxis. However, our results that IgE-dependent passive cutaneous anaphylaxis was significantly reduced in the immediately after the intravenous injection of 0.5% Evans blue dye. At 10, 30, 60, and 120 min, peritoneal lavage was performed. The cells in the lavage fluid were removed by centrifugation, and the absorbance of the lavage fluid was measured at 610 nm, which was measured to be 0.5% Evans blue dye extravasation. Error bars indicate S.E. (n = 3–4). *, p < 0.05 as compared with LTC₄S⁴/⁴. B, RP-HPLC analysis at 280 nm of the peritoneal lavage fluid supernatants from LTC₄S⁴/⁴ (left) and LTC₄S⁴/⁴ (right) 120 min after zymosan A injection. Arrows depict the retention times for PGB₂ and LTE₄. *, a peak detected at a retention time of about 25.2 min in the LTC₄S⁴/⁴ lavage fluid was not a cysLT as assessed by its UV spectrum. Results are representative of four independent experiments summarized in panel C. C, leukotrienes in the peritoneal lavage fluid supernatants 120 min after zymosan injection of LTC₄S⁴/⁴ (white column) and LTC₄S⁴/⁴ (black column) were resolved by RP-HPLC and quantitated for LTE₄ by UV absorbance at 280 nm relative to the PGB₂ standard and for LTB₄ by enzyme immunoassay of the fraction eluting at the correct time as defined by the [³H]LTB₄. Error bars indicate S.E. (n = 4).
LTC₄S⁻/⁻ mice as compared with the wild-type littermates indicate that the cysLTs play an important role in the increased vascular permeability in the IgE-dependent allergic reactions in the skin. Thus, the LTC₄S⁻/⁻ mice, generated by targeted disruption of the gene, do have a discrete phenotype that becomes apparent during the initial phase of altered vascular permeability that accompanies an inflammatory reaction elicited by either an innate or a specific immune stimulus.

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