American Journal of Pathology, Vol. 161, No. 2, August 2002

γ-Glutamyl Leukotrienase, a Novel Endothelial Membrane Protein, Is Specifically Responsible for Leukotriene D_4 Formation *in Vivo*

Bing Han,* Guoyang Luo,* Zheng-Zheng Shi,* Roberto Barrios,* Donna Atwood,* Weili Liu,* Geetha M. Habib,* Richard N. Sifers,* David B. Corry,† and Michael W. Lieberman*‡

From the Departments of Pathology, Medicine,† and Molecular and Cellular Biology,‡ Baylor College of Medicine, Houston, Texas*

The metabolism of cysteinyl leukotrienes *in vivo* and the pathophysiological effects of individual cysteinyl leukotrienes are primarily unknown. Recently we identified an additional member of the γ-glutamyl transpeptidase (GGT) family, γ-glutamyl leukotrienase (GGL), and developed mice deficient in this enzyme. Here we show that *in vivo* GGL, and not GGT as previously believed, is primarily responsible for conversion of leukotriene C_4 to leukotriene D_4, the most potent of the cysteinyl leukotrienes and the immediate precursor of leukotriene E_4. GGL is a glycoprotein consisting of two polypeptide chains encoded by one gene and is attached at the amino terminus of the heavy chain to endothelial cell membranes. In mice it localizes to capillaries and sinusoids in most organs and in lung to larger vessels as well. In contrast to wild-type and GGT-deficient mice, GGL-deficient mice do not form leukotriene D_4 *in vivo* either in blood when exogenous leukotriene C_4 is administered intravenously or in bronchoalveolar lavage fluid of *Aspergillus fumigatus* extract-induced experimental asthma. Further, GGL-deficient mice show leukotriene C_4 accumulation and significantly more airway hyperreponsiveness than wild-type mice in the experimental asthma, and induction of asthma results in increased GGL protein levels and enzymatic activity. Thus GGL plays an important role in leukotriene D_4 synthesis *in vivo* and in inflammatory processes. *(Am J Pathol 2002, 161:481–490)*

Cysteinyl leukotrienes (Cyst LTs) are important mediators of some inflammatory and immune disorders including anaphylaxis, Zymosan A-induced peritonitis, and asthma.[1–4] The pathophysiological effects of Cyst LTs include stimulation of smooth muscle contraction leading to broncho- and vasoconstriction, edema formation, and mucus production. Synthesis of leukotriene C_4 (LTC_4), the parent Cyst LT, from leukotriene A_4 and glutathione is catalyzed by leukotriene C_4 synthase in macrophages, eosinophils, mast cells, and some leukemic cell lines.[5–7] Conversion of LTC_4 to leukotriene D_4 (LTD_4) involves loss of a γ-glutamyl residue. Although γ-glutamyl transpeptidase (GGT) is known to catalyze LTD_4 formation in the test tube,[8,9] the mechanism of LTD_4 formation *in vivo* is unknown. Because LTD_4 is more potent than its precursor and binds with much higher affinity to the cysteinyl LT1 receptor than LTC_4,[10] understanding LTD_4 metabolism is important in clarifying its role in disease. In addition, clearance of Cyst LTs in the urine as leukotriene E_4 (LTE_4) requires LTD_4 formation because it is the immediate precursor of LTE_4.[11–13]

It is generally accepted that GGT is responsible for LTC_4/LTD_4 conversion *in vivo*.[13,14] However, we recently identified and cloned a mouse GGT family member designated γ-glutamyl leukotrienase (GGL) because its primary natural substrate appears to be LTC_4.[15,16] The enzyme also cleaves several other *S*-substituted glutathiones, but not GSH itself. The GGL gene is located ~3-kb upstream of GGT, and the two cDNAs share a 57% nucleotide identity.[16] Because all known substrates for GGL are also substrates for GGT,[16,17] it is not possible to assay for GGL in tissues from wild-type (WT) mice without also detecting GGT. To circumvent this problem we have used GGT-deficient mice for assays of GGL activity and developed antibodies specific for GGL.[4,15–18] GGL is expressed at highest levels in the spleen and uterus.[4,16] This observation is puzzling because it is not readily apparent what physiological function an enzyme such as GGL would have in tissues as different as spleen and uterus. Although the relative contributions of GGL and GGT to Cyst LT metabolism are unknown, our previous data indicate that no other enzymes in the mouse cleave LTC_4.[4] Thus it is possible to take advantage of mice deficient in GGL, GGT, or both enzymes to evaluate their roles in Cyst LT metabolism *in vivo* and in pathophysiology.[4,17]

Supported by National Institutes of Health grant ES 07827 and a grant from the Sandler Program for Asthma Research.

Accepted for publication April 29, 2002.

Address reprint requests to Michael W. Lieberman, M.D., Department of Pathology, Baylor College of Medicine, Houston, TX 77030. E-mail: mikel@bcm.tmc.edu.

Differences in organ distribution of the two enzymes suggest both different functions and different substrate specificities *in vivo*.[4,15–18] For the most part, GGT is expressed on absorptive and secretory epithelium (the luminal surface of the proximal tubular cells of the kidney, the villous epithelium of the small intestine, the ductular epithelium of the pancreas, and the secretory epithelial cells of the seminal vesicle).[9,19] These locations are consistent with the generally held idea that the major function of GGT is GSH cleavage.[9] Although LTC_4 is a substrate for GGT in the test tube,[8] the distribution of the enzyme contrasts with the known distribution and function of Cyst LTs.[20] Using GGL-deficient mice, we have recently found that in Zymosan A-induced peritonitis most of the initial neutrophil accumulation is dependent on GGL activity and that GGL is responsible for most or all of the cleavage of endogenous LTC_4 that accumulates in the peritoneal cavity.[4] These findings underscore the potential role of GGL in inflammation and provide evidence that it functions separately from GGT. Further studies of the characteristics of GGL and its roles in different pathophysiological conditions are essential for the understanding of its biological function.

Here we demonstrate that GGL is a glycoprotein composed of two polypeptide chains and is located on the surface of endothelial cells. GGL converts exogenously administered LTC_4 in the circulation, as well as formed in *A. fumigatus* extract (CF)-induced experimental asthma, to LTD_4 to limit potentially life-threatening airway hyperreponsiveness (AHR). These findings suggest the importance of GGL in the pathophysiology of disease.

Materials and Methods

Chemicals

LTC_4, LTD_4, and LTE_4 were from Cayman Chemical Company (Ann Arbor, MI). Papain and *N*-glycosidase F were purchased from Sigma Chemical Co. (St. Louis, MO). Endoglycosidase H was from Boehringer Mannheim Co. (Indianapolis, IN). Anti-mouse CD31 monoclonal antibody was from Pharmingen (San Diego, CA). Texas Red-conjugated anti-rat antibody and Oregon Green conjugated anti-rabbit antibody were from Molecular Probes (Eugene, OR). The immunohistochemistry kit was from BioGenes (San Ramon, CA).

Animals

All GGT-, GGL-, and GGT/GGL-deficient mice were developed in our laboratory.[4,17] All mice including WT controls were on a C57BL/129SvEv background and were used between 6 to 8 weeks of age. They were supplied with *N*-acetyl cysteine in the drinking water.[17]

Generation of Anti-GGL Polyclonal Antibody

Rabbit anti-mouse GGL was generated by using an N-terminus truncated recombinant GGL protein. GGL cDNA fragment (nucleotides 426 to 1721) was expressed in-frame in an expression vector, pET-30a (+) (Novagen, Madison, WI) in *Escherichia coli*. The truncated protein with 100 amino acids at the N-terminus removed was then purified by using the Xpress System Protein Purification kit (Invitrogen, Carlsbad, CA). New Zealand White rabbits were injected with 200 μg of the protein in complete Freund's adjuvant and boosted with 100 μg of protein in incomplete Freund's adjuvant every 2 weeks. Rabbits made high-titer-specific anti-GGL antibodies as judged by Western blot analysis.

Induction of Experimental Asthma

A. fumigatus culture filtrate allergen (CF) (lot no. DC980809) was prepared and used as previously described.[21] Mice were challenged as previously described;[21] briefly, 50 μl of CF or saline control was administered intranasally to mice anesthetized with Metofane (Janssen, Toronto, Canada). Mice were challenged five times with 4 days between each challenge. Fifteen hours after the final CF challenge, airway resistance was measured and AHR determined by C_{200}, and bronchoalveolar lavage fluid (BALF), serum, and lung tissue were collected. BALF total and differential cell counts, BALF mucine, and lung histology were evaluated as described.[21] Data are representative of two independent experiments with seven to eight mice in each groups.

Western Blot Analysis

Tissue homogenates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence or absence of 0.1 mol/L of dithiothreitol and electrophoretically transferred to nitrocellulose membrane. The anti-serum was used at 1:20,000 dilution. The detecting system was a Phototope-HRP Detection Kit (New England BioLabs, Beverly MA).

For deglycosylation of GGL with endoglycosidases, tissue homogenates from spleen and uterus were subjected to endoglycosidase H (Boehringer Mannheim Co.) or *N*-glycosidase F (Sigma Chemical Co.) treatment as previously described.[22–24] The reactions were performed at 37°C for 18 hours followed by Western blot analysis.

For dissociation of GGL from cell membranes, homogenates of WT uterus were centrifuged at 43,000 $\times$ *g* for 30 minutes at 4°C, and the membrane fraction was subjected either to dithiothreitol reduction or to papain digestion. The samples were reduced with 0.1 mol/L of dithiothreitol in the presence of 0.02% SDS at 60°C for 1 hour or incubated with papain at a final concentration of 1 mg of papain/1.5 g membrane protein at 25°C for 30 minutes. The reactants were centrifuged at 43,000 $\times$ *g* at 4°C for 10 minutes, and both supernatant and pellet were analyzed by Western blot.

For assaying GGL protein level in the lungs after asthma induction, lung homogenates were directly analyzed by Western blot and differences were quantified by scanning densitometry.

LTC_4/LTD_4 Conversion Assay

LTC_4 conversion activity was assayed by high performance liquid chromatography (HPLC) as described previously.[15] Specific activity was expressed as nmol of LTC_4 converted/mg protein/hour by measuring the formation of LTD_4 and LTE_4; the latter is formed by the action of membrane-bound dipeptidase on LTD_4.[12,15,16]

To assay LTC_4/LTD_4 conversion activity in phosphate-buffered saline (PBS)-treated and *A. fumigatus*-treated lungs, organs were homogenized in 0.1 mol/L of Tris-HCl, pH 8.0, and the homogenates were directly incubated with LTC_4 for activity assay.

To assay the activity in different compartments of the spleen, we prepared spleen fractions. The organs were teased apart with fine forceps in PBS to release free cells. The cell suspension and the initial residues were separated by allowing the preparation to settle on ice for 5 minutes. The cells were collected by centrifugation at 1000 × *g* for 5 minutes. The supernatant was combined with the initial residues and this was designated the residual stroma. Both the cell suspension and the residual stroma were homogenized for the assay.

To examine the intravascular metabolism of exogenous LTC_4, LTC_4 at 100 μg/ml in ethanol was diluted in normal saline to 40 μg/ml, and 50 μl was injected intravenously through the tail veil (2 μg/mouse). Three mice from each of the WT, GGT-, GGL-, and GGT/GGL-deficient group were treated. Ten minutes after the injection, blood samples were harvested by heart puncture and plasma was collected using Brand Serum Separators (Becton Dickinson, Franklin Lakes, NJ). Plasma from control mice (no LTC_4 administration) was used as a negative control. For inhibition of LTD_4/LTE_4 conversion, D-penicillamine (Sigma Chemical Co.) was injected intraperitoneally 5 minutes before the administration of LTC_4 at 5 mmol/kg body weight. Cyst LTs in plasma were analyzed by HPLC as described above.

LTC_4 and LTE_4 Measurement in BALF

BALF was collected in PBS and centrifuged. Supernatants were collected and stored at −80°C until assayed. BALF was first extracted and Cyst LTs concentrated using Sep-Park cartridges (Waters Corp., Milford, MA). They were assayed for LTC_4 and LTE_4 using leukotriene C_4 and leukotriene E_4 EIA kits (Cayman Chemical Company). To rule out the cross-reactivity among Cyst LTs, in some experiments samples were further fractionated by HPLC based on the retention times established with standards after extraction by Sep-Park cartridges[4] and assayed for LTC_4 and LTE_4 by enzyme-linked immunosorbent assay. Data are representative of two independent experiments with seven to eight mice in each group.

Immunohistochemistry and Immunofluorescence Analysis

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized and rehydrated using a standard protocol. The endogenous peroxidase was quenched by incubating the slides in 3% H_2O_2 in methanol for 5 minutes followed by two 5-minute PBS washes. The slides were incubated with trypsin (Trypsin 3-Pack; BioGenex, San Ramon, CA) for 20 minutes at 37°C, blocked with 1.5% normal goat serum in PBS at room temperature for 30 minutes, and incubated with protein A-purified anti-GGL polyclonal antibody at 10 μg/ml at 25°C for 1 hour. Peroxidase staining was achieved using BioGenex's Supersensitive detection kit and liquid diaminobenzidine. The sections were counterstained with hematoxylin. For immunofluorescence co-localization of mouse CD31 and GGL, we used zinc-fixed sections because the rat anti-mouse CD31 does not react well with the formalin-fixed antigen. The zinc fixative solution was 0.1 mol/L Tris buffer at pH 7.4, containing 0.05% calcium acetate, 0.5% zinc acetate, and 0.5% zinc chloride. Both rabbit anti-GGL and rat anti-CD31 (Pharmingen, San Diego, CA) antibodies were used at a concentration of 10 μg/ml. The fluorescence-labeled secondary antibodies were Texas Red-conjugated goat anti-rat IgG and Oregon Green conjugated goat anti-rabbit IgG (Molecular Probes). The fluorescence was detected with a fluorescence microscope (Nikon Optiphot-2; Nikon Inc., Nelville, NY) with a 568-nM filter for Texas Red and a 488 nM filter for Oregon Green.

Statistical Analysis

Statistical analyses were performed using Student's *t*-test. Data are presented as mean ± SEM.

Results

Characteristics of GGL Protein

Because the mature GGT protein consists two disulfide-linked peptide chains generated from a single precursor polypeptide and GGL is a membrane protein with similarities to GGT,[16,25] we examined the structure of GGL protein in tissue homogenates from spleen and uterus. Using SDS-PAGE and Western blotting with anti-GGL antibodies, we found that GGL protein consists of two polypeptides with molecular weights of 57 kd and 20 kd under reducing conditions and runs as a single band of 75 kd under nonreducing conditions (Figure 1A). These bands were not detected in homogenates of spleen and uterus from GGL-deficient mice, confirming the specificity of our antibodies. These results indicate that, like GGT, GGL consists of two polypeptides chains bonded through one or more S-S linkages

We found that GGL is an N-linked glycoprotein, glycosylated on the heavy chain. Because the apparent molecular mass of 75 kd (Figure 1A) is greater than the predicted molecular weight of ~63 kd (573 amino acids) for the heavy chain, we examined protein glycosylation. For this purpose, tissue homogenates were incubated with either of two endoglycosidases, *N*-glycosidase F and endoglycosidase H. *N*-glycosidase F hydrolyzes all N-linked carbohydrate chains from glycoproteins

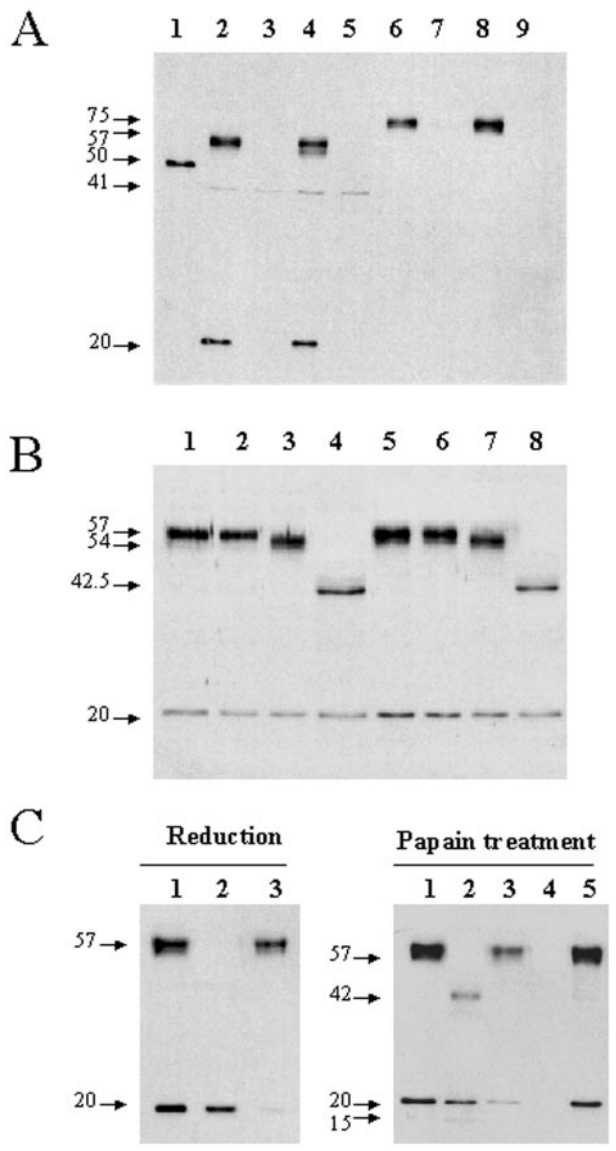

Figure 1. Characterization on GGL protein by SDS-PAGE and Western blotting. **A:** Structural analysis on GGL. **Lane 1** shows the truncated recombinant GGL protein used for immunization (reduced). Samples in **lanes 2** to **5** were run under reducing conditions and those in **lanes 6** to **9** were run under nonreducing conditions. **Lanes 2** and **6** were samples from WT spleen, **lanes 3** and **7** from $GGL^{-/-}$ spleen. **Lanes 4** and **8** were samples from WT uterus, **lanes 5** and **9** from $GGL^{-/-}$ uterus. **B:** Deglycosylation of GGL with endoglycosidase H and *N*-glycosidase F. Samples in **lanes 1** to **4** were WT spleen homogenates and those in **lanes 5** to **8** were WT uterus homogenates. **Lanes 1** and **5** show untreated samples. **Lanes 2** and **6** show samples incubated with the buffer in the absence of enzymes. **Lanes 3** and **7** show samples treated with endoglycosidase H, and **lanes 4** and **8** show samples treated with *N*-glycosidase F. **C:** Dissociation of GGL from the cell membrane. The **left** panel shows the result of releasing GGL protein from a uterus membrane preparation with dithiothreitol. **Lane 1** is untreated sample, **lane 2** is the supernatant after dithiothreitol treatment, and **lane 3** is the pellet after dithiothreitol treatment. The **right** panel shows the result of limited papain digestion on a uterus membrane preparation. **Lane 1** is the untreated sample, **lane 2** is the supernatant from a papain-digested sample, **lane 3** is the pellet from a papain-digested sample, **lane 4** is the supernatant of the sample incubated with buffer in the absence of papain, and **lane 5** is the pellet. The numbers at the **left** side of the figures indicate the molecular weights in kd. Twenty μg of total protein from spleen homogenates and 10 μg of total protein from uterus homogenates were analyzed in all of the experiments.

whereas hydrolysis by endoglycosidase H is restricted to high mannose and hybrid types.[22–24] The deduced amino acid sequence of GGL heavy chain has six potential N-linked glycosylation sites.[16] If each site is glycosylated, the predicted molecular weight is ~60 kd (assuming an average of 3 kd per complex type of N-linked sugar), whereas the predicted molecular weight of the unglycosylated heavy chain is ~42.8 kd.[16] After *N*-glycosidase F treatment, the apparent molecular weight of the heavy chain decreased from 57 kd to ~42.5 kd, indicating the removal of most or all carbohydrate (Figure 1B, lane 4). After treatment with endoglycosidase H, the heavy chain decreased from 57 kd to 54 kd (Figure 1B, lane 3), indicating that most carbohydrate chains of the protein are the complex type, which is a feature of plasma membrane glycoproteins.[26] Neither of the enzymes changed the migration of the light chain (Figure 1B, lanes 3 and 4), indicating the absence of N-linked glycosylation.

To characterize the physical anchoring of GGL to cells, we reduced a membrane preparation with dithiothreitol; this treatment released only the light chain, indicating that GGL is anchored to the membrane through the heavy chain (Figure 1C, left). As a second approach, we released GGL from the membrane with limited papain digestion.[27] Papain treatment released the intact GGL light chain and a protein of 42 kd representing a shortened heavy chain. An ~15-kd band cleaved from the heavy chain was also detected in the supernatant (Figure 1C, right). These results provide additional evidence that GGL is anchored in the membrane through the heavy chain. An analysis of the amino acid hydropathy revealed a hydrophobic region (amino acids 11 to 29) located at the N-terminus of GGL,[13] suggesting that like GGT, membrane association of GGL involves the N-terminus of the heavy chain.

Tissue Localization of GGL

We examined the distribution of GGL in spleen by separating it into a cell suspension and residual stroma. By light microscopy, the suspension contained red blood cells, lymphocytes, neutrophils, a small number of macrophages and fibroblasts, and a few endothelial cells, whereas the residual stroma contained predominantly sinusoidal and reticular material with attached endothelial cells, collagen fibers (with fibroblasts), macrophages, and a few lymphocytes. In both WT and GGT-deficient spleen, the residual stroma was ~10-fold enriched in LTC_4 cleavage activity (Figure 2). In GGL-deficient spleen, LTC_4 cleavage is approximately eightfold lower than in WT spleen and is associated with the suspended cells; this cleavage represents residual GGT activity.[15,16] Thus in contrast to GGT, which is known to be expressed on lymphocytes in spleen (at relatively low levels),[28] GGL activity is associated with nonlymphoid cells in stroma.

Using immunohistochemistry, we found that GGL is expressed by the endothelial cells of sinusoids and capillaries in most organs. Sinusoid endothelial cells in WT spleen stained intensely (Figure 3B). As expected, GGL-

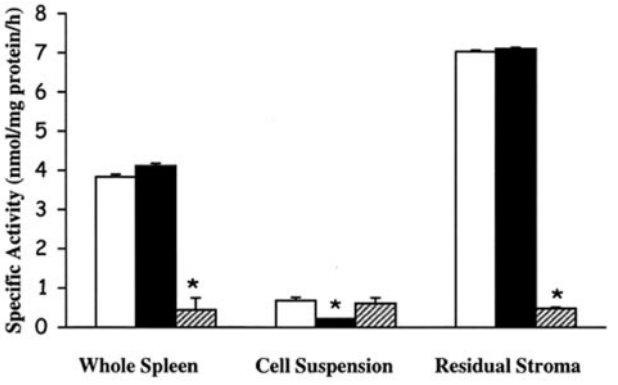

Figure 2. LTC_4/LTD_4 conversion activity in different fractions of spleen from WT (□), $GGT^{-/-}$ (■), and $GGL^{-/-}$ (▨) mice. The cleavage of LTC_4 was analyzed by HPLC as described in Materials and Methods. The specific activity is expressed as nmol/mg protein/hour ($n = 3$). Values are mean ± SEM. *, Significant difference from the other two groups ($P < 0.01$) by Student's *t*-test.

deficient spleen did not stain (Figure 3A). Sinusoid endothelial cells in WT liver were also stained (Figure 3C). Capillary endothelial cells in brain (Figure 3D), glomerulus (Figure 3E), and myocardium (Figure 3F) also expressed GGL, as did endothelial cells in endometrium and the lamina propria of the small intestine (data not shown). Larger blood vessels in most organs, as exemplified by the central veins in liver (Figure 3C), small arteries in kidney (Figure 3E), and aorta and vena cava (data not shown), were negative. The lungs were an exception, and we found that in addition to capillary endothelium, endothelium in larger vessels was also positive (data not shown). In the organs examined, we did not detect staining in endothelium of lymphatics. To confirm the endothelial localization of GGL we performed fluorescent microscopy and found that CD31 (PECAM-1), an integral membrane protein constitutively expressed on the surface of endothelial cells,[29,30] and GGL co-localized (Figure 3; G to L).

The Role of GGL in Intravascular LTC_4 Metabolism

In mice, GGT and GGL are the only enzymes known to generate LTD_4 from LTC_4 in the test tube.[4,15,16] The endothelial location of GGL suggests that GGL may perform this function *in vivo*. To test this hypothesis, we administered exogenous LTC_4 intravenously to WT, GGT-, GGL-, and GGT/GGL-deficient mice and analyzed the Cyst LT profile in blood by HPLC.[4,12] Ten minutes after injection, 25 to 30% of the LTC_4 had been converted to LTE_4 in both WT (Figure 4A) and GGT-deficient mice (Figure 4B), whereas little, if any, conversion was detected in blood from GGL-deficient mice (Figure 4C). Similarly, LTE_4 was also absent from the blood of GGT/GGL-deficient mice (Figure 4D). When mice were pretreated with D-penicillamine, a specific inhibitor of membrane-bound dipeptidase,[31] LTD_4 was also detected in the blood from WT and GGT-deficient mice (Figure 4, E and F), indicating that the LTD_4 is quickly converted to LTE_4 in blood. These findings demonstrate that GGL is responsible for LTD_4 synthesis in blood.

The Role of GGL in Experimental Asthma

Because asthma is one of the most pressing medical problems associated with Cyst LT production, we examined the role of GGL in CF-induced experimental asthma in mice. Although it is generally believed that GGT is responsible for LTC_4 to LTD_4 conversion in asthma,[3] our results on LTC_4 metabolism in blood (Figure 4) and in Zymosan A-induced peritonitis[4] suggest that GGL might catalyze LTC_4 to LTD_4 conversion in asthma. To resolve this discrepancy and to assess the relative roles of GGL and GGT, we induced asthma in mice using CF and studied mice deficient in GGL, GGT, or both GGL and GGT. We found that the level of LTC_4 in BALF increases sixfold to eightfold in CF-treated GGL- and GGL/GGT-deficient mice compared to saline-treated controls whereas little change is seen in WT mice and GGT-deficient mice (Figure 5A). These data indicate that during the asthmatic response GGL is the principal enzyme that converts LTC_4 to LTD_4. Because the antibody used to measure LTC_4 has some cross reactivity with LTD_4, we separated Cyst LTs in BALF by HPLC and verified that in GGL- and GGL/GGT-deficient mice only LTC_4 is present (data not shown). In parallel with this finding, we found that there was a threefold to fivefold increase in LTE_4 levels in CF-treated WT mice and GGT-deficient mice (Figure 5B). We also detected what appear to be very low levels of LTE_4 in all PBS-treated mice, including mice deficient in GGL and both GGL and GGT. It is likely that this result stems from cross-reactivity with other arachidonic derivatives because LTE_4 was not detectable in these samples after HPLC fractionation (data not shown). Our data indicate that GGL deficiency prevents the generation of LTD_4 and LTE_4 in experimental asthma and are further confirmation that GGL is the principle enzyme for LTC_4 to LTD_4 conversion *in vivo*.

To examine the pathophysiological role of GGL in CF-induced experimental asthma, we evaluated the cellular composition in BALF and severity of AHR in WT and mutant mice by evaluating PC_{200} after CF challenge. The results show that mice of all types challenged with CF developed a stereotypic asthma syndrome, characterized by an increase in the AHR as revealed by enhanced sensitivity to acetylcholine challenge (Figure 5C) and increased eosinophil infiltration and goblet cell metaplasia with mucus overproduction (data not shown). All mice exhibited similar total and differential cell counts and mucin levels in BALF at the time of BALF collection (data not shown). These results indicate that CF can effectively induce asthma in mice whether or not LTD_4 is present. We found that in all groups of mice CF treatment resulted in a greater than twofold decrease in PC_{200} compared to saline treatment (Figure 5C). This result is not surprising because both LTC_4 and LTD_4 are known to be involved in asthma.[2,3] It is interesting that the PC_{200} values in CF-

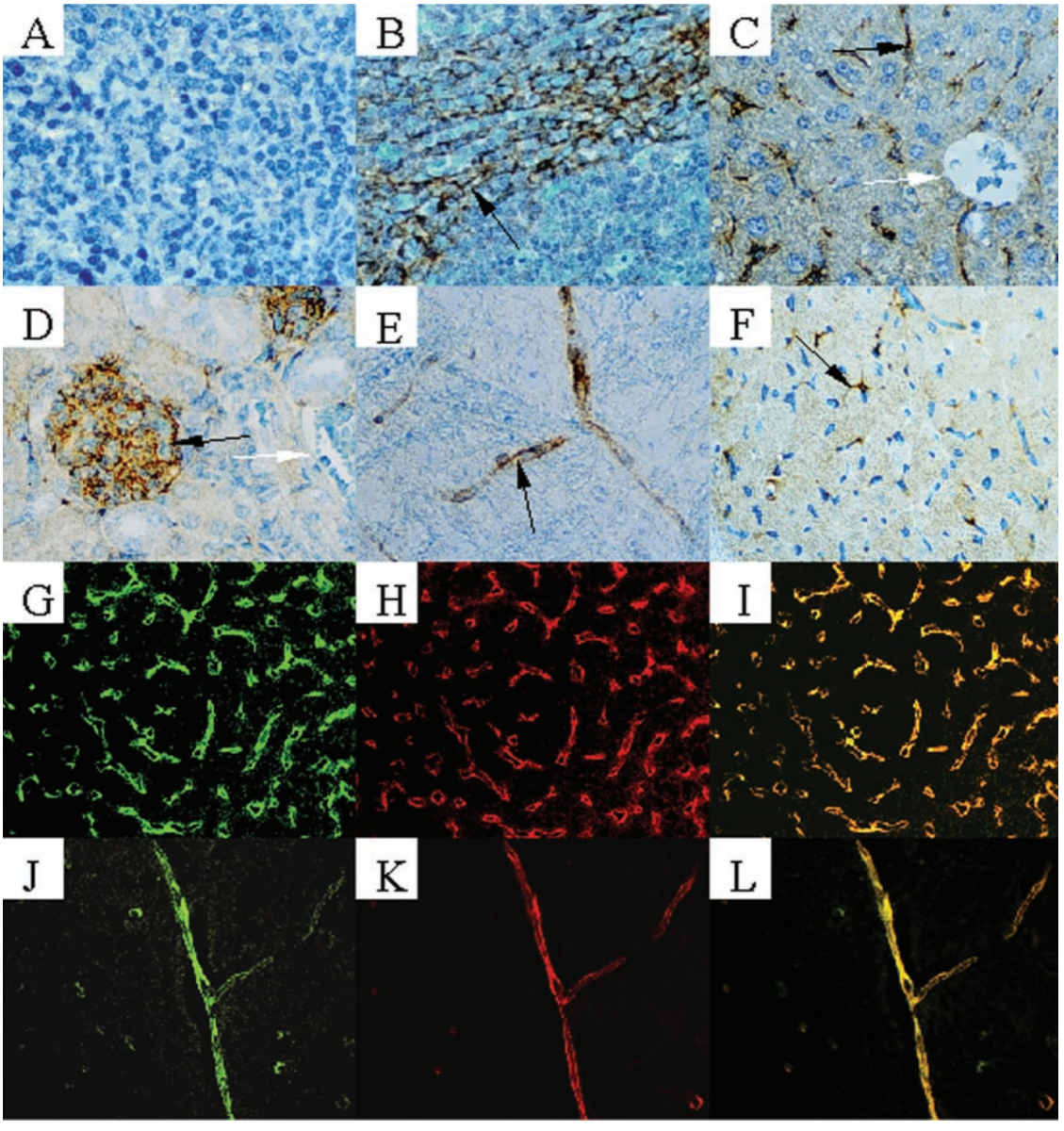

Figure 3. Localization of GGL by immunohistochemistry and immunofluorescence. **A:** Section of GGL-deficient spleen. **B:** Section of WT spleen. The **black arrow** points to positive staining on endothelium of a sinusoid. **C:** Section of WT liver. The **black arrow** points to positive staining on endothelium of a sinusoid; the **white arrow** points to lack of staining on endothelium of a central vein. **D:** Section of WT kidney. The **black arrow** points to positive staining on endothelium of capillaries in a glomerulus; the **white arrow** points to lack of staining on endothelium of a small artery. **E:** Section of WT brain. The **black arrow** points to positive staining on endothelium of a capillary. **F:** Section of WT heart. The **black arrow** points to positive staining on endothelium of a capillary in myocardium. **G** to **L:** Results of immunofluorescence co-localization of GGL and CD31. **G** to **I:** Sections of WT liver. **J** to **L:** Sections of WT brain. **G** and **J:** GGL expression detected with Oregon Green. **H** and **K:** CD31 expression detected with Texas Red. **I** and **L:** GGL and CD31 co-localization with Oregon Green and Texas Red merged. Original magnifications, ×400.

treated GGL- and GGL/GGT-deficient mice are significantly lower ($P < 0.01$) than that for CF-treated WT mice; this finding indicates that deficiency in GGL further exacerbates AHR. Although the difference between the PC_{200} for GGT-deficient mice and that for WT mice after CF treatment is not statistically significant (Figure 5C), it shows a trend in that direction. Whether this result indicates involvement of GGT functions unrelated to Cyst LT metabolism in asthma or is not a real difference remains to be determined.

Response of GGL Enzymatic Activity and Protein to Asthma Induction

The observation that LTC_4 does not accumulate in CF-treated WT and GGT-deficient mice led us to examine GGL protein levels and LTC_4/LTD_4 conversion activity in lungs after CF-treatment. We used Western blot to determine GGL protein levels in lungs in WT and GGT-deficient mice, and found that after CF treatment these mice exhibit a twofold increase in GGL protein as quantified by

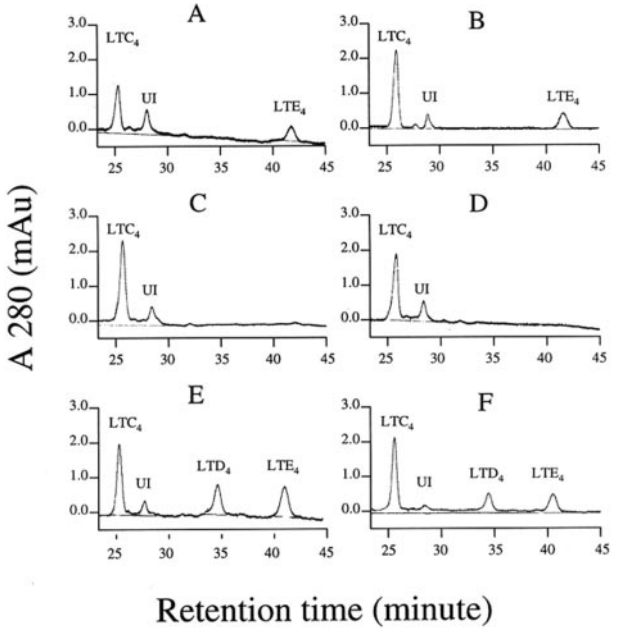

Figure 4. Analysis of plasma leukotrienes after exogenous administration of LTC_4. HPLC was applied as described in Materials and Methods. **A,** WT; **B**, GGT-deficient; **C**, GGL-deficient; **D**, GGT/GGL-deficient; **E**, WT pretreated with D-penicillamine; **F**, GGT-deficient pretreated with D-penicillamine. LTC_4, LTD_4, and LTE_4 peaks were authenticated by comparing them with retention times of external standards.[12,13] Peak UI is an unidentified peak that is also observed in the control plasmas (no exogenous LTC_4 administration) (data not shown). Three mice were used for each group, and all mice in the same group showed similar results.

scanning densitometry (Figure 6A). This result indicates that expression of GGL protein is increased in response to asthma development.

To evaluate the changes in LTC_4/LTD_4 conversion activity of GGL and GGT, we used HPLC to examine the enzyme activities in fresh lung homogenates from mice after saline or CF treatment. In agreement with previously reported data, in saline-treated mice LTC_4/LTD_4 conversion activity in lung homogenates is predominantly the result of GGT activity.[4,16] We found that compared to saline treatment, CF treatment resulted in a twofold increase in GGL activity in GGT-deficient mice (Figure 6B). (Recall that GGL enzyme activity can only be measured directly in GGT-deficient mice.) This increase is similar to the increase in GGL protein levels (Figure 6A). As expected, there was no detectable LTC_4 to LTD_4 conversion activity in GGL/GGT-deficient mice (data not shown).[16] With respect to GGT, we found a decrease in enzyme activity in CF-treated GGL-deficient mice (Figure 6B). Why GGT enzyme activity is reduced after CF treatment in our experiments remains to be determined. The total LTC_4/LTD_4 conversion activity in CF-treated mice was the same as that for saline-treated mice, reflecting the increase in GGL and decrease in GGT activity. These data provide further evidence that GGL is the enzyme responsible for the initiation of Cyst LT metabolism in asthmatic inflammation. They also raise the questions of the physiological role of GGT in lung.

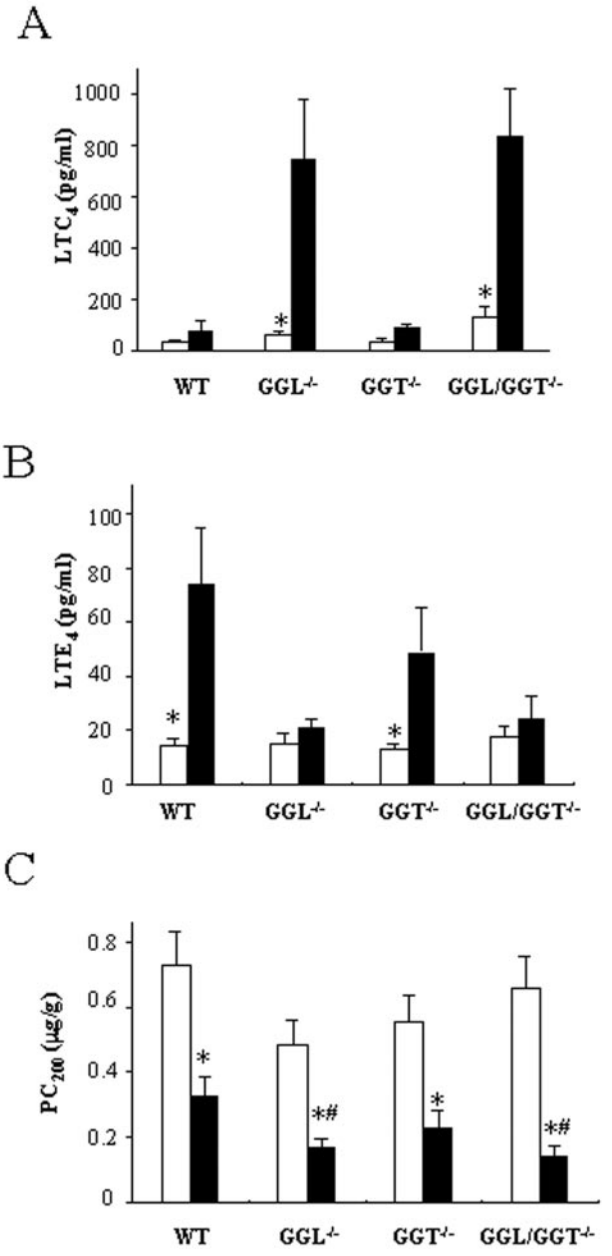

Figure 5. Analysis on BALF Cyst LTs levels in mice with experimental asthma. **A:** LTC_4 levels in BALF. **B:** LTE_4 levels in BALF. **C:** AHR after acetylcholine challenge. AHR was measured as the amount of acetylcholine that induced a 200% rise in pulmonary resistance from the baseline during challenge (PC_{200}). WT, $GGL^{-/-}$, $GGT^{-/-}$, and $GGL^{-/-}/GGT^{-/-}$ mice were treated with PBS (□) or CF (■). Data are representative of two separate experiments with seven to eight mice per group and are plotted as means ± SEM *, Significant differences ($P < 0.01$) between PBS treatment and CF treatment; #, significant differences ($P < 0.01$) from the WT group, by Student's *t*-test.

Discussion

Our results demonstrate that GGL, like GGT, is processed into two S-S linked peptide chains and is anchored in the plasma membrane by the heavy chain through its amino terminus (Figure 1). In contrast to GGT, which is located primarily on the luminal surface of epithelial cells,[9,19] GGL is expressed on the surface of endothelial cells of capillaries and sinusoids in most organs

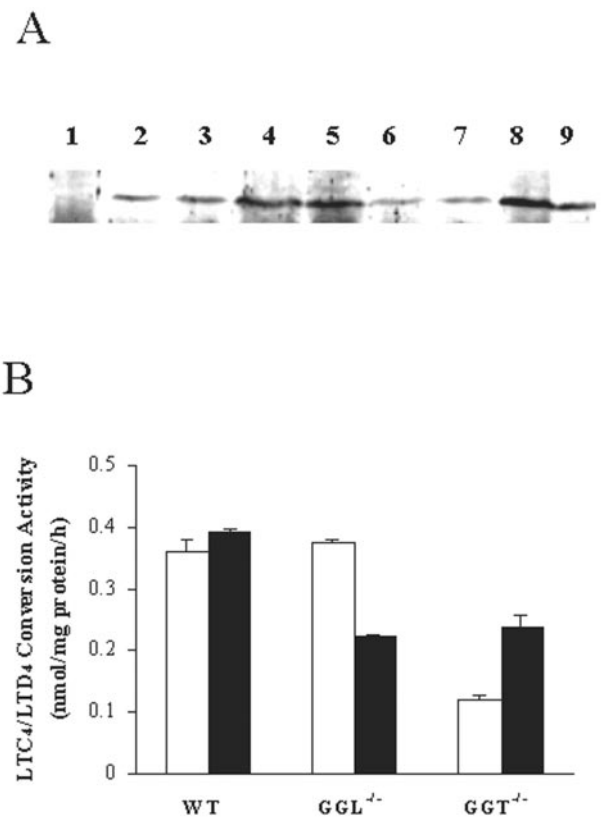

Figure 6. Response of GGL to asthma induction. **A:** Western blot analysis of GGL protein in lungs from mice treated with PBS or CF. The figure shows the light chain of GGL protein with a molecular weight of 20 kd (see Materials and Methods). **Lane 1** is from the lung of a PBS-treated GGL-deficient mouse, used as a negative control for GGL; **lanes 2** and **3**, samples from PBS-treated WT mice; **lanes 4** and **5**, samples from CF-treated WT mice; **lanes 6** and **7**, samples from PBS-treated GGT-deficient mice; and **lanes 8** and **9**, samples from CF-treated GGT-deficient mice. **B:** LTC_4/LTD_4 conversion activity in lungs. WT, $GGT^{-/-}$, and $GGL^{-/-}$ mice were treated with either PBS (□) or CF (■). Conversion of LTC_4 to LTD_4 and to LTE_4 was analyzed by HPLC as described in Materials and Methods. The specific activity is expressed as nmol/mg protein/hour (n = 3 to 6). Values are mean ± SEM. For GGT (assayed in GGL-deficient mice) the difference between PBS treatment and CF treatment, $P < 0.05$**;** for GGL (assayed in GGT-deficient mice) the difference between PBS treatment and CF treatment, $P < 0.001$; both tested by Student's t-test.

and larger vessels in the lung (Figure 3 and data not shown). Our results do not rule out low-level GGL expression on other endothelial cells or even other cell types at levels below the sensitivity of our techniques, but it is likely that any such expression is quantitatively minor.[15,16] Our data also indicate that GGL plays a key role in regulating conversion of intravascular LTC_4 as well as those formed in CF-induced asthma, to LTD_4 in mice (Figures 4 and 5).

Previously we found that spleen and uterus expressed the highest levels of GGL.[4,15] Our current study provides an explanation for this phenomenon: the abundant sinusoidal endothelium in spleen and the rich capillary network in endometrium contribute to the high levels of GGL in these organs (Figure 3B and data not shown). The fact that endothelium of large vessels in most organs expresses little, if any, of GGL protein highlights variations in its expression among different types of endothelial cells and the physiological importance of GGL. Using *in situ* hybridization we have previously reported tissue distribution of GGL;[16] these results show some differences with our current results. The reason for this discrepancy is not readily apparent, but because our chemical and immunohistochemistry results agree, we believe that the results reported here accurately reflect the distribution of GGL.

Our data also clarify the relation between GGL and GGT function. GGT has long been thought to be the enzyme that catalyzes LTC_4/LTD_4 conversion *in vivo*.[13] However, our results on LTC_4 metabolism in the blood and in experimental asthma indicate that GGL performs much, if not all this function *in vivo* (Figures 4 and 5). In addition to its expression on secretory and absorptive epithelium, GGT has been reported to be expressed on capillary endothelial cells in the brain.[32] However, it is absent from capillaries in other organs and from brain capillaries that lack a blood-brain barrier.[33] Functionally, GGT in brain capillaries is apparently involved in the transport of neutral amino acids across blood-brain barrier.[34] Lymphocytes also express low levels of GGT.[28] Thus it is theoretically possible that GGT could function to a limited extent in some brain capillaries or lymphocytes to convert circulating LTC_4 to LTD_4; however, our data argue against any significant participation of GGT in the intravascular metabolism of Cyst LTs in that we could not detect metabolism of LTC_4 in GGL-deficient mice (which express GGT) (Figure 4). Because GGL has a more limited substrate spectrum than GGT and does not cleave GSH,[15] its presence on endothelium allows synthesis of LTD_4 without interfering with circulating GSH. In addition, the K_m of GGL for LTC_4 is 10-fold lower than that of GGT,[15] further implicating the role of GGL in LTD_4 synthesis *in vivo*. All of these observations argue for a central role for GGL in LTD_4 synthesis and in the regulation of responses to injury.

In humans Cyst LTs are important agonists of AHR in asthma, and blocking the action of Cyst LTs by Cyst LT receptor antagonists is an important part of asthma therapy.[35–37] Our findings elucidate the mechanism by which Cyst LTs are metabolized during the development of experimental asthma and demonstrate that GGL plays a key role in the conversion of LTC_4 to LTD_4 during asthmatic inflammation. This finding is significant because it clarifies the mechanism of how LTD_4 is synthesized *in vivo*. In our study, AHR shows significant increase in GGL-deficient mice (Figure 5C). Because LTC_4 formed is quickly converted to LTD_4 by GGL and further to LTE_4 by membrane-bound dipeptidase in WT and GGT-deficient mice (Figure 5, A and B),[12] we reason that the increase in AHR in GGL- and GGL/GGT-deficient mice results from LTC_4 accumulation. This result indicates that GGL not only converts LTC_4 to more potent LTD_4, but also serves as a critical enzyme to promote the chemical clearance of Cyst LTs, thus to limit potentially deleterious airway obstruction. This finding is pathophysiologically important because defect and insufficiency on GGL function caused by any pathological factors may lead to the development and/or exacerbation of asthma. The fact that GGL expression is increased during asthma underscores the importance of this enzyme in regulating the metabolism of Cyst LTs (Figure 6).

It seems unlikely that GGT functions in Cyst LT metabolism during asthma. GGT enzyme activity falls during asthma (Figure 5B), and there is no evidence of conversion of LTC_4 to LTD_4 (as measured by LTE_4 accumulation, Figure 4B; in Figure 4B, GGT activity is assessed in GGL-deficient mice). We have previously reported that lung homogenates from untreated mice contain more GGT activity than GGL activity (see also Figure 6B).[4,16] Although this finding may seem counterintuitive in terms of asthma, GGT probably functions primarily in GSH homeostasis, which is known to be critical for protection against oxidative damage in lung.[9,37]

Previous studies have shown that LTD_4 can mediate eosinophil infiltration, mucus production, and bronchial contraction.[3] Because GGL- and GGL/GGT-deficient mice do not synthesize LTD_4 after CF challenge (Figure 5) and develop eosinophil infiltration and mucus production similar to those in CF-treated WT mice and GGT-deficient mice (data not show), it follows that LTC_4 plays a role similar to that of LTD_4 in asthma. We previously observed a delayed neutrophil infiltration in Zymosan A-induced peritonitis in the GGL-deficient mice.[4] In the current study, we found similar total and differential cell counts and mucin levels in BALF from WT, GGL-, GGT-, and GGL/GGT-deficient mice. The difference between these two studies may reflect differences in inducing agent, physiological location, or the inflammatory process.

In summary, GGL appears to have evolved from GGT to cleave a substrate related to inflammatory processes. Most of the structural features of GGT have been preserved in the structure of GGL, but its expression appears to be restricted to some endothelial cells. The importance of GGL in pathophysiological events is underscored by its role in regulating metabolism of Cyst LTs *in vivo*.

Acknowledgments

We thank Drs. Mark W. Majesky and Subbarao V. Kala for their advice and valuable discussions.

References

1. Henderson Jr WR: The role of leukotrienes in inflammation. Ann Intern Med 1994, 121:684–697
2. Nicosia S, Capra V, Rovati GE: Leukotrienes as mediators of asthma. Pulm Pharmacol Ther 2000, 14:3–19
3. Henderson Jr WR, Lewis DB, Albert RK, Zhang Y, Lamm WJ, Chiang GK, Jones F, Eriksen P, Tien YT, Jonas M, Chi EY: The importance of leukotrienes in airway inflammation in a mouse model of asthma. J Exp Med 1996, 184:1483–1494
4. Shi ZZ, Han B, Habib GM, Matzuk MM, Lieberman MW: Disruption of γ-glutamyl leukotrienase results in disruption of leukotriene D_4 synthesis in vivo and attenuation of the acute inflammatory response. Mol Cell Biol 2001, 21:5389–5395
5. Galli SJ: New concepts about the mast cell. N Engl J Med 1993, 328:257–265
6. Williams JD, Czop JK, Austen KF: Release of leukotrienes by human monocytes on stimulation of their phagocytic receptor for particulate activators. J Immunol 1984, 132:3034–3040
7. Weller PF, Lee CW, Foster DW, Corey EJ, Austen KF, Lewis RA: Generation and metabolism of 5-lipoxygenase pathway leukotrienes by human eosinophils: predominant production of leukotriene C4. Proc Natl Acad Sci USA 1983, 80:7626–7630
8. Anderson ME, Allison RD, Meister A: Interconversion of leukotriene catalyzed by purified γ-glutamyl transpeptidase; concomitant formation of leukotriene D_4 and γ-glutamyl amino acids. Proc Natl Acad Sci USA 1982, 79:1088–1091
9. Lieberman MW, Barrios R, Carter BZ, Habib GM, Lebovitz RM, Rajagopalan S, Sepulveda AR, Shi ZZ, Wan DF: γ-Glutamyl transpeptidase: what does the organization and expression of a multipromoter gene tell us about its functions? Am J Pathol 1995, 147:1175–1185
10. Lynch KR, O'Neill GP, Liu Q, Im D, Sawyer N, Metters KM, Coulombe N, Abramovitz M, Figueroa DJ, Zeng Z, Connolly BM, Bai C, Austin CP, Chateauneuf A, Stocco R, Greig GM, Kargman S, Hooks SB, Hosfield E, Williams Jr DL, Ford-Hutchinson AW, Caskey CT, Evans JF: Characterization of the human cysteinyl leukotriene CysLT1 receptor. Nature 1999, 399:789–779
11. Kumlin M, Dahlen SE: Characteristic of formation and further metabolism of leukotrienes in the chopped human lung. Biochem Biophys Acta 1990, 1044:201–210
12. Habib GM, Shi ZZ, Cuevas AA, Guo Q, Matzuk MM, Lieberman MW: Leukotriene D4 and cystinyl-bis-glycine metabolism in membrane-bound dipeptidase-deficient mice. Proc Natl Acad Sci USA 1998, 95:4859–4863
13. Maycock AL, Pong SS, Evans JF, Miller DK: Leukotrienes and lipoxygenase: chemical, biochemical and clinical aspect. Leukotrienes and Lipoxygenase: Chemical, Biochemical and Clinical Aspect. Edited by J Rokach. Amsterdam, Elsevier, 1989, pp 143–208
14. Mayatepek E: Leukotriene C_4 synthesis deficiency: a member of probably underdiagnosed new group of neurometabolic diseases. Eur J Pediatr 2000, 159:811–818
15. Carter BZ, Wiseman AL, Orkiszewski R, Ballard KD, Ou C, Lieberman MW: Metabolism of leukotriene C4 in γ-glutamyl transpeptidase-deficient mice. J Biol Chem 1997, 272:12305–12310
16. Carter BZ, Shi Z, Barrios R, Lieberman MW: γ-Glutamyl leukotrienase, a γ-glutamyl transpeptidase gene family member, is expressed primarily in spleen. J Biol Chem 1998, 273:28277–28285
17. Lieberman MW, Shields JE, Will Y, Reed DJ, Carter BZ: γ-Glutamyl leukotrienase cleavage of leukotriene C_4. Eicosanoid and Other Bioactive Lipids in Cancer, Inflammation, and Injury, ed 4. Edited by Honn KV, Marrett LJ, Nigam S, Dennis EA. New York, Kluwer Academic/Plenum Publisher, 1999, pp 301–306
18. Lieberman MW, Wiseman AL, Shi ZZ, Carter BZ, Barrios R, Ou CN, Chevez-Barrios P, Wang Y, Habib GM, Goodman JC, Huang SL, Lebovitz RM, Matzuk MM: Growth retardation and cysteine deficiency in γ-glutamyl transpeptidase-deficient mice. Proc Natl Acad Sci USA 1996, 93:7923–7926
19. Meister A, Larsson A: The Metabolic Basis of Inherited Diseases, ed 7. Edited by CR Scriver, AL Beaudet, WS Sly, D Valle. New York, McGraw-Hill, 1995, pp 1461–1477
20. Lewis RA, Austen KF, Soberman RJ: Leukotriene and other products of the 5-lipoxygenase pathway, biochemistry and relation to pathobiology in human diseases. N Engl J Med 1990, 323:645–655
21. Corry DB, Grunig G, Hadeiba H, Kurup VP, Warnock ML, Sheppard D, Rennick DM, Locksley RM: Requirements for allergen-induced airway hyperreactivity in T and B cell-deficient mice. Mol Med 1998, 4:344–355
22. Kobata A: Use of endo- and exoglycosidases for structural studies of glycoconjugates. Anal Biochem 1997, 100:1–14
23. Trimble RB, Maley F: Optimizing hydrolysis of N-linked high-mannose oligosaccharides by endo-beta-N-acetylglucosaminidase H. Anal Biochem 1984, 141:515–522
24. Tarentino AL, Gomez CM, Plummer Jr TH: Deglycosylation of asparagine-linked glycans by peptide: N-glycosidase F. Biochemistry 1984, 24:4665–4671
25. Meister A, Tate SS, Ross LL: Enzymes of Biological Membranes. Edited by A Martonosi. New York, Plenum Publishing Corp., 1976, pp 315–347
26. Kornfeld R, Kornfeld S: Assembly of asparagine-linked oligosaccharides. Annu Rev Biochem 1985, 54:631–664
27. Meister A, Tate SS, Griffith OW: γ-Glutamyl transpeptidase. Methods Enzymol 1981, 77:237–253
28. Novogrodsky A, Tate SS, Meister A: γ-Glutamyl transpeptidase, a

lymphoid cell-surface marker: relationship to blastogenesis, differentiation, and neoplasia. Proc Natl Acad Sci USA 1976, 73:2414–2418
29. Vecchi A, Garlanda C, Lampugnani MG, Resnati M, Matteucci C, Stoppacciaro A, Schnurch H, Risau W, Ruco L, Mantovani A: Monoclonal antibodies specific for endothelial cells of mouse blood vessels. Their application in the identification of adult and embryonic endothelium. Eur J Cell Biol 1994, 63:247–254
30. Baldwin HS, Shen HM, Yan HC, DeLisser HM, Chung A, Mickanin C, Trask T, Kirschbaum NE, Newman PJ, Albelda SM, Buck CA: Platelet endothelial cell adhesion moleculae-1 (PECAM-1): alternatively spliced, functionally distinct isoforms expressed during mammalian cardiovascular development. Development 1994, 120:2539–2553
31. Huber M, Keppler D: Inhibition of leukotriene D4 catabolism by D-penicillamine. Eur J Biochem 1987, 167:73–79
32. Frey A: γ-Glutamyl transpeptidase: molecular cloning and structural and functional features of a blood-brain barrier marker protein. The Blood-Brain Barrier. Edited by WM Pardiridge. New York, Raven, 1993, pp 339–369
33. Lawrenson JG, Reid AR, Finn TM, Orte C, Allt G: Cerebral and pial microvessels: differential expression of γ-glutamyl transpeptidase and alkaline phosphatase. Anat Embryol 1999, 199:29–34
34. Jankásková B, Lisý V, Šťastný F: Effect of γ-glutamyl transpeptidase inhibitors on the transport of glutamate into neuronal and glial primary cultures. Int J Dev Neurochem 1992, 10:225–230
35. Adkins JC, Brogden RN: Zafirlukast. A review of its pharmacology and therapeutic potential in the management of asthma. Drugs 1998, 55:121–144
36. Reiss TF, Sorkness CA, Stricker W, Botto A, Busse WW, Kundu S, Zhang J: Effects of montelukast (MK-0476); a potent cysteinyl leukotriene receptor antagonist, on bronchodilation in asthmatic subjects treated with and without inhaled corticosteroids. Thorax 1997, 52: 45–48
37. Kemp JP: Role of leukotriene receptor antagonists in pediatric asthma. Pediatr Pulmonol 2000, 30:177–182
38. Barrios R, Shi ZZ, Wiseman AL, Weity S, Kala G, Bahler AA, Ou CN, Lieberman MW: Oxygen-induced pulmonary injury in γ-glutamyl transpeptidase-deficient mice. Lung 2002, 179:1–12