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Disruption of γ-Glutamyl Leukotrienase Results in Disruption of Leukotriene D_4 Synthesis In Vivo and Attenuation of the Acute Inflammatory Response

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To study the function of γ-glutamyl leukotrienase (GGL), a newly identified member of the γ-glutamyl transpeptidase (GGT) family, we generated null mutations in GGL (GGL^{tm1}) and in both GGL and GGT (GGL^{tm1}-GGT^{tm1}) by a serial targeting strategy using embryonic stem cells. Mice homozygous for GGL^{tm1} show no obvious phenotypic changes. Mice deficient in both GGT and GGL have a phenotype similar to the GGT-deficient mice, but ~70% of these mice die before 4 weeks of age, at least 2 months earlier than mice deficient only in GGT. These double-mutant mice are unable to cleave leukotriene C_4 (LTC_4) to LTD_4, indicating that this conversion is completely dependent on the two enzymes, and in some organs (spleen and uterus) deletion of GGL alone abolished more than 90% of this activity. In an experimental model of peritonitis, GGL alone is responsible for the generation of peritoneal LTD_4. Further, during the development of peritonitis, GGL-deficient mice show an attenuation in neutrophil recruitment but not of plasma protein influx. These findings demonstrate an important role for GGL in the inflammatory response and suggest that LTC_4 and LTD_4 have distinctly different functions in the inflammatory process.

Leukotrienes (LT) are a group of biologically active metabolites of arachidonic acid and have been implicated in the pathophysiology of many inflammatory diseases, including asthma, arthritis, psoriasis, and inflammatory bowel disease (8, 17, 20). Unlike many other inflammatory mediators, LT are not stored but synthesized de novo in response to inflammatory stimuli (17). Synthesis of LT is an intracellular process initiated by the conversion of free arachidonic acid to LTA_4, an epoxide intermediate, by the key enzyme 5-lipoxygenase and the accessory protein 5-lipoxygenase-activating protein. LTA_4 can be converted to LTB_4 by LTA_4 hydrolase or conjugated with glutathione (GSH) by LTC_4 synthase to form LTC_4. LTC_4 is then transported to the extracellular microenvironment where it is converted to LTD_4 (the cysteinyl glycine conjugate of LTA_4) and then to LTE_4 (the cysteinyl conjugate of LTA_4).

LTC_4 and its metabolites, LTD_4 and LTE_4, are referred to as cysteinyl LT and were originally identified as the active components of the slow reacting substance of anaphylaxis. They are known to stimulate a wide spectrum of inflammatory processes, including vasoconstriction, increases in postcapillary venule permeability, local recruitment of eosinophils, bronchoconstriction, and mucous secretion (8, 9, 17, 19, 20). LT have been the focus of extensive investigation in recent years because of the potency of their inflammatory effects, particularly in asthma, and promising therapeutic results with novel inhibitors of their synthesis and/or antagonists of their receptors (9, 19).

Cysteinyl LT exert their effects through specific receptors; however, there has been significant uncertainty about the interaction of cysteinyl LT with their receptors and the relative potencies of individual cysteinyl LT. In vivo studies have shown that the onset of the bronchoconstriction response to inhaled LTC_4 generally occurs later than the response to inhaled LTD_4, suggesting that LTC_4 is metabolized to LTD_4 before acting on the receptor (11). These observations suggest that a block in LTC_4 metabolism or a deficiency of LTD_4 formation could significantly diminish or abolish the inflammatory effects of cysteinyl LT. On the other hand, a block in LTC_4 clearance could cause an accumulation of LTC_4 that might compensate for the lack of formation of LTD_4 or even accelerate the inflammatory response. Recently the human cysteinyl LT_1 receptor has been cloned (26, 31). The affinity of LTD_4 for the receptor is ~350-fold higher than that of LTC_4 and 200-fold higher than that of LTE_4. LTD_4 is about 10-fold more potent than LTC_4 in activating the receptor while LTE_4 is a weak agonist (26). However, in another study on the same receptor, LTC_4 and LTD_4 were found to produce similar degrees of calcium mobilization in receptor-transfected human embryonic kidney (HEK-293) cells (31). Moreover, in guinea pig and rat, evidence suggests the presence of an LT receptor (probably cysteinyl LT_2) that is highly selective for LTC_4 (18, 29). A human cysteinyl LT_2 receptor has recently been cloned (16) and found to have binding properties and a tissue distribution different from those of cysteinyl LT_1.

Recent studies of two enzymes that metabolize LTC_4 provide an opportunity to assess these issues (4, 5, 23, 24). A second member of the γ-glutamyl transpeptidase (GGT) gene family, termed γ-glutamyl leukotrienase (GGL), has recently been cloned and characterized (4, 5). GGT and GGL share 41% amino acid sequence identity and are tightly linked on the same chromosome (4, 5, 24). LTC_4 (the GSH conjugate of

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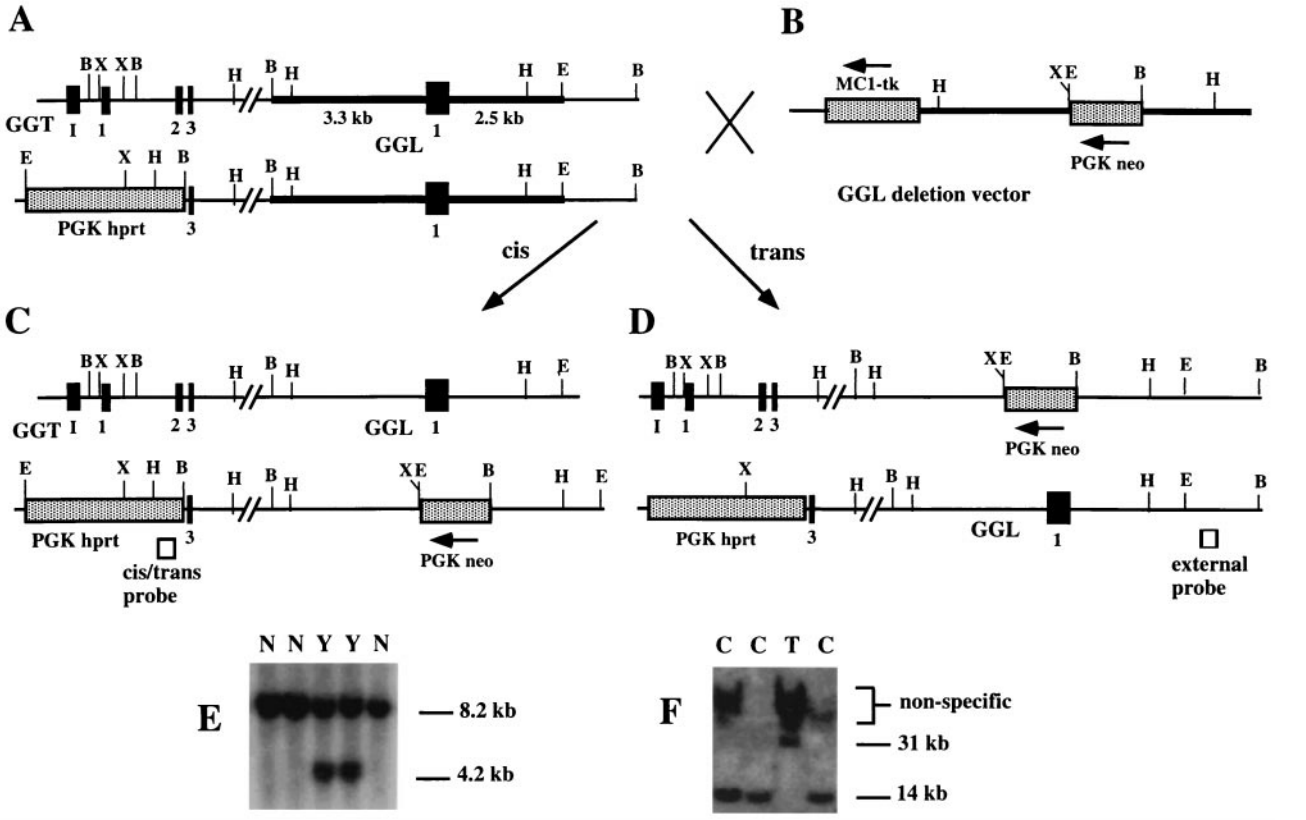

FIG. 1. Generation of ES cells carrying GGT^{tml} and GGL^{tml} mutations at both *cis* and *trans* locations. (A) An ES clone heterozygous for GGT^{tml} was used for gene targeting at the GGL locus. In one of the GGT alleles, the first noncoding exon (I) and coding exons 1 and 2 had been replaced by *PGK-hprt* cassette (23). Note that the two genes are only ~3 kb apart. On the map, only the first exon of GGL is depicted. B, *Bam*HI; X, *Xho*I; H, *Hin*dIII. (B) A targeting construct was designed such that the first exon of GGL was replaced by a *PGKneo* selective marker cassette; the *MC1tk* cassette was used for negative selection. (C and D) Predicted mutant alleles after homologous recombination. When the targeted deletion of GGL (GGL^{tml}) occurs on the same chromosome as GGT^{tml}, the two mutations are in *cis* (C); when GGT^{tml} and GGT^{tml} are on different chromosomes, they are in *trans* (D). (E) Southern blot analysis of ES cells using an external probe for the GGL gene (a 0.25-kb *Pfl*MI/*Sac*I fragment). The probe identifies an 8.2-kb *Bam*HI band (wild type) as well as a 4.2-kb *Bam*HI band (mutant). ES cell clones positive for both bands may carry GGT-GGL double mutations (either in *cis* or in *trans*). Y, GGL locus targeted; N, GGL locus not targeted. (F) Southern blot analysis to distinguish *cis* and *trans* double mutations. A probe isolated from the 3′ *hprt* sequence was used to hybridize *Xho*I-digested ES cell DNA. A 31-kb band represents the allele carrying only GGT^{tml} (GGL^{tml} is on the other allele; thus, the two mutations are in *trans* [T]); the 14-kb band carries both alleles (the two mutations are in *cis* [C]).

LTA_4) is known to be cleaved by GGT to form LTD_4 by removal of a γ-glutamyl group. Although the older literature suggests that GGT is the only enzyme capable of performing this function (for an example, see reference 27), it has been demonstrated that GGL can also cleave LTC_4 (hence the name, γ-glutamyl leukotrienase) (4, 5, 24). Because GGT is widely expressed and more abundant than GGL (4, 5, 23, 24), it is unclear what role, if any, GGL plays in vivo. Yet GGL is expressed at a relatively higher level than GGT in some organs, such as the liver and spleen, and has a 10-fold-lower K_m for LTC_4 than GGT, suggesting that GGL may function in vivo to convert LTC_4 to LTD_4.

We reasoned that a genetic approach in which GGL alone or GGL and GGT together were disrupted would clarify the function of GGL and its role in LTC_4-to-LTD_4 conversion in the mouse. Further, such single- or double-mutant mice might be more useful for analyzing the biological functions of individual cysteinyl LT than standard methods employing inhibitors (2, 28). In this communication, we describe the generation and characterization of GGL null mice and GGT-GGL null mice. Our findings demonstrate that GGT and GGL are the only two enzymes capable of cleaving LTC_4 to LTD_4, that the disruption of GGL alone is sufficient to diminish LTD_4 synthesis during peritoneal inflammation, and that the absence of GGL results in attenuation of the acute inflammatory response.

MATERIALS AND METHODS

Construction of the targeting vector and generation of double-mutant mice. The replacement targeting vector for the GGL locus contained 3.3 kb of sequence of the 5′ homology arm, a reverse-oriented *PGKneo* selectable marker cassette, a 2.5-kb genomic fragment as the 3′ homology arm, and an *MC1tk* expression cassette for negative selection (Fig. 1A to D). Correct targeting of this vector would replace an ~500-bp region of the locus, including all of exon 1. The vector was linearized and electroporated into an AB2.1 embryonic stem (ES) cell line heterozygous for the GGT mutant allele previously generated in this laboratory (23). The mutant ES cells were selected in G418 and 1-(2′-deoxy-2′-flouro-β-D-arabinofuransyl)-5′-iodouracil (FIAU), and the homologous recombination events subsequent to the preexisting GGT^{tm1} locus were determined by Southern blot analysis. In the analysis, as shown in Fig. 1E and F, a GGL external

probe was first used to screen ES clones carrying the second targeted mutation (GGL^{tm1}); further, an ~300-bp *Hin*dIII/*Hin*cII fragment isolated from the 3′ region of *PGK-hprt* cassette was used as a probe to distinguish different positions of the mutant alleles in *Xho*I-digested genomic DNA. Clones carrying double mutations (GGT^{tm1} and GGL^{tm1}) in both *cis* and *trans* orientations were expanded and injected into blastocysts of C57BL/6 mice to produce chimeras. Seven male chimeras derived from GGT^{tm1}-GGL^{tm1} (*cis*) ES cells were mated with C57BL/6 females to produce F_1 heterozygous mice. Germ line transmission of the mutant alleles was confirmed by Southern analysis. Among chimeras derived from GGT^{tm1}-GGL^{tm1} (*trans*) ES cells, only one female was found to transmit the two mutant alleles through the germ line after breeding with a C57BL/6 male mouse (like the case in reference 25). Heterozygous crossing of offspring from the above chimeras resulted in mice homozygous for GGT^{tm1}-GGL^{tm1} (*cis*) and the single mutation. In a separate experiment, the GGL targeting vector was electroporated into AB2.1 ES cells (wild type), and the mutant clones were selected and subjected to blastocyst injections. In this case, four male chimeras were generated and three of them were found to transmit the GGL^{tm1} allele through the germ line. Matings of these GGL^{tm1} heterozygotes were also performed to produce $GGL^{tm1/tm1}$ mice. Genotypes of F_1 and F_2 mice were routinely determined by Southern blot analysis of tail DNA using a GGT 5′ external probe (23) and a GGL 3′ external probe (for the GGT^{tm1}-GGL^{tm1} allele) or a GGL 3′ external probe (for the single GGL^{tm1} allele) (Fig. 1E and F). All mice were maintained on a C57BL/6/129SvEv background.

GGL activity assay. LTC_4-to-LTD_4 converting activity in mouse tissues and peritoneal lavage fluid was determined by high performance liquid chromatography (HPLC) as previously described (4). Fresh homogenates were used for the assay.

Zymosan-induced peritonitis. Adult female homozygous mice (18 to 22 grams, 8 to 12 weeks) were used in this inflammatory model (10, 30). Briefly, mice were injected intraperitoneally with 1 mg of zymosan A (Sigma) in 1 ml of sterile saline. At different time points, mice were sacrificed by CO_2 asphyxiation; peritoneal cavities were lavaged with 4 ml of phosphate-buffered saline (PBS). After centrifugation, supernatants were collected for measurement of total protein concentration and LT, and the cell pellets were saved for measurement of myeloperoxidase (MPO) activity. To monitor recovery of the LT, an internal control (prostaglandin B_1, 0.1 μg) was included in the 4 ml of PBS prior to lavage. Three to six mice from each genotype were analyzed at each time point.

Assays for MPO and LT in peritoneal fluid. Cell pellets from 1.5 ml of peritoneal cavity lavage fluid were disrupted in 0.5 ml of 0.5% HTAB (hexadecyltrimethylammonium bromide) solution by sonication and freeze-thawing (three times). MPO assays were performed as previously described (1). LT metabolites in the lavage supernatant were isolated and measured by HPLC by procedures used for the tissue GGL assay (see above and reference 4).

RESULTS

Targeted disruption of GGL in mice. The generation of a GGT null allele in mice by homologous recombination has been previously described (23). This allele, GGT^{tm1mwl} (hereafter designated GGT^{tm1}), was created by the replacement of a GGT sequence containing exons I, 1, and 2 with a *PGK-hprt* targeting vector. The first exon of GGL is only ~3 kb downstream of the last exon of GGT (5). To generate mice with a null mutation in GGL and mice in which both GGT and GGL were absent, we first constructed a targeting vector in which a 0.5-kb genomic fragment of GGL containing the TATA box and the first exon (encoding the first 58 amino acids) was replaced by a *PGKneo* cassette (Fig. 1). This vector was introduced into ES cells carrying the GGT^{tm1} allele (previously targeted [23]) to produce a secondary targeting event. We predicted that this second homologous recombination event would generate two sets of chromosomes with respect to the mutant alleles: a *cis* set, if the targeting of the GGL locus (GGL^{tm1mwl}, hereafter referred to as GGL^{tm1}) occurred on the same chromosome as the previously targeted GGT locus (GGT^{tm1}); and a *trans* set, if the GGL^{tm1} targeting occurred on the chromosome containing the wild-type allele (Fig. 1). By standard methods, we isolated several ES clones carrying both sets of chromosomes, and germ line transmission was achieved. By crossing F_1 mice heterozygous for the mutations in *cis* (GGT^{tm1}-GGL^{tm1}), we obtained homozygous double-mutant mice ($GGT^{tm1/tm1}$-$GGL^{tm1/tm1}$), which were presumably lacking in both GGT and GGL. Similarly, we also generated mice homozygous for GGL^{tm1} alone ($GGL^{tm1/tm1}$) by selective breeding of the offspring of a chimeric mouse derived from ES cells in which the GGT and GGL mutations were in *trans*. In addition, we also generated another line of GGL^{tm1} mice de novo through a traditional strategy, i.e., targeting the GGL locus in wild-type ES cells using the same targeting vector, and by breeding F_1 heterozygous mice from male chimeric founders. Mice homozygous for GGL^{tm1} created using the above two strategies showed no phenotypic differences.

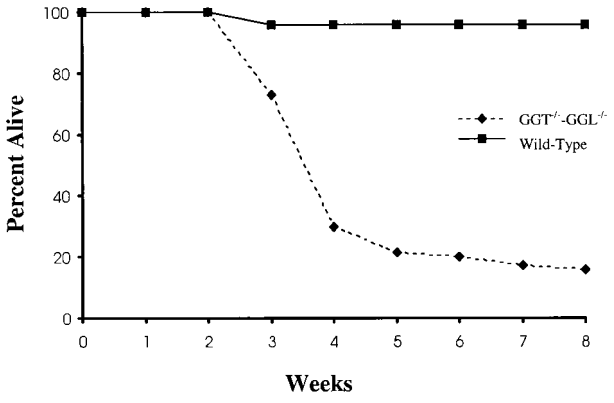

FIG. 2. Survival of $GGT^{tml/tml}$-$GGL^{tml/tml}$ mice ($n = 70$) compared to that of wild-type mice ($n = 73$).

Phenotypes of $GGL^{tm1/tm1}$ and $GGT^{tm1/tm1}$-$GGL^{tm1/tm1}$ mice. When the offspring of heterozygous crosses were genotyped at 2 to 3 weeks of age, all lines showed the expected Mendelian ratio of 1:2:1 (data not shown), indicating that GGL and/or GGT is not required for embryogenesis and is not lethal to embryonic or neonatal mice. Mice homozygous for GGL^{tm1} were grossly undistinguishable from their wild-type littermates and survived for at least a year, indicating that $GGL^{tm1/tm1}$ alone did not affect growth and development. However, mice homozygous for the double mutation ($GGT^{tm1/tm1}$-$GGL^{tm1/tm1}$) failed to thrive and ~70% died by 4 weeks of age (Fig. 2). The phenotype of double-mutant mice that survived was similar to that of mice deficient in GGT alone, including growth retardation, sexual immaturity, gray coat color, and development of bilateral cataracts (22, 23). Death in the double-mutant mice occurred much earlier than in GGT-null mice, which died between 10 and 18 weeks (reference 23 and unpublished observations). From these data it appears that there is partial functional complementation.

NAC rescue of double-mutant mice. Previous studies from our group demonstrated that administration of *N*-acetyl cysteine (NAC) in drinking water can rescue GGT-null mice from the lethal effects of cysteine deficiency that occurs as a result of cysteine loss (as GSH) in the urine (23). When NAC was provided to $GGT^{tm1/tm1}$-$GGL^{tm1/tm1}$ mice in the drinking water beginning at weaning (10 mg/ml), it failed to rescue them. However, when NAC was available in the drinking water from

TABLE 1. Conversion of LTC_4 to LTD_4 in wild-type mice and mice deficient in GGT and/or GGL

Organ	Mean conversion (nmol/h/mg of protein) ± SEM for mouse type			
	Wild type[a]	GGT deficient[a]	GGL deficient[a]	GGT and GGL deficient[b]
Spleen	3.85 ± 0.102	4.12 ± 0.119	0.63 ± 0.105	ND
Uterus	7.19 ± 0.305	7.38 ± 0.296	0.37 ± 0.019	ND
Lung	0.45 ± 0.014	0.12 ± 0.007	0.48 ± 0.019	ND
Liver	0.40 ± 0.023	0.43 ± 0.096	0.05 ± 0.008	ND

[a] $n = 6$.
[b] $n = 3$; ND, not detectable.

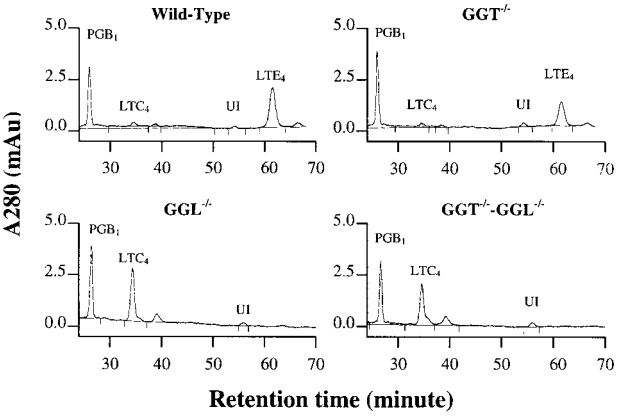

FIG. 3. Analysis of endogenous cysteinyl LT in lavage fluid from zymosan A-induced peritonitis by HPLC. Three hours after zymosan A injection, cysteinyl LT in the supernatants of the lavage fluid were isolated. Prostaglandin B_1 (PGB_1) (Sigma) was used to correct for recovery of the LT. Peaks of PGB_1, LTC_4, and LTE_4 were authenticated by comparing them with retention times of commercial standards (Cayman Chemical Co.). Peak UI is an unidentified nonspecific peak.

birth, survival of the $GGT^{tm1/tm1}$-$GGL^{tm1/tm1}$ mice was dramatically enhanced. Under these circumstances, we found that ~84% (52 of 62) of the double-mutant mice supplemented with NAC were alive at 28 days and ~50% were alive at 12 weeks of age. Presumably when treatment was begun at birth, pups received NAC initially through the milk and later by drinking the water on their own. Similar to rescued GGT-null mice, the growth curve of $GGT^{tm1/tm1}$-$GGL^{tm1/tm1}$ mice fed with NAC was parallel to that of wild-type mice (see reference 23; also data not shown). At 8 to 12 weeks of age, the average weight of $GGT^{tm1/tm1}$-$GGL^{tm1/tm1}$ mice fed NAC was 90% of that of wild-type littermates. In addition, similar to observations with GGT-deficient mice, administration of NAC to $GGT^{tm1/tm1}$-$GGL^{tm1/tm1}$ mice partially prevented formation of cataracts as well as the dark coat color (7, 23).

The reproductive activities of these mutant lines were also tested by setting up matings between homozygous mutant males and females or between homozygous mutants and wild-type mice. Mice homozygous for GGL^{tm1} were fertile and indistinguishable from wild-type littermates with respect to reproduction. $GGT^{tm1/tm1}$-$GGL^{tm1/tm1}$ mice rescued by NAC had reduced fertility: during a 6-month term of observation, four of five double-mutant females gave birth to litters of normal size after mating with wild-type males; however only two out of seven double-mutant males mated with wild-type females sired offspring. In contrast, mice lacking only GGT (and maintained on NAC) show little if any reproductive loss (22, 23).

LTC_4 cleavage in mutant mice. Earlier studies showed that LTC_4-to-LTD_4 conversion occurs in GGT-null mice (4). However, these studies did not resolve the issue of how many enzymes in addition to GGT can catalyze this process. To resolve this issue we measured LTC_4-to-LTD_4 conversion in single- and double-mutant mouse lines in different organs (Table 1). To maximize the likelihood of detecting additional enzyme activity, we examined LTC_4-to-LTD_4 conversion in homogenates of organs known to have low GGT levels (4, 23). Among the four organs examined, the spleen and uterus had very high levels of LTC_4-to-LTD_4 conversion in wild-type mice. In the absence of GGT these activities remained high. However, in mice lacking GGL alone, only low levels of activity were detected, suggesting that GGL (not GGT) catalyzes most of the LTC_4-to-LTD_4 conversion activities in these two organs. In mice lacking both GGL and GGT, no LTC_4-to-LTD_4 conversion activity was detected. The lung and liver had much lower enzyme activity than the spleen and uterus in wild-type mice. Analysis of individual mutant phenotypes revealed that in the liver, GGL contributed a higher percentage of the total activity while the opposite was true in the lung. Our data demonstrate that GGL and GGT are responsible for all of the LTC_4-to-LTD_4 conversion in these organs and that the uterus has the highest levels of GGL activity of all the organs examined (see also references 4 and 23).

The absence of LTC_4-to-LTD_4 conversion in double-mutant mice (Table 1) also indicates that GGL^{tm1} is a null allele. Since GGT^{tm1} is known to be a null allele (23) and the double knockout abolishes LTC_4-to-LTD_4 conversion, it follows that GGL^{tm1} is also a null allele. In addition, Western blot analysis with an anti-GGL antibody failed to detect any GGL protein in tissues from $GGL^{tm1/tm1}$ mice (data not shown).

LTC_4-to-LTD_4 conversion during the inflammatory response in mutant mice. Both GGT and GGL cleave LTC_4 to form LTD_4 in standard in vitro enzyme assays (Table 1 and reference 4). To investigate the in vivo role of each enzyme in LTC_4 metabolism during the inflammatory response, we induced peritonitis by injection of zymosan A into the peritoneal cavity of wild-type and mutant mice (10, 30). We employed our standard HPLC-based method to analyze endogenously formed LT metabolites in peritoneal lavage fluid (4, 15). One would expect in zymosan A-exposed wild-type mice that newly synthesized LTC_4 would be rapidly converted first to LTD_4 by GGT and/or GGL and then to LTE_4 by membrane-bound dipeptidase(s) (14, 15). As shown in Fig. 3, 3 h after intraperitoneal injection of zymosan A, LTE_4 was readily detectable in the peritoneal lavage fluid, while LTC_4 and LTD_4 were almost undetectable. We found the same pattern of rapid clearance of LTC_4 in GGT-null mice. In contrast, in both GGL-null mice and GGL-GGT-null mice, high levels of LTC_4 accumulated and LTD_4 and LTE_4 were undetectable. Taken together, these data indicate that in response to intraperitoneal zymosan A administration, GGL alone catalyzes LTC_4-to-LTD_4 conversion in this model of peritoneal inflammation.

To determine the source of GGL activity that was responsible for conversion of LTC_4 to LTD_4 in this model of perito-

neal inflammation, we analyzed the cells recovered by lavage from the peritoneal cavity of mice treated with zymosan A. We failed to detect any conversion of LTC_4 to LTD_4 in standard assays of cell homogenates from any of the wild-type or mutant mice. Nor were we able to detect GGL protein in these cells by Western blot analysis using an anti-GGL antibody (data not shown). These data rule out both resident free macrophages and extravasated inflammatory cells as the source of GGL activity. They suggest that GGL activity is present in unharvestable resident (tissue) peritoneal cells and/or peritoneal or mesenteric blood vessels. Recent studies from our laboratory indicate that capillary endothelial cells are a major location of GGL (data not shown).

Attenuation of zymosan A-induced peritonitis in GGL-null mice. The biochemical data presented above encouraged us to examine the biological profile of the inflammatory response in these mutant mice. We again employed the zymosan A-induced model of peritonitis. The literature suggests that LT are the major mediators of inflammation in this model (10, 30), and mice on a C57BL/6/129SV genetic background are suitable for this analysis (12). As expected, in wild-type mice the intraperitoneal injection of zymosan A induced a marked inflammatory response consisting of a rapid accumulation of protein (extravasation of plasma) (Fig. 4A). Plasma protein leakage into the peritoneal cavity in response to the zymosan A was identical at all time points examined in GGL-mutant mice and in wild-type mice.

We also measured MPO activity, a quantitative marker for neutrophils, in lysates prepared from cell pellets of the lavage fluid to estimate the extravasation of neutrophils. We found that zymosan A-induced MPO activity in GGL-deficient mice was markedly lower than in wild-type mice during the first 4 h after treatment (Fig. 4B). In fact, at 2, 3, and 4 h, peritoneal MPO activity in zymosan A-treated GGL-deficient mice was only 28, 33, and 63%, respectively, of the activity seen in treated wild-type mice. Subsequently (at 6 and 24 h), MPO activities in GGL-null and wild-type mice were similar, suggesting that other factors control neutrophil accumulation at later times. This reduction in MPO activity in zymosan A-induced peritonitis in GGL-deficient mice was verified by differential cell counting using the lavage fluid collected 3 h after injection.

These results demonstrate that GGL attenuates recruitment of neutrophils during zymosan A-induced peritonitis. Since the only known in vivo activity of GGL is LTC_4-to-LTD_4 conversion, and LT are well-documented mediators of inflammation, it is likely that failure to generate LTD_4 following intraperitoneal injection of zymosan A results in failure to accumulate neutrophils during acute inflammation in this model. It is conceivable that LTE_4 formation is responsible for stimulation of neutrophil influx, but we view this possibility as unlikely given that LTE_4 is known to be 2 orders of magnitude less potent than LTC_4 and LTD_4 (8, 17, 20).

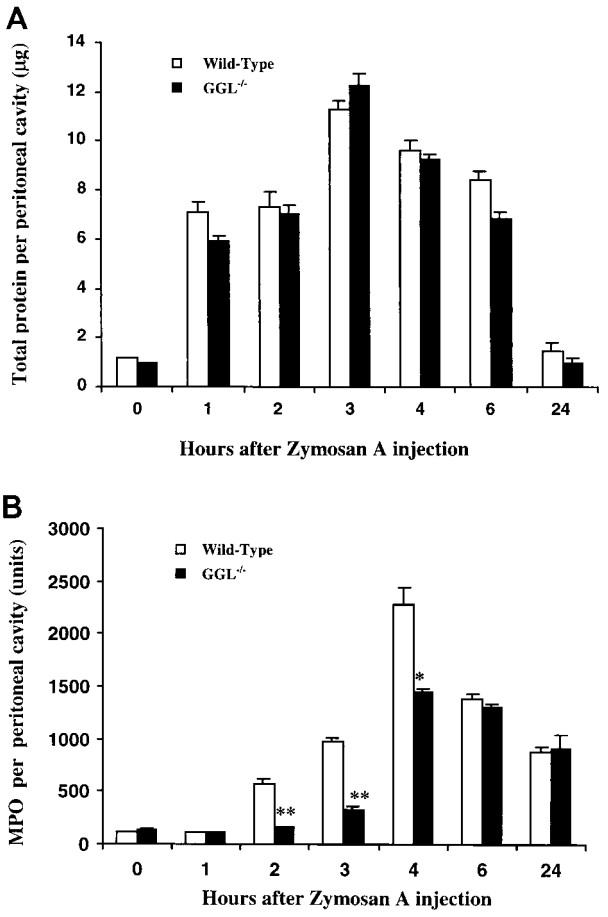

FIG. 4. Effect of GGL deficiency on plasma protein extravasation (A) and neutrophil infiltration (B) in a model of experimental peritonitis. GGL-null and wild-type mice were injected intraperitoneally with 1 mg of zymosan A. At 0, 1, 2, 3, 4, 6, and 24 h, mice were euthanatized and peritoneal lavage was performed with 4 ml of PBS. To evaluate plasma extravasation, the total protein concentration in the supernatant of the lavage fluid was measured (10); for quantification of neutrophil accumulation, MPO activity was determined in the cell pellets of the lavage fluid. Data shown are means ± standard errors of the mean. $n = 3$ *to* 8; *, $P < 0.05$; **, $P < 0.01$.

DISCUSSION

We describe here the generation of mice with null mutations in the GGL gene (GGL^{tm1}) and in both the GGL and GGT genes (GGT^{tm1}-GGL^{tm1}). Our studies indicate that GGL and GGT are the only enzymes capable of converting LTC_4 to LTD_4 in the mouse and that during chemically induced peritoneal inflammation, GGL alone is responsible for the accumulation of LTD_4 in peritoneal fluid. In this model, LTD_4 participates in the acute phase of inflammation by stimulating an ingress of neutrophils.

Both GGL and GGT are membrane-bound γ-glutamyl peptidases that cleave S-substituted GSHs, including LTC_4 (24, 27); however, GGL has a narrower spectrum of substrates and does not cleave GSH (4, 5, 24). In addition, GGL is expressed at very low levels in most organs (4). Our findings indicate that GGL functions independently of GGT in at least one inflammatory response. It is known that the GGL and GGT genes are tightly linked, and it is possible that GGL evolved from GGT by gene duplication to execute specialized γ-glutamyl cleavage functions (5). At present, the only known natural substrate for GGL is LTC_4, but it is not inconceivable that GGL participates in other restricted cleavage activities with important biological consequences.

The mechanism by which the absence of GGL enhances the mortality induced by the absence of GGT deficiency is unclear. Whether this lethality relates to LTC_4 metabolism or some other as yet unknown function of GGL cannot be determined at present. Previous studies indicate that the lethality of GGT deficiency is largely the result of cysteine deficiency secondary to massive loss of GSH in the urine (23). Since GGL does not cleave GSH (4), it cannot be involved in the reabsorption of cysteine in the renal proximal tubules. It is possible that accumulation of LTC_4 or failure to generate LTD_4 and LTE_4 is somehow critical to sustained growth and development. However, because deletion of all LT synthesis is not lethal (6, 13), it is not clear how this influence could occur. The most likely conclusion is that there is some as yet unidentified reaction catalyzed by GGL and GGT that is important for sustained growth and development. This contention is supported by the fact that NAC administration, which completely compensates for cysteine loss in GGT-null mice (23), only partially rescues GGL-GGT double-mutant mice.

Our data also demonstrate an important role for GGL in a model of peritoneal inflammation. In the absence of GGL, zymosan A-treated mice fail to convert LTC_4 in peritoneal fluid to LTD_4 and show marked attenuation of neutrophil accumulation in the acute phase of peritonitis (Fig. 4). The importance of these findings is that they suggest that differences between LTC_4 and LTD_4 are not merely quantitative; rather, the two LT have at least some distinct functions.

Based on our data and those in the literature, it seems likely that LTD_4 works synergistically with the well-recognized chemotactant LTB_4 in the recruitment of neutrophils. The contribution of cysteinyl LT to the influx of neutrophils to the inflammatory site (peritoneal cavity) has been previously shown in a study using 5-lipoxygenase knockout mice (deficient in all LT) and LTA_4 hydrolase knockout mice (deficient in LTB_4 only) (3). Our data indicate that LTD_4 rather than LTC_4 mediates this response (Fig. 3 and 4). These data are also consistent with pharmacologic studies showing that LTD_4, rather than LTC_4, is responsible for neutrophil extravasation (21).

In summary, the data presented in this paper establish that GGL functions independently of GGT and demonstrate the importance of GGL in inflammation. Furthermore, our findings suggest that the ability of GGL to catalyze the conversion of LTC_4 to LTD_4 has pathophysiologic consequences and that other biologically important substrates for GGL may also exist.

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