High-affinity pan-specific monoclonal antibodies that target cysteinyl leukotrienes and show efficacy in an acute model of colitis

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Abstract Cysteinyl leukotrienes (CysLTs) are a small family of biological signaling lipids produced by active leukocytes that contribute to diverse inflammatory disease states as a consequence of their engagement with dedicated G protein-coupled receptors. Immunization of mice with a CysLT-modified hapten carrier protein yielded novel monoclonal antibodies that display variable binding affinity to CysLTs. Solution binding assays indicated differing specificities among the antibodies tested, with antibody 10G4 displaying a preference for leukotriene C₄ (LTC₄). X-ray crystallography of a humanized 10G4 Fab fragment in complex with LTC₄ revealed that binding induces a hook-like conformation within the hydrocarbon tail of the lipid arachidonic acid moiety. Specific hydrogen bonding to the LTC₄ carboxylate groups further stabilized the complex, while a water molecule mediated a hydrogen bond network that connected the N-terminal arm of L-glutathione to both the arachidonyl carboxylate of LTC₄ and the antibody heavy chain. Prophylactic administration of two anti-CysLT antibodies in mice followed by challenge with LTC₄ demonstrated their in vivo efficacy against acute inflammation in a vascular permeability model. 10G4 ameliorated the effects of acute dextran sulfate sodium-induced colitis, suggesting that anti-CysLT antibodies could provide a therapeutic benefit in the treatment of inflammatory diseases.

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The cysteinyl leukotrienes (CysLTs), leukotriene (LT) C₄, LTD₄, and LTE₄, are biologically active lipids involved in both acute and chronic inflammatory responses that have been shown to contribute to inflammatory bowel disease and asthma (1, 2). CysLTs were initially identified as the slow reacting substances of anaphylaxis (3). These pro-inflammatory factors are produced from arachidonic acid through the 5-lipoxygenase (5-LO) pathway. Arachidonic acid is enzymatically converted to LTA₄ by 5-LO. LTC₄ synthase or, to a much lesser extent, glutathione-S-transferase II, then catalyzes the production of LTC₄ from LTA₄ and L-glutathione. LTC₄ is next converted through removal of the glutamate residue of its glutathione moiety to LTD₄ (Fig. 1A). Hydrolysis of the glutathionyl glycine then converts LTD₄ to LTE₄, the final stable metabolite in the pathway (4–7).

CysLTs act differentially through the G protein-coupled receptors (GPCRs), CysLT₁R, CysLT₂R, and CysLT₃R (also known as OXGR1 or GPR99), to mediate the inflammatory response (8–10). In transfected cells, CysLT₁R exhibits affinity for all three CysLTs where the potency rank order is LTD₄ > LTC₄ > LTE₄ (11). CysLT₂R exhibits a potency rank order where LTC₄ = LTD₄ >> LTE₄, while CysLT₃R responds to LTE₄ >> LTC₄ > LTD₄ (12, 13). Binding by CysLTs to their GPCRs causes a signaling cascade that promotes calcium release leading to cell proliferation and smooth muscle contraction. Engagement of CysLT₂R has further been shown to mediate an increase in vascular permeability and promote fibrosis in atopic dermatitis and chronic pulmonary inflammation (14, 15). Therefore, the three receptors appear to play overlapping, but distinct, roles in translating the inflammatory response effects of CysLTs.

Abbreviations: BCP, blue carrier protein; BS3, bis(sulfosuccinimidyl) suberate; CDR, complementarity-determining region; CsA, cyclosporine A; CysLT, cysteinyl leukotriene; DSS, dextran sulfate sodium; FAF, fatty acid-free BSA; GPCR, G protein-coupled receptor; Hu10G4, humanized version of 10G4; KinExA, kinetic exclusion assay; LT, leukotriene; 5-LO, 5-lipoxygenase; PG, prostaglandin; TX, thromboxane.

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In support of efforts aimed at developing therapeutics to treat inflammatory diseases mediated by CysLT signaling, we have produced pan-specific murine monoclonal antibodies (10G4, 2G9, 9B12, and 14H3) and carried out in vitro binding studies to measure their binding affinity and specificity for CysLTs. In preparation for assessing its clinical potential as a treatment for inflammatory diseases, a humanized version of 10G4 (Hu10G4) was produced and shown to preserve the LTC₄ binding profile of its murine precursor. To understand the source of specificity and to gain insight into its mode of antigen recognition, we have determined the 1.75 Å X-ray cocrystal structure of a Hu10G4 Fab fragment in complex with LTC₄. Treatment of mice with 10G4 or 2G9 serves to inhibit LTC₄-dependent movement of fluid across blood vessels into peritoneal tissues. Furthermore, 10G4 administration mitigates disease symptoms of mice in a dextran sulfate sodium (DSS)-induced model of acute colitis. This study illustrates that humanized monoclonal antibodies can be produced that target specific CysLTs and convey unique physiological properties that are of potential therapeutic value.

**MATERIALS AND METHODS**

**Chemicals and reagents**

All lipids used in this study were obtained from Cayman Chemical Co. (Ann Arbor, MI). The l-cysteiny1-glycine was purchased from Sigma-Aldrich (St. Louis, MO). Imject blue carrier protein (BCP), bis(sulfosuccinimidyl)suberate (BS3), Zeba desalting columns, Imject purification buffer, Imject BSA, Freund’s adjuvant, l-cysteine, EZ-link NHS-LC-LC-biotin kits, and activated papain were purchased from Thermo Scientific (Waltham, MA). Detection antibodies for hybridoma screening were purchased from Southern Biotech (Birmingham, AL). HEK293 cells were purchased from Invitrogen (Carlsbad, CA). ClonartCell°Hy hybridoma cloning kit was obtained from Stenell Technologies (Vancouver, Canada). Fatty acid-free BSA (FAF-BSA) and ProSep–A resin were purchased from Millipore (San Diego, CA). Iscove’s DMEM, 1x PBS, Gibco FBS, and Cellgro supplements were purchased from Corning (Corning, NY). NucleaseSpin RNA kits were purchased from New England Biolabs (Ipswich, MA). The expression vectors, pFUSE-Chig-mG1 and pFUSE2-CLG-mK, were obtained from Invivogen (San Diego, CA). CHO-M cells were obtained from Selexis (San Francisco, CA). SFM4CHO media, ActiCHO, protein A resin, and protein G resin were purchased from GE Healthcare Life Sciences (Pittsburgh, PA). PMMA beads for the kinetic exclusion assay (KinExA) experiments were purchased from SediBionics Instruments (Boise, ID). Coating antibodies for cross-reactivity studies, as well as detection antibodies for cross-reactivity and KinExA experiments, were purchased from Jackson Immunoresearch (West Grove, PA).

**Preparation of LTC₄-protein conjugates**

The immunogen was prepared by incubating 0.22 mg of LTC₄, 2.5 mg of BCP, and 2.9 mg of BS3 in 90% PBS/10% DMSO for 2 h at room temperature, followed by purification of the protein-lipid conjugate using 7 kDa MWCO Zeba desalting columns equilibrated with Imject purification buffer. The LTC₄-BCP flow through material was used directly for immunization. LTC₄ was also cross-linked to Imject BSA through BS3 according to the manufacturer’s recommendations. This LTC₄-BSA conjugate was used as the coat material for competition ELISA and KinExA experiments.

**Immunization and hybridoma screening**

Six 8-week-old female Swiss Webster mice were initially immunized by two subcutaneous injections of 0.025 mg (0.05 mg total) of immunogen (LTC₄-BCP) emulsified in complete Freund’s adjuvant. After 21 days, the mice were boosted with a single intraperitoneal injection of 0.05 mg of LTC₄-BCP emulsified in incomplete Freund’s adjuvant. Every week thereafter, the mice received a single intraperitoneal injection of 0.05 mg LTC₄-BCP emulsified in incomplete Freund’s adjuvant for an additional 8 weeks.

Serum samples were collected 3 days after the second, third, fifth, and ninth boosts and screened for the presence of potential anti-CysLT antibodies by direct ELISA. Briefly, 96-well ELISA plates were coated with 1 µg/ml LTC₄-BSA conjugate diluted in 0.1 M carbonate buffer (pH 9.5) at 4°C overnight. Plates were

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**Fig. 1.** Characterization of antibody binding to CysLTs. A: Chemical structures of the CysLTs targeted in this study (LTC₄, LTD₄, and LTE₄); numbering consistent with crystal structure. B–D: Direct competition ELISA of four anti-CysLT antibodies (14H3, black; 9B12, green; 10G4, blue; 2G9, red) binding to native LTC₄, LTD₄, and LTE₄, respectively.
blocked with 1% BSA in 1× PBS plus 0.1% Tween-20 (blocking buffer) for 1 h at room temperature. Then the plates were washed with 1× PBS and serum samples serially diluted in blocking buffer were added to the wells and incubated for 1 h at room temperature. The plates were washed and a 1:5,000 dilution of HRP conjugated to goat anti-mouse IgG (#103045; Southern Biotech) in blocking buffer was added to the wells. After 1 h, the plates were washed and developed using TMB (Invitrogen). The reaction was quenched after 5 min by adding 1.0 M H2SO4. The absorbance at 450 nm was measured using a Perkin Elmer plate reader (#1420) and the data were analyzed using GraphPad Prism software.

Spleens from mice that displayed high titers were harvested and used to generate hybridomas with the ClonaCell 5-HY hybridoma cloning kit. Once the hybridomas were grown to confluency and the data were analyzed using GraphPad Prism software.

Antibody sequencing, production, and purification

After three rounds of limiting dilution and growth in medium E, the hybridoma subclones were transferred to Iscove’s DMEM plus Gibco FBS and Cellgro supplements (no penicillin/streptomycin). Total RNA was isolated from 5 × 10^6 cells using the Nu-cleospin RNA kit. mRNA was isolated from total RNA using oligo d(T)25 magnetic beads and used to generate first strand cDNA followed by TdT tailing and PCR amplification, according to the manufacturer’s protocol for 5′RACE cloning (Invitrogen). The immunoglobulin heavy chain variable region cDNA was generated using a mouse IgG1 isotype-specific primer (5′-TATG-CAAGGGTTACACAGCAG-3′). The TdT-tailed cDNA was PCR amplified using a 5′ anchor primer (5′-G GCCACCGGTGAC-TAGTACGGGIGGGIGGGIG-3′) with a nested 3′ primer (5′-CA-CAAATTTCTTGCACACCTGTTG-3′). The product of the reaction was purified using a NucleoSpin gel and PCR clean-up kit and sequenced using a reverse primer (5′-CTTGCACAGGATCCACA-3′). The variable domain of the heavy chain was then amplified and inserted into an Agd/Ajf-digested fragment and ligated into the expression vector, pFUSE2-CHIg-mG1. The immunoglobulin kappa chain variable region cDNA was generated using a mouse IgG1k isotype-specific primer (5′-TATGC-GAAGCTCTTGACAATGGG-3′) and ligated into the expression vector, pFUSE2-CLlg-mK.

Murine monoclonal antibodies were produced from stable CHO-M cell lines transfected with the heavy and light chain expression vectors described above. E1000 shake flasks were seeded at 0.3 × 10^6 cells/ml in SFM4CHO media and supplemented with ActiCHO feeds. After ~10 days in culture, the harvest was clarified by centrifugation (4,000 g) followed by in-line filtration (0.45–0.20 μm; Sartorius). The Hu10G4 was produced from transient transfection HEK293 cells, and the supernatant was collected after 5 days in culture.

Antibody sequencing, production, and purification

Humanization of 10G4

The variable domains of the murine anti-CysLT monoclonal antibody, 10G4, were humanized by grafting the murine complementarity-determining regions (CDRs) into human framework regions. Suitable acceptor human framework sequences were selected using IgBLAST (16). Human immunoglobulin heavy variable 4-39 (accession number AB019438) was selected for the humanized version of the 10G4 heavy chain variable domain and JH6 (accession number J00256) was used for the heavy chain J region. For the light chain, VK1 O12 (accession number X59315) was the human framework selected for the 10G4 light chain variable domain. JK2 (accession number J00242) was used for the J region. The CDR sequences were those of the murine antibody 10G4. Humanized 10G4 variants containing various combinations of mutations to the murine amino acid in the framework regions (back mutations) of both the heavy and light chains were constructed, expressed, and evaluated for binding to LTC4, LTD4, and LTE4 using ELISA and KinExA. The variant that showed the highest affinity for the CysLTs (Hu10G4) was selected for cross-reactivity and crystallization studies.

Cross-reactivity

Aliquots of LTE4 and LTC4 were dried down under argon. Each lipid was biotinylated at a ratio of 20:1 biotin:lipid using Pierce EZ-link NHS-LC-Biotin kit according to manufacturer’s instructions (Thermo). ELISA plates (96-well; Greiner) were coated with 0.5 μg/ml coating material (Hu10G4: goat anti-human IgG, 10G4: mouse anti-human IgG, for the heavy chain and Hu10G4: mouse anti-human IgG, for the light chain) were blocked with 1% BSA in 1× PBS and 0.1% Tween-20 (blocking buffer). After washing, the plates were incubated with 1:5,000 dilution of HRP-mouse IgG. The plates were washed and developed using TMB (Invitrogen). The reaction was quenched after 5 min by adding 1.0 M H2SO4. The absorbance at 450 nm was measured using a Perkin Elmer plate reader (#1420) and the data were analyzed using GraphPad Prism software.

Measurement of antibody:CysLT binding affinity by KinExA

The equilibrium dissociation constant for individual CysLTs was determined by KinExA (Sapidyne Instruments) using a KinExA 3200 instrument equipped with an autosampler. The LT conjugate used to capture the free antibody was prepared by linking 5S-hydroxy-6R-(S-cysteinyl)-7E,9E,14Z-eicosatetraenoic acid to BSA in 0.1 M sodium phosphate and 0.15 M NaCl (pH 7.2) as described above. The purified LTE4-n-sub-BSA conjugate was diluted with running buffer (PBS without calcium and magnesium with 0.002% azide) to 30 μg/ml, adsorbed to PMMA beads, and blocked with 150 μM fraction V FAF-BSA (Calbiochem).

Aliquots of LTE4, LTC4, and LTD4 were transferred to new amber glass vials and the methanol was evaporated using a dry argon stream. The dried CysLT aliquots were resuspended in running buffer containing 15 μM FAF-BSA by repeated sonication and vortex mixing to a final CysLT concentration of 0.3 mM. Samples containing antibody and FAF-BSA (1.5 μM final) were prepared with running buffer in silanized glass tubes and the CysLT was added and serially diluted over 14 fractions. Antibody and starting CysLT concentrations varied depending on experiment and ranged from 25 to 500 μg IgG and 5 nM to 3 μM, respectively. Sample fractions were equilibrated for >1 h at room temperature before performing equilibrium affinity experiments at 0.5–1 ml/min. Antibody captured on the beads was detected using an AlexaFluor or Dylight goat anti-mouse or human secondary (Jackson ImmunoResearch) depending on the antibody being tested. Each fraction was sampled and analyzed in duplicate using the KinExA Pro software version 3.6.3 (Sapidyne Instruments).
Fcγ-specific Jackson #109-005-008; 10G4 and 2G9: goat anti-mouse IgG. Fcγ-specific Jackson #115-005-071 diluted in 0.1 M carbonate buffer (pH 9.5). Plates were sealed with thermal adhesive and allowed to incubate at 4°C overnight. Plates were washed with 1× PBS plus 0.05% Tween-20 and then blocked with 1% BSA in 1× PBS plus 0.1% Tween-20 for 1 h at room temperature. The plates were washed and primary antibody diluted in 1× PBS (Hu10G4, 100 ng/ml; 10G4, 50 ng/ml; 2G9, 50 ng/ml) was added to the plate and allowed to incubate for 1 h at room temperature. The plates were washed. All reference and test competitors were purchased from Cayman Chemicals with the exception of t-cysteine (Pierce), and t-cysteine-ε-glucine (Sigma). A 12 point 3-fold dilution series of reference competitor starting at 1 μM LTC4, and 10 μM LTE4, was used for 10G4/Hu10G4 and 2G9, respectively. A 12 point 3-fold dilution series of test competitor starting at 30 μM was used for Hu10G4, 10G4, and 2G9. All reference and test competitors were diluted in 0.5 μm tracer in 0.5× PBS plus 1% mg/ml BSA (Hu10G4 and 10G4, LTC4-LC-LC-biotin; 2G9, LTE4-LC-LC-biotin). Diluted competitor solution was applied to the plate and allowed to incubate for 21 h at room temperature. The plates were washed and a 1:60,000 dilution of secondary antibody in blocking solution (peroxidase-conjugated streptavidin Jackson #016-030-084) was allowed to incubate on the plates for 15 min. The plates were washed and developed by allowing cold TMB (Invitrogen) to incubate on the plates for approximately 5 min before the reaction was quenched by addition of 1.0 M H2SO4. Plates were read at 450 nm on a Perkin Elmer plate reader (#1420) and the data were analyzed using Graphpad Prism software.

**Fab production and complex formation**

The full-length humanized 10G4 monoclonal antibody was produced from transient 293-F cells (Invitrogen) using a 3:1 light:heavy chain ratio of plasmid. Cultures were collected after 5 days. Antibodies were purified by protein-A affinity chromatography using MabSelect resin, dialyzed against 1× PBS, and concentrated to 11.25 mg/ml using Centricron-30 centrifugal concentrators (Millipore). Concentration was determined by absorbance at 280 nm. Purified full-length antibody was incubated for 24 h at 37°C in a 160:1 ratio with activated papain (Thermo) in digestion buffer [20 mM cysteine-HCl, 50 mM sodium phosphate (pH 7.2), 20 mM EDTA]. The Fab was purified by protein-A affinity chromatography using ProSep-A resin (Millipore) and purity was confirmed by SDS-PAGE. The purified Fab was dialyzed against 50 mM Tris-HCl and 150 mM sodium chloride (pH 7.5). The purified Fab was concentrated to 14.4 mg/ml using Centricon-30 centrifugal concentrators (Millipore), sterile filtered, and stored at 4°C until ready for use. Eighty nanomoles of LTC4 (Cayman) were dried down in a glass vial under argon gas. The sample was then resuspended in 30 μl 1× sample buffer [50 mM Tris-HCl, 150 mM sodium chloride (pH 7.5)]. The sample was vortexed vigorously for 2 min, then sonicated 5–10 min and vortexed for an additional 2 min to completely resuspend lipid. Thirty-two nanomoles of total Fab were added to lipid solution and allowed to incubate on a rotator for 24 h at 4°C. The Fab:LTc4 complex was collected through a 0.22 μm cellulose acetate filter (Corning) and stored at 4°C until use. The final complex concentration was 11.3 mg/ml.

**Complex co crystallization**

Fab:LTc4 complex co crystals were grown by the vapor diffusion hanging drop method using the microcrystal additive screening approach (17). Briefly, 1 μl of antibody complex was combined with 1 μl reservoir containing 0.2 M ammonium sulfate, 0.1 M sodium cacodylate trihydrate (pH 6.5), and 30% PEG 8000 and incubated in the sealed well of a tissue culture plate at room temperature over 0.5 ml of reservoir solution. Needle-like crystals grew after 7 days. A microseed stock was prepared by resuspending the entire crystallization drop in 100 μl fresh reservoir solution followed by vigorous vortexing and storage at −20°C. The microseed stock (0.6 μl) was then added to a drop containing 2.4 μl of FabLTC4 complex and 0.8 μl of a new reservoir solution composed of 0.15 M zinc acetate dihydrate and 20% PEG 3350 and the resulting drop was sealed and incubated at room temperature over 0.5 ml of this second reservoir solution. Single crystals of dimensions 0.2 × 0.1 × 0.04 mm3 formed in roughly 5 days.

**X-ray crystallography**

Crystals were harvested directly from the mother liquor using nylon loops and transferred to a cryopreservation solution containing 20% ethylene glycol and flash-cooled by plunging into liquid nitrogen. X-ray diffraction data were collected on an ADSC Quantum 200 CCD detector at ALS synchrotron beamline 5.0.2 at LBNL. A complete set of diffraction data was collected and processed in HKL2000 (18). The data indexed to a primitive monoclinic lattice and the clear absence of reflection intensities at the 2n+1 values in 000 indicated a P2₁ space group. Matthew’s coefficient analysis suggested that with one complex in the asymmetric unit, the solvent content was 42%. Statistics for the scaled intensity data are presented in Table 3.

The Hu10G4 Fab:LTc4 X-ray cocrystal structure was solved by molecular replacement. Atomic coordinates for LT1009, the humanized anti-sphingosine-1-phosphate antibody (PDB ID: 3I9G), were modified by removal of all nonbonded atoms and deletion of the six CDR loops. The resulting model was employed as a probe using Phaser from the CCP4i package (19, 20). Breaking the probe into two rigid ensembles, one containing both the heavy and light chain variable domains and the other composed of the two constant domains, produced a single clear solution after rotation and translation functions were run against working data within the 10.0–4.0 Å range. Statistics for molecular replacement are included in Table 3.

Rigid body maximum likelihood refinement with REFMAC5 was carried out against 50.0–3.40 Å data resulting in a starting Rfree of 43.6% and Rwork of 43.2% (21). Initial restrained refinement with overall restraint of B factors was next carried out to 2.70 Å and CDR loops and side chains were built using 2Fo-Fc and Fo-2Fc difference electron density maps in COOT (22). The resulting model was refined to 2.10 Å resolution and density for the ligand was clearly seen in the resulting Fc/2Fc electron density map. Idealized coordinates and a library file for LTc4 were created using the Sketcher module in CCP4i and placed in the map density by hand in COOT. Refinement against working data to 2.0 Å with individual B factor refinement clearly revealed that the two ends of the glutathione moiety were incorrectly placed. Moreover, three strong spherical peaks could be observed with σ > 10 in the resulting Fc/2Fc map, each corresponding to a zinc ion. The ligand was manually rebuilt and three zinc ions, as well as individual water molecules, were placed by hand and refined against all working data to 1.90 Å. Model building was completed and refined by maximum likelihood with individual isotropic B factor refinement against all working data to 1.75 Å. Model stereochemistry was validated by MolProbity in Phenix (23, 24). The final model contained light chain amino acids 1-214 and heavy chain residues 1-214, one LTc4 molecule, three zinc ions, one molecule each of ethylene glycol, polyethylene glycol, and Tris, and 363 waters. The final model refinement statistics and geometry are reported in Table 3. Figures were prepared using PyMOL (25).

**Vascular permeability**

C57BL/6 mice were obtained from Charles River Breeding Laboratories. Test antibody (1.5 nmol) (2G9 or 10G4) was
combined with 1.5 nmol LTC₄ and left to incubate overnight. Mice were injected intravenously with 0.2 ml 0.2% Evan’s Blue in PBS (Sigma) and then immediately injected intraperitoneally with saline containing no antibody (control), nonspecific antibody plus LTC₄, or either 2G9 or 10G4 antibody plus LTC₄. After 30 min, mice were euthanized by isoflurane overdose and the peritoneum was washed with 1.5 ml PBS. The wash was then centrifuged at 500 g for 10 min. The optical density of the wash was read at 610 nm (Spectronic Genessys 2).

For prophylactic subcutaneous injection experiments, mice were injected with vehicle (0.5% DMSO in PBS), nonspecific antibody, or 10G4 (30 mg/kg) 24 h prior to lipid treatment. Mice were injected intravenously with 0.2 ml 0.2% Evan’s Blue in PBS. Immediately after this step, mice were injected with 1.5 nmol LTC₄. After 30 min, mice were euthanized by isoflurane overdose and the peritoneum was washed and prepared for spectrophotometry analysis, as described previously.

**Acute DSS-induced colitis**

Female C57BL/6 mice (17–20 g) (Shanghai SLAC Laboratory Animal Co. Ltd.) were housed five to a cage in a standard vivarium (PharmaLegacy Laboratories) at 20–26°C at a relative humidity of 40–70%. The animals had ad libitum access to rodent food (PharmaLegacy Laboratories) and water (PharmaLegacy Laboratories). Animals were randomly divided into seven treatment groups (n = 10 per group) on day 1: group 1, control; group 2, vehicle (PBS); group 3, cyclosporine A (CsA); group 4, LT1014; group 5, LT1002; group 6, 10G4; and group 7, 2G9. Groups 2–7 received 2% DSS in their drinking water for 6 days followed by regular drinking water for 4 days. Group 1 received regular drinking water for 10 days. Animals in group 1 remained the untreated controls while mice in groups 2–7 received respective vehicle, reference article, or test article.

Body weight and stool consistency (0, normal; 2, loose stools; 4, diarrhea) were monitored daily throughout the study. All animals were euthanized by CO₂ asphyxiation followed by cervical dislocation on day 10. The colon was harvested free from surrounding tissue. Colon length (from the ileocecal valve to the anus) and weight were measured. The colon was longitudinally bi-sectioned, one piece processed as Swiss-roll and immersed in neutral buffered 10% formalin, and the other piece snap-frozen in liquid nitrogen and stored at −80°C. The frozen colon tissue was minced on ice and homogenized in 0.5 M phosphate buffer to measure myeloperoxidase activity. The fixed colon tissue was embedded in paraffin, sectioned at 4 μm thickness, and stained with hematoxylin and eosin. In a blinded fashion that treatment information was unknown to the slide reader, the stained slides were examined by a histopathologist (PharmaLegacy Laboratories) and assessed with histopathological scoring for inflammatory cell infiltration (0, occasional or no infiltrate; 1, infiltration into lamina propria; 2, infiltration into submucosa; 3, transmural infiltration) and tissue damage (0, no mucosal damage; 1, focal crypt lesions; 2, mucosal erosions or ulcerations; 3, extensive damage affecting the submucosa). Data were expressed as group means ± SEM for body weight and colon length. Statistical analyses were performed on each parameter using GraphPad Prism, SPSS, or Sigmaplot. A value of P < 0.05 was considered statistically significant. All animal studies were conducted in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, incorporated in the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals and approved by the Institutional Review Board of BTS Research, San Diego under IACUC 1111-03.

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**RESULTS**

**Monoclonal antibodies bind LTC₄, LTD₄, and LTE₄ with high affinity**

Four monoclonal hybridomas (10G4, 2G9, 9B12, and 14H3) were generated by screening mice immunized with LTE₄ conjugated to protein carriers via disuccinimidyl homobifunctional cross-linking (26). LTE₄ was chosen on the basis that it is the stable end product of CysLT extracellular metabolism (27). Interestingly, IgG secreted from these hybridomas displayed markedly different binding properties to un conjugated LTC₄, LTD₄, and LTE₄, based on a competition ELISA with LTE₄-BSA-coated plates (Fig. 1). Comparison of the four antibodies for target binding revealed that 10G4 bound LTC₄ with the highest affinity, while 2G9 and 9B12 appeared to exhibit a preference for binding LTE₄. Monoclonal antibodies from these three clones showed similar binding to native LTD₄ using this competition format. The 14H3 clone displayed relatively weak binding to all three lipids, so it was excluded from further characterization.

The apparent high-affinity CysLT binding observed with the competition ELISA above was confirmed in solution by KinExA. In this assay, the amount of free antibody present in an antibody:lipid complex solution at equilibrium was measured after capture on lipid-coated beads. Equilibrium dissociation binding constants (Kᵰ) for the purified mouse monoclonal antibodies 10G4, 2G9, and 9B12, as well as Hu10G4, prepared by grafting the six CDR loops from 10G4 onto a human IgG scaffold and then mutating seven additional residues back to the murine sequence are reported in Table 1. The 10G4 and Hu10G4 antibodies demonstrated picomolar range binding affinity for each of the three CysLTs tested. 10G4 bound with highest affinity to LTC₄, with a measured Kᵰ value of less than 10 picomolar. As observed previously by ELISA, the 2G9 and 9B12 antibodies exhibited different target binding profiles. The 2G9 bound preferentially to LTE₄ with a measured Kᵰ value of roughly 50 picomolar. The Kᵰ values for 9B12 binding to LTC₄, LTD₄, and LTE₄ were all higher than those of 2G9, so 9B12 was excluded from further target specificity studies.

**The 2G9 and 10G4 are pan-specific antibodies against CysLTs**

Using a competition ELISA with a biotinylated tracer molecule, we next evaluated the ability of a wide range of related lipids and CysLT₁R receptor antagonist compounds to bind the immobilized antibodies (supplemental Table S1). The IC₅₀ values for each unlabeled competitor normalized as a percentage relative to unlabeled LTE₄ and LTC₄ for 2G9 and 10G4, respectively, are listed in Table 2. In addition to LTD₄ and LTE₄, 2G9 and 10G4 bound to CysLTs, LTF₄, and N-acetyl LTE₄, with high affinity (Fig. 2). However, no detectable binding was observed for 5-LO products that did not contain the sulfidopeptide moiety [LTB₄, 5(5)-HETE] or any other eicosanoids tested [12(S)-HETE, 20-HETE, prostaglandin (PG)E₂]. Finally, neither 2G9 nor 10G4 demonstrated binding to isolated 1-cysteine,
Characterization and efficacy of novel anti-CysLT antibodies

1-cysteine-1-glycine dipeptide, 1-glutathione, or to any of the commercially available CysLT1R antagonists that we tested (Table 2).

Expansion of the study to include all of these compounds paints a clear picture of target binding specificity and reveals that, although 2G9 and 10G4 were generated using the same immunization and screening processes, these monoclonal antibodies exhibited different relative binding preferences for LTC4 and its metabolites. Antibody 2G9 bound with highest affinity to LTF4 and N-acetyl LTE4, followed by LTE4 > LTD4 > LTC4, while 10G4 bound these compounds in the following order: LTC4 > LTF4 > N-acetyl LTE4 > LTD4 > LTE4. The specificity profile suggests that both 2G9 and 10G4 recognize the fatty acid hydrocarbon chain and the sulfidopeptide group on the respective CysLTs, and that both substituents of the CysLTs must be present in a proper conformation for high affinity binding. However, it is also clear that the mode of CysLT binding differs between 2G9 and 10G4.

In addition to the rank order of binding affinity for the main CysLTs, 2G9 and 10G4 also showed differences for other sulfidopeptide-containing LTs. Antibody 2G9 exhibited weak, but measurable, binding to eoxin E4 and C4, whereas 10G4 failed to display any detectable binding to these compounds. Likewise, 2G9 suffered an ~10-fold loss in binding when the C11 cis double bond in LTC4 isomerized to the trans conformation, where 10G4 showed a >100-fold loss of binding due to this alteration. Methyl esterification of the C1 carboxylate group resulted in a significant decrease in the ability of LTE4 or LTC4 to cross-react with preformed CysLT complexes with either 2G9 or 10G4, suggesting that this carboxylate group contributes significantly to antigen binding by both antibodies. This limited structure-activity-relationship study suggests that, although both antibodies

### Table 1. Binding dissociation constants ($K_d$) for CysLTs measured by KinExA

| Anti-CysLT Antibodies | Hu10G4 (pM) | 10G4 (pM) | 2G9 (pM) | 9B12 (pM) |
|-----------------------|------------|-----------|----------|-----------|
| LTC4                  | 1.5 (UD–4.28) | 5.39 (3.93–7.19) | 1,520 (1,250–1,790) | 2,600 (1,800–3,480) |
| LTD4                  | 101.36 (67.27–138.97) | 37.95 (15.57–69.71) | 575.27 (116.8–1,460) | 3,000 (1,150–5,020) |
| LTE4                  | 300.24 (222.98–367.97) | 310.67 (241.79–391.04) | 51.51 (3.68–142.44) | 628.9 (481.99–798.77) |

Ninety-five percent confidence intervals are given within parentheses. UD, unable to be determined.

### Table 2. Percentage cross-reactivity of diverse structurally related eicosanoid, sulfidopeptide, or CysLT receptor agonist compounds

| Compound          | Hu10G4 | 10G4 | 2G9       |
|-------------------|--------|------|-----------|
| LTC4              | 100.0  | 100.0| 3.8       |
| LTD4              | 4.1    | 4.0  | 5.4       |
| LTE4              | 0.6    | 0.3  | 100.0     |
| LTF4              | 76.6   | 37.3 | 424.8     |
| N-acetyl LTE4     | 27.6   | 11.4 | 440.9     |
| Eoxin C4          | <0.1   | <0.1 | 0.2       |
| Eoxin E4          | <0.1   | <0.1 | 0.8       |
| 11-trans-LTC4     | <0.1   | <0.1 | 0.3       |
| LTE4 methyl ester| <0.1   | <0.1 | 6.7       |
| LTC4 methyl ester| 1.7    | 1.5  | 0.2       |
| Montelukast       | <0.1   | <0.1 | <0.1      |
| Pranlukast        | <0.1   | <0.1 | <0.1      |
| BayCysLT2         | <0.1   | <0.1 | <0.1      |
| HAMI3579          | <0.1   | <0.1 | <0.1      |
| BAY-u9773         | <0.1   | <0.1 | 0.4       |
| Zafirlukast       | <0.1   | <0.1 | <0.1      |
| MK-571            | <0.1   | <0.1 | <0.1      |
| LTB4              | <0.1   | <0.1 | <0.1      |
| 5(S)-HETE         | <0.1   | <0.1 | <0.1      |
| 12(S)-HETE        | <0.1   | <0.1 | <0.1      |
| 20-HETE           | <0.1   | <0.1 | <0.1      |
| PGE2              | <0.1   | <0.1 | <0.1      |
| 1-cysteine-HCl-H2O| <0.1   | <0.1 | <0.1      |
| 1-cysteine-t-glycine| <0.1 | <0.1 | <0.1      |
| 1-glutathione, reduced | <0.1 | <0.1 | <0.1      |
| 1-glutathione, oxidized | <0.1 | <0.1 | <0.1      |

Fig. 2. Binding affinities of anti-CysLT antibodies measured by ELISA. A: Binding curves for interaction of murine antibody 10G4 with LTC4 (red), LTD4 (blue), LTE4 (green), LTF4 (black), N-acetyl LTE4 (orange), and LTB4 (light blue). B: Similar curve for a humanized version of the murine 10G4 antibody (Hu10G4). C: Binding data for murine antibody 2G9.
specifically target CysLTs, 10G4 shows a preference for LTC₄ and displays greater selectivity for its preferred target antigen than does 2G9.

**Hu10G4 Fab:LTC₄ complex crystal structure**

Humanization of 10G4 resulted in a monoclonal antibody, Hu10G4, with similar affinity and specificity properties as compared with the parental murine 10G4 antibody (Table 2, Fig. 1). In order to elucidate the mechanism of CysLT selective binding by 10G4, we crystallized and determined the three-dimensional structure of the Hu10G4 Fab fragment in complex with LTC₄ (Fig. 3A). The X-ray co-crystal structure was solved by molecular replacement and refined to 1.75 Å resolution resulting in a model with excellent geometry and stereochemistry (Table 3).

The Hu10G4:LTC₄ complex crystallographic model reveals that LTC₄ binds to the antigen binding site primarily by burying its hydrocarbon tail. The combination of trans double bonds at carbons 7 and 9 followed immediately by cis double bonds at carbons 11 and 14 serves to support adoption by the lipid hydrocarbon tail of a hook-like structure that inserts deep within the antigen binding site between the heavy and light chain variable domains. The glutamate residue of the LTC₄ glutathione moiety extends away from the antigen binding site. Analysis of relative temperature (B) factors and poor electron density map quality at this residue suggests that the glutamate α-carbon exhibits conformational flexibility relative to the remainder of the bound lipid antigen molecule (Fig. 3B). This is not too surprising, as this region corresponds to the end of the LTE₄ immunogen that was anchored to its hapten carrier protein during immunization. Both the arachidonate and glutathionyl glycine carboxylic acid groups participate in hydrogen bond networks involving the side chains of amino acid residues from CDR loops of both the antibody heavy and light chains. In its bound conformation, the LTC₄ lipid presents 542.1 Å² molecular surface area (probe radius 1.00 Å). Binding of LTC₄ to Hu10G4 excludes 275.5 Å² (50.83%) of its molecular surface (Fig. 3C) (28).

**Structural determinants of LTC₄ binding affinity and specificity by Hu10G4**

A detailed analysis of the noncovalent interactions between atoms of the Hu10G4 antibody Fab fragment and its LTC₄ lipid antigen reveals the chemical details of its binding affinity and selectivity. In all, side chains of sixteen amino acids from each of the six heavy and light chain CDR loops come within close contact of the bound LTC₄ antigen [the Hu10G4 antibody heavy and light chains are numbered according to the system of Kabat et al. (30); the letters “L” and “H” immediately prior to amino acid numbers indicate that they derive from the light or heavy chain, respectively]. Nine of these (TyrH33, SerH34, IleH37, TrpH47, TyrH52, AlaH96, ProH98, ArgH99, and TrpH101) emanate from the heavy chain and seven (LeuL36, ArgL46, TyrL49, LeuL89, TyrL91, ArgL96, and PheL98) are from the light chain. Two additional heavy chain amino acids (AsnH35A and AsnH50) contact the antigen indirectly through water-mediated hydrogen bonds (Fig. 4A).

A complex hydrogen bond network stabilizes the carboxylate group of the LTC₄ arachidonate moiety (Fig. 4B). This supports our observations that methylation at this carboxylate group decreases affinity of LTC₄ binding to Hu10G4. ArgL96 and TyrH52 are both positioned within hydrogen bonding distance of the same carboxylate oxygen atom.
An ordered water molecule, bonded to the side chains of AsnH35A and AsnH50, is also positioned to share a hydrogen atom with this oxygen atom. The second arachidonyl carboxylate oxygen atom is located within hydrogen bonding distance of the SerH34 side chain. Interestingly, the amide nitrogen of SerH34 positions a second water molecule to form hydrogen bonds with both the arachidonyl carboxylate group and the amide group oxygen from the glutamyl isopeptide linkage of the LTC$_4$. This second water molecule links CDR-H3 to the arachidonate carboxylate and glutamate of LTC$_4$. As removal of this glutamate converts LTC$_4$ to LTD$_4$, a single score, normalized to the same scale as X-ray resolution (24)

### Anti-CysLT antibodies inhibit vasopermeability during acute inflammation

LTC$_4$ has been shown to induce Evan’s Blue dye extravasation into a variety of tissues. This includes the respiratory and urogenital tracts and into the peritoneal cavity after either intravenous or intraperitoneal administration, respectively (31, 32). Shortly following intraperitoneal injection in mice, LTC$_4$ induces vascular permeability in a dose-dependent manner and likely helps to mediate the fluid accumulation phases of the acute inflammatory response (31). Using the mouse model, we evaluated the potency of 2G9 and 10G4 to inhibit the activity of LTC$_4$ to
induce plasma exudation into the peritoneal cavity by monitoring dye extravasation (Fig. 5).

Initially, LTC_4 was premixed with equimolar or molar excess of antibody 2G9 and then injected into the peritoneum of mice that had received prior intravenous administration of 0.2% Evan’s Blue dye. Dye extravasation was measured 30 min following injection. A statistically significant decrease of dye extravasation was observed for the groups of mice that received 2G9 (P = 0.0011 for equimolar and P < 0.0001 for excess 2G9) compared with the isotype-matched control antibody group (Fig. 5A). 10G4 also appeared to be effective at blocking LTC_4-induced plasma exudation and restricting dye extravasation to levels similar to the naïve group (Fig. 5B).

After demonstrating that preincubation of 2G9 and 10G4 with LTC_4 neutralized the biological action of LTC_4, we next investigated to determine whether 10G4 showed activity when administered prophylactically. To this end, two groups of mice were dosed subcutaneously with 30 mg/ml 10G4 or control antibody 24 h prior to LTC_4 treatment and dye extravasation was monitored as above. A significant (P < 0.0001) decrease in measured peritoneal dye was observed for the mice receiving 10G4 pretreatment compared with the control antibody treatment group (Fig. 5B). These data suggest that our anti-CysLT antibodies hold potential as therapeutic agents that effectively block the action of LTC_4 in modulating vascular permeability during the acute phase of inflammation.

10G4 protects against DSS-induced acute colitis in mice

In order to further assess the in vivo efficacy of anti-CysLT antibodies to treat acute inflammation, we next tested to determine whether treatment of mice with 10G4 or 2G9 could provide protection against acute inflammation brought on by administration of DSS, which induces colitis in mice (33, 34). C57BL/6 mice that were fed with a 2% DSS water solution for six days followed by regular drinking water during days 7–10 showed a significant decrease in body weight, increased colon weight, increased colon weight versus colon length versus body weight ratio, and increased colon weight versus body weight relative to...
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mice from which DSS was withheld. Pretreatment of the mice with antibody 10G4 served to significantly lessen the symptoms of acute colitis relative to DSS-treated mice that received either no antibody or an isotype control antibody (LT1014) that did not target CysLTs. 10G4 protected the DSS-treated mice from weight loss to the same extent as administration the immunosuppressive drug, CsA (Fig. 6A).

The same correlations were observed when mice were analyzed and assessed by their disease activity index score, which takes into account body weight, stool consistency, and severity of rectal bleeding (Fig. 6B).

Histopathological analysis of extracted colon tissue revealed decreased infiltration of inflammatory cells in DSS-treated mice that were administered 10G4 relative to vehicle- or isotype control antibody-treated mice (Fig. 6C). Finally, decreased tissue damage was measured in colon tissue extracted from the cohorts of DSS-treated mice that were administered 10G4 or CsA (Fig. 6D).

DISCUSSION

The elevated production of CysLTs is associated with diverse inflammatory disease states. Syslová et al. (35) measured a 2-fold increase of LTE4 and LTC4 in the exhaled breath condensate of patients with moderate asthma relative to healthy controls. LTC4 levels were detected at 3-fold higher levels in the urine of Crohn’s disease patients relative to healthy subjects and a 2-fold increase was measured in patients with ulcerative colitis (36). Urinary LTE4 levels increase by eight-fold in patients suffering from chronic lung disease of extreme prematurity (37). A 4-fold increase in serum LTC4 has been measured in eczema patients, while 16-fold increased LTD4 levels were reported in the serum of patients with hepatocellular carcinoma (38, 39). As CysLTs are known to promote inflammatory signaling upon binding to GPCRs, it is likely that these molecules directly promote inflammatory disease states and that therapeutic strategies aimed at interfering with CysLTs before they engage their receptors might be beneficial to clinicians and the patients they treat.

To date, drug discovery in the field of inflammatory diseases has remained focused primarily on development of small molecule therapeutics. For example, there are currently several small molecule therapeutics on the market for the treatment of asthma, including montelukast (Singulair), zafirlukast (Accolate), and zileuton (Zyflo) (40). Each of these drugs interferes with LT signaling or production as montelukast and zafirlukast target CysLT1R and zileuton inhibits the enzyme, 5-LO. However, in recent years, interest in antibody-based therapeutics has increased due to characteristics that set them apart from small molecule and natural product therapies. Antibodies can bind immutable targets with high affinity and specificity, which can be further optimized through antibody engineering. Although antibody therapeutics have many potential advantages, there are still challenges that need to be overcome that include limited penetration of membrane barriers and their potential for immunogenicity.

Past attempts at development of anti-CysLT antibodies have met with mixed success. In one study, LTD2-BSA conjugates were used to immunize BALB/c mice. The resulting anti-LTD2 antibody did not cross-react with arachidonic acid, PGD2, PGE2, PGF2, 6-keto-PGF2, thromboxane (TX) B2, l-cysteine, glutathione, 5-HETE, or LTB4. However, it displayed nearly equal specificity toward LTC4, LTE4, and N-acetyl-LTE4 with percentage cross-reactivity values of 95.7, 88.7, and 89.7, respectively, and cross-reacted significantly with 5-epi-LTC4, 6-epi-LTC4, and 11-trans-LTC4 (50, 13.2, and 5.16%, respectively) (26). In another study, anti-LTC4 and anti-LTE4 antibodies were produced by immunizing rabbits with LTC4 or LTE4 conjugated to BSA. The resulting antibodies exhibited minimal cross-reactivity to LTB4. The anti-LTC4 antibody produced from that study was 76% cross-reactive with LTD4 and 7.4% cross-reactive with LTE4, while the anti-LTE4 antibody showed 22% cross-reactivity with LTC4 and 20% cross-reactivity with LTD4 (41). In a third study, a more specific anti-LTC4 antibody was produced by immunizing BALB/c mice with an
LTC₄ binding affinity by supporting interactions with an amide carbonyl oxygen N-terminal to the glutathionyl cysteine residue is N-acetylated (supplemental Table S1). Therefore, antibody 2G9 appears to display increased antigen binding affinity by supporting interactions with an amide carbonyl oxygen N-terminal to the glutathionyl cysteine, while the presence of a glutathionyl glycine disrupts this and other 2G9:antigen binding interactions. Surprisingly, 10G4 exhibited strong preferential binding to LTC₄ over LTE₄ (4.0% cross-reactivity) or LTD₄ (0.3% cross-reactivity). The cross-reactivity of other molecules was less for 10G4 than for 2G9. Moreover, the binding affinity of LTC₄ for 10G4 was remarkably tight, with observed binding dissociation constants derived from KinExA in the low picomolar range.

As described in the present study, the X-ray crystal structure of the Fab fragment of a humanized version of 10G4 was reported. Of the antibodies tested, 2G9 displayed the strongest binding preference for LTE₄ with 3.8% cross-reactivity with LTC₄ and 5.4% cross-reactivity with LTD₄. When tested against a battery of modified CysLT and related compounds, 2G9 was found to bind with even greater preference to LT₆₄ (a version of LTC₄ in which the glutathionyl glycine has been removed, but the glutamate remains intact), as well as a version of LTE₄ in which glutathionyl cysteine residue is N-acetylated (supplemental Table S1). Therefore, antibody 2G9 appears to display increased antigen binding affinity by supporting interactions with an amide carbonyl oxygen N-terminal to the glutathionyl cysteine, while the presence of a glutathionyl glycine disrupts this and other 2G9:antigen binding interactions. Surprisingly, 10G4 exhibited strong preferential binding to LTC₄ over LTE₄ (4.0% cross-reactivity) or LTD₄ (0.3% cross-reactivity). The cross-reactivity of other molecules was less for 10G4 than for 2G9. Moreover, the binding affinity of LTC₄ for 10G4 was remarkably tight, with observed binding dissociation constants derived from KinExA in the low picomolar range.

In this study, we describe a set of antibodies that were isolated after immunization of mice with an LTE₄ protein conjugate. Of the antibodies tested, 2G9 displayed the strongest binding preference for LTE₄, with 3.8% cross-reactivity with LTC₄ and 5.4% cross-reactivity with LTD₄. When tested against a battery of modified CysLT and related compounds, 2G9 was found to bind with even greater preference to LT₆₄ (a version of LTC₄ in which the glutathionyl glycine has been removed, but the glutamate remains intact), as well as a version of LTE₄ in which glutathionyl cysteine residue is N-acetylated (supplemental Table S1). Therefore, antibody 2G9 appears to display increased antigen binding affinity by supporting interactions with an amide carbonyl oxygen N-terminal to the glutathionyl cysteine, while the presence of a glutathionyl glycine disrupts this and other 2G9:antigen binding interactions. Surprisingly, 10G4 exhibited strong preferential binding to LTC₄ over LTE₄ (4.0% cross-reactivity) or LTD₄ (0.3% cross-reactivity). The cross-reactivity of other molecules was less for 10G4 than for 2G9. Moreover, the binding affinity of LTC₄ for 10G4 was remarkably tight, with observed binding dissociation constants derived from KinExA in the low picomolar range.

As described in the present study, the X-ray crystal structure of the Fab fragment of a humanized version of 10G4 in complex with LTC₄ revealed the source of the specificity of this antibody for its lipid antigen. Antigen binding induced a unique conformation within the unsaturated hydrocarbon tail of LTC₄, while antibody side chains contributed to a water-mediated network of hydrogen bonds that linked the lipid fatty acyl group to both the glutamate and glycine arms of the covalently attached glutathione moiety. Analysis of the amino acid changes that were likely to have occurred during affinity maturation offered insight into the manner by which 10G4, which was raised by immunization against LTE₄, bound with such exceptional affinity to LTC₄. Using the NCBI/IgBLAST server to identify likely mouse germine variable domain sequences that gave rise to 10G4 revealed that all of the mutated amino acids mediated contacts with atoms that were common between LTE₄ and LTC₄ (16). For example, conversion of Phe90 in the murine kappa light chain variable domain germline sequence IgkV9-124*01 to TyrL91 in 10G4 provided a hydrogen bond donor that stabilized a water-mediated contact to the C5-OH of the LTC₄ arachidonic acid moiety, while mutation in the IgHV3-1*02 heavy chain variable domain of HisS2 to TyrH52 supported hydrogen bonding with the arachidonate C1 carboxylate group. These mutations served to make 10G4 a good antibody (Kₐ = 0.3 nM) against LTE₄. The additional placement of glutamate and glycine amino acids in the glutathionyl moiety of LTC₄ provided additional beneficial binding interactions that did not result from affinity maturation. For instance, the N atom of 10G4 amino acid SerH34 mediated a water-mediated hydrogen bond network that connected the glutamate with the arachidonic C1 carboxylate and heavy chain CDR loop 1 and ArgH99 lay within hydrogen bonding distance of the carboxylate of glycine. We speculate that these fortuitous positive interactions resulted in the “good” LTE₄ antibody, 10G4, functioning as an exceptional (Kₐ = 5 pM) antibody against LTC₄.

Prior to this analysis, only one crystallography study of CysLTs bound to a protein was reported. In 2016, Jablonka et al. (44) published X-ray crystal structures of the lipocalin family protein, LTBP1, from saliva of the “kissing bug,” Rhodnius prolixus, a vector for the Chagas’ disease pathogen, Trypanosoma cruzi, in complex with either LTD₄ or LTC₄. The protein, which is thought to sequester CysLTs in...
order to inhibit skin inflammation around the site of a bug bite, binds with nearly equal affinity to LTC₄, LTD₄, and LTE₄. The crystallographic models reveal that LTBP1 binds a CysLT by burying its fatty acyl component at the center of the lipocalin β-barrel domain, while the glutathionyl moiety causes conformational change in a loop to “lock” the lipid in place. The extended conformation adopted by the hydrocarbon tail of the bound lipid seems unlikely to convey too much specificity. However, the authors report that TXA₂, PGD₂, and arachidonic acid each fail to bind to LTBP1 and they go on to show that the loop conformational change is responsible for the CysLT binding affinity.

On the topic of antibody recognition of small molecule lipid antigens: it is interesting to note that each of the three anti-lipid antibodies we have succeeded in analyzing by complex crystallization and X-ray crystallography in this and previous studies used a significantly different approach for selective binding of its specific antigen. Whereas Hu106G4 bound LTC₄ by combining complementarity to a unique conformation available to the lipid unsaturated hydrocarbon tail with side chain- and water-mediated hydrogen bonds, the LT3015 anti-lyso phosphatidic acid antibody relied primarily on amino acid side chains from both heavy and light chain CDR loops to bury the unique phospho headgroup of its lysophosphatidic acid antigen, while leaving the hydrocarbon tail largely exposed to solvent (45). The LT1009 humanized anti-sphingosine-1-phosphate antibody used amino acids from its light chain to partially coordinate a pair of calcium ions that bound with high affinity to an otherwise highly solvent-exposed phosphophosphingoine head group, while the heavy chain nearly completely engulfed the hydrocarbon tail (46). These differences are partially explained by the various strategies used to anchor the different lipid antigens to their carrier proteins for immunization. In all three cases the atom through which the lipid was anchored to hapten is exposed to solvent. However, it seems likely that the systems studied thus far illustrate only a few of the possible chemical strategies by which antibodies can selectively bind small molecule lipids. While the relatively few existing structural models show convincingly that selective binding of lipids by antibodies is possible, future studies to identify and structurally characterize additional novel high-specificity antibody:lipid complexes are needed to expand this field and serve as structural templates for theoretical and engineering projects on specific lipid binding.

We show by two in vivo models of acute inflammation that anti-CysLT antibodies can convey beneficial outcomes. The decrease in LTC₄-dependent dye extravasation observed in mice treated with antibody 2G9 was, on the surface, surprising because we had previously found that antibody to bind preferentially to LTE₄. However, we did observe that 2G9 bound with somewhat significant affinity to LTC₄ (Table 1). Moreover, LTC₄ was metabolized to LTE₄, which is a potent activator of inflammatory signaling through CysLT₄R. We suspect that the observed effect of 2G9 relative to control antibody on dye extravasation is a result of a combination of its nanomolar binding affinity for LTC₄ and its ability to compete with the CysLT₄R for binding to trace amounts of LTE₄. Although not tested directly against 2G9, the LTC₄-specific antibody, 10G4, provided a stronger and more statistically powerful protection against the effects of LTC₄ injection on dye extravasation when measured either after subcutaneous or intraperitoneal injection of the antibody relative to saline vehicle or nonspecific antibody. Consequently, 10G4 was further tested in the acute colitis model, where it was found to convey improvement to overall health and colon tissue histopathology in DSS-treated mice.

Our biochemical, structural, and in vivo observations support the development of potential clinical applications for anti-CysLT antibodies. We propose that by binding to free CysLTs, the antibodies prevent their LT antigens from engaging their cognate GPCRs, thus preventing activation of signaling pathways that lead to increased vascular permeability, acute inflammation, and eventually tissue remodeling and fibrosis. It bears mentioning that studies aimed at determining the efficacy of anti-CysLT antibodies against chronic inflammatory conditions did not yield conclusive results.

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