Bifunctional Lipocalin Ameliorates Murine Immune Complex-induced Acute Lung Injury

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Results: The C5 and LTB4 binding activities of OmCI are functionally and structurally independent, and OmCI potently inhibits immune complex-induced acute lung injury (IC-ALI). We describe two crystal structures of bacterially expressed OmCI: one binding a C16 fatty acid and the other binding LTB4 (C20). We show that the C5 and LTB4 binding activities of the molecule are independent of each other and that OmCI is a potent inhibitor of experimental IC-ALI, equally dependent on both C5 inhibition and LTB4 binding for full activity. The data highlight the importance of LTB4 in IC-ALI and activation of C5 by the complement pathway C5 convertase rather than by non-C proteases. The findings suggest that dual inhibition of C5 and LTB4 may be useful for treatment of human immune complex-dependent diseases.

The immune response depends upon coordinated release and orchestration of diverse mediators. Molecules that simultaneously inhibit mediators that have independent or co-dependent proinflammatory effects may have advantages over conventional therapeutics such as mAb, which normally target single components of the immune system. Possible advantages of targeting more than one pathway include higher specificity, lower effective dose (1), and the ability to counteract compensation mechanisms within biological networks (2, 3).

The 17-kDa lipocalin *Ornithodoros moubata* complement inhibitor OmCI (4), originally isolated from an ectoparasitic tick (*Acari*), is a bifunctional protein that may have such therapeutic advantages. It captures the proinflammatory eicosanoid leukotriene B4 (LTB4)8 within an internal binding cavity (data presented herein) and also prevents complement (C)-mediated activation of C component 5 (C5) in a wide range of mammalian species including humans (5). By binding directly to C5 in the vicinity of the C5-C345C domain OmCI prevents cleavage of C5 by the C5 complement convertases, thereby preventing release of anaphylatoxin C5a and formation of the terminal 5b-9 C complex (TCC) (6–8). OmCI therefore cir-

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Molecules that simultaneously inhibit independent or co-dependent proinflammatory pathways may have advantages over conventional monotherapeutics. OmCI is a bifunctional protein derived from blood-feeding ticks that specifically prevents complement (C)-mediated C5 activation and also sequesters leukotriene B4 (LTB4) within an internal binding pocket. Here, we examined the effect of LTB4 binding on OmCI structure and function and investigated the relative importance of C-mediated C5 activation and LTB4 in a mouse model of immune complex-induced acute lung injury (IC-ALI). We describe two crystal structures of bacterially expressed OmCI: one binding a C16 fatty acid and the other binding LTB4 (C20). We show that the C5 and LTB4 binding activities of the molecule are independent of each other and that OmCI is a potent inhibitor of experimental IC-ALI, equally dependent on both C5 inhibition and LTB4 binding for full activity. The data highlight the importance of LTB4 in IC-ALI and activation of C5 by the complement pathway C5 convertase rather than by non-C proteases. The findings suggest that dual inhibition of C5 and LTB4 may be useful for treatment of human immune complex-dependent diseases.

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8 The abbreviations used are: LTB4, leukotriene B4; LT, leukotriene; C, complement; TCC, terminal 5b-9 C complex; AA, arachidonic acid; cysLT, cysteinyl leukotriene; IC-ALI, immune complex-induced acute lung injury; hC5, human C5; OmCI, *Ornithodoros moubata* complement inhibitor; bOmCI, bacterially expressed OmCI; yOmCI, OmCI expressed in yeast; OmClI, *Ornithodoros moubata* cysteinyl leukotriene inhibitor; RaHBP2, *Rhipicephalus appendiculatus* histamine-binding protein 2; 12(S)-HETE, 12(S)-hydroxyeicosatetraenoic acid; TXA2, thromboxane A2; TxB2, thromboxane B2; AP, alkaline phosphatase; PDB, Protein Data Bank; OVA, ovalbumin; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; SPR, surface plasmon resonance.
cumvents the effects of the TCC, and the cell surface G protein-coupled receptors activated by LTB₄ (BLT1 and BLT2 receptors) and C5α (C5αR). OmCI may also prevent activation of the non-G protein-coupled C5L2 receptor for C5α. The function, and even the cellular location, of C5L2 is subject to ongoing debate with both pro- and anti-inflammatory activities described (9).

The established downstream effects of the TCC and C5αR, BLT1, and BLT2 signaling are numerous and interconnected. LTB₄, derived like all eicosanoids from arachidonic acid (AA), and activated C5 both have rapid and vital roles in the initiation and coordination of the early inflammatory and adaptive immune responses (reviewed in Refs. 10–14). Among other effects, TCC formation on self-cells induces release of inflammatory mediators including IL-6, synthesis of AA derivatives, transendothelial migration of polymorphonuclear leukocytes, and production of active oxygen metabolites (reviewed in Ref. 15). Both C5α and LTB₄ rapidly recruit and activate granulocytes (in particular neutrophils) and monocytes and trigger oxidative burst and degranulation (14–17), resulting in the release of numerous preformed proinflammatory and vasoactive mediators (histamine, serotonin, tryptase, and defensins) and proteases that can generate C5α independently of C (18, 19). These actions stimulate the production of proinflammatory cytokines (IL-1, IL-2, IL-6, IL-8, and TNFα), chemokines (eotaxin, RANTES, and MIP2), growth factor (TGFβ), LTB₄, and other eicosanoids that augment and prolong tissue inflammation (20, 21). C5α alone induces vasodilation and smooth muscle cell contraction, whereas both C5α and LTB₄ increase microvascular permeability (10, 13). LTB₄ amplifies the neutrophil chemotactic effect of C5α in inflammatory processes, and conversely, the release of AA and synthesis of LTB₄ can be stimulated by both TCC and C5α (10, 15, 22–24).

Marketed therapies target C5 or leukotrienes. C is the focus of much recent drug research and development (10, 25), and a humanized anti-C5 mAb (ecluzumab) successfully treats nocturnal paroxysmal hemoglobinuria (26). Eculizumab is in clinical trials for the treatment of a variety of other pathologies including atypical hemolytic uremic syndrome and kidney transplant rejection (60, 61). Therapies targeting leukotrienes including atypical hemolytic uremic syndrome and kidney transplantation (31). The effect of the combined inhibition of C5 and LTB₄ in a mouse model (9, 32–37) of immune complex-induced acute lung injury (IC-ALI).

**EXPERIMENTAL PROCEDURES**

**Materials—**[5,6,8,9,11,12,14,15-3H(n)]LTB₄ (lot 3589956; total activity 5 μCi or 185 kBq; specific activity 190 Ci/mmol) was from PerkinElmer Life Sciences. Fatty acid ELISA kits were from Assay Designs, Inc. The C5α assay kit was from DRG Diagnostica (catalog No. RE59292). All surface plasmon resonance (SPR) reagents were from Biacore (Uppsala, Sweden). Sheep RBC were from Tissue Culture Services. Hemolysin, pooled normal human sera, GVB²⁺, and human thrombin (catalog No. T6884) were from Sigma. Guinea pig serum and the control proteins RaHBP2 (Rhipecephalus appendiculatus histamine-binding protein 2) and OmCl (O. moubata cysteinyl leukotriene inhibitor) were derived in-house. Human C5 (hC5) was purchased from Calbiochem (catalog No. 204888). The 5-lipoxygenase-activating enzyme inhibitor MK886 was purchased from Tocris Bioscience (Bristol, UK), and LTB₄, leukotriene C₄ (LTC₄), 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE), and AA were bought from Cayman Chemical (Ann Arbor, MI) and Biomol International (Exeter, UK).

**Recombinant OmCl—**Soluble bacterially expressed OmCl (bOmCl) was manufactured using the proprietary technology of Wacker GmbH (38). *Escherichia coli* strain WCM105 containing a plasmid for expression and secretion of OmCl was cultivated in a 30-liter bioreactor (type Biostat C-20; Sartorius AG, Melsungen, Germany). Fermentation was performed at 30 °C in a mineral salt medium including trace elements, yeast extract, and phytone at pH 7.0 under aerobic conditions with a continuous glucose feed. Prior to inducing expression with IPTG, the temperature was dropped to 20 °C. Approximately 36 h after induction, the supernatant was harvested by centrifugation (CEPA Z41, Padberg GmbH, Germany) and filtered through a 0.25 mm, 0.25 μm membrane filter (Sartopure PP2) and then a 0.2-μm membrane filter (Sartopore 2HF). OmCl was purified from the supernatant as described (7) with a final additional chromatographic step in which the active fractions were adjusted to 2 m ammonium sulfate and then loaded onto phenyl-Sepharose (GE Healthcare) and eluted using a 2–0.5 m ammonium sulfate gradient. Active fractions were diazoyed against PBS, analyzed by denaturing SDS-PAGE, and concentrated to 8.4 mg/ml by ultrafiltration. A predicted A₂₈₀ of 1.3 was used to estimate the protein concentration. The pure protein (94% by RP-HPLC (supplemental Fig. S1) had a final yield of ~0.4 g/liter of fermentation broth. The identity and activity of the protein were confirmed by ESI-Q-TOF MS and by inhibition of a classical complement pathway assay used in accordance with the manufacturer’s instructions (CH50 Eq ELISA from Quidel). The experimentally estimated Mr of 16,779.0 g/mol matched the calculated Mr of 16,779.5 g/mol.

**GC-MS Analysis of Fatty Acids from Recombinant bOmCl—**Extraction and analysis of fatty acids from the recombinant protein was performed as described (6). Mass spectrometric analysis was performed on a Trace MS (Thermo Finnigan, D-63329, Egelsbach, Germany) equipped with fused silica Alltech EC5 (D-82008, Unterhaching, Germany) capillary (15 μm × 0.25 mm, 0.25 μm) using helium at 1.5 ml min⁻¹ as the carrier gas.
Preparation of LTB₄-saturated OmCI (bOmCI-LTB₄)—bOmCI (4.5 mg) was incubated with 2 ml of LTB₄ (50 ng/µl stock in ultrapure ethanol) in 39 ml of PBS, pH 7.2, at room temperature with shaking for 10 min. This 1:1.1 molar ratio mixture was concentrated to 200 µl in a 5-kDa cut-off Vivaspin ultrafiltration device (Sartorius). The retentate was washed with 30 ml of PBS, pH 7.2, and re-concentrated to 200 µl. In parallel, the same amount (4.5 mg) of bOmCI was incubated with 2 ml of ultrapure ethanol in 39 ml of PBS and then washed and concentrated as described.

Measurement of Eicosanoid Binding by Enzyme Immunoassay—Correlate-EIA™ kits (Assay Designs, Inc.) for solution measurement of LTB₄, thromboxane B₂ (TXB₂), and cysLT were used. To examine direct binding, 0.1–30 µg of pure OmCI, OmCI saturated with LTB₄, RaHBP2 (histamine binding), and OmCLI (cysteinyl leukotriene binding) were incubated with 150 µl of the eicosanoids conjugated to alkaline phosphatase (AP). After 20 min of shaking (500 rpm) at room temperature, free eicosanoid-AP was detected by mixing the lipocalin-eicosanoid solution with anti-eicosanoid-specific polyclonal rabbit Ab and adding the solution to a plate coated with goat anti-rabbit Ab. After a 2-h incubation, the plate was washed in accordance with the manufacturer’s instructions; at this stage the tick lipocalins including those bound to eicosanoid-AP were washed away. Anti-eicosanoid Ab bound to AP-labeled fatty acids was detected by addition of substrate. The results for each lipocalin were compared with PBS controls.

UV Absorption Spectroscopy of bOmCI Bound to LTB₄—bOmCI and LTB₄ in ethanol were mixed in PBS at molar ratios of 0:1, 0.03:1, 0.06:1, 0.0125:1, 0.25:1, 0.5:1, 1:1, 2:1, 4:1, and 8:1 and incubated for 5 min at room temperature. UV absorption spectra (200–400 nm) were recorded using a Nanodrop ND-1000 spectrophotometer. The average spectrum (five replicates) for each protein dilution with ultrapure ethanol but without LTB₄ was subtracted from the average spectrum for each protein dilution in the presence of LTB₄. The same assay was undertaken using a 4:1 molar excess of RaHBP2, OmCLI, and OmCI saturated with LTB₄. Spectroscopy of LTB₄ with and without OmCI was also undertaken in the presence of a large molar excess of AA (60×) or 12(S)-HETE (40×). The average spectrum (five replicates) for each protein dilution with AA or 12(S)-HETE but without LTB₄ was subtracted from the average spectrum for each protein dilution in the presence of LTB₄ mixed with excess AA or 12(S)-HETE.

bOmCI Crystallization, Data Collection, and Structure Determination—bOmCI and bOmCI-LTB₄ crystal 4s were grown by vapor diffusion in 400 nl of sitting drops at 20 °C. The crystallization drops were obtained by mixing 0.2 µl of protein solution with 0.2 µl of the crystallization screen and were equilibrated against 100 µl of mother liquor.

For bOmCI, screens were set up using a TECAN robot (Tecan Group Ltd.). Initial crystallization screens using the molecular dimensions structure screens were set up with bOmCI at 65 mg/ml. Initial crystals grew in conditions 23, 33, and 37 of MD Structure Screen 1; the best crystals grew after setting up repeats of condition 33 (30% PEG 4000, 0.2 m magnesium chloride, and 0.1 m Tris, pH 8.5).

For bOmCI-LTB₄, bOmCI loaded with LTB₄ (see “Experimental Procedures”) was concentrated to 25 mg/ml in Tris-HCl, pH 7, 30 mM NaCl. Screens were set up using an Oryx-Nano robot (Douglas Instruments Ltd., UK). Initial crystallization screens using Hampton Research crystal screens were set up with a bOmCI-LTB₄ mix in 1:1 molar ratios (see above for concentration). Initial crystals grew in conditions 14 and 22 of the Hampton Research Crystal Screen HT; better crystals were grown after screening around condition 22 (21% PEG 4000, 0.2 m sodium acetate, 0.1 m Tris, pH 8.4).
For bOmCl, a 1.9 Å x-ray diffraction data set was collected from a crystal (orthorhombic P2$_1$2$_1$2$_1$, $a = 44.4$ Å, $b = 51.9$ Å, $c = 68.40$ Å, 1 molecule/asymmetric unit) on beamline ID14-2 at the ESRF. For bOmCl/LTB$_4$ a 1.9 Å x-ray diffraction data set was collected from a crystal (monoclinic P2$_1$, $a = 41.76$ Å, $b = 112.81$ Å, $c = 62.40$ Å, $\beta = 101.89$ degrees, 4 molecules/asymmetric unit) on beamline BM14 at the ESRF. All x-ray data integration and scaling were done using the computer programs Mosflm (39) and Scala (40). Supplemental Table S1 shows the crystallographic data collection and processing statistics. Both structures were phased by molecular replacement with the computer program CCP4-Phaser, built in Coot (41), and refined in Buster-TNT (42) using alternating cycles of full B cycles. The structures were deposited in the Protein Data Bank and have accession codes 3ZUI and 3ZUO.

**Surface Plasmon Resonance**—Measurements were conducted on a Biacore 3000 instrument. 600–1000 resonance units of bOmCl or 200–300 resonance units of bOmCl/LTB$_4$ (which coupled less efficiently than bOmCl) were immobilized by amine coupling at pH 4.5 to separate CM5 sensor chips as recommended by Biacore. A control channel was mock coupled with buffer. All experiments were performed at 25 °C in 10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20 with various concentrations of hC5 with and without 10 nM LTB$_4$. Analyte solutions were passed through the flow cells sequentially at a flow rate of 40 \mu{l}/min. The chip surface was regenerated using 10 mM glycine, pH 1.5, between each injection. Kinetic parameters were evaluated in accordance with the manufacturer's instructions.

**Classical Hemolytic Assay**—Sensitized sheep erythrocytes were prepared in GVB$^{2+}$ buffer. Assays were carried out using 50 \mu{l} of 1:320 GVB$^{2+}$ diluted guinea pig serum and 50 \mu{l} of $2 \times 10^8$ activated erythrocyte cells ml$^{-1}$. This was the maximum serum dilution (1:640 final) that gave $\sim 100\%$ lysis without inhibitor after 30 min of incubation. The GVB$^{2+}$ buffer was supplemented with a 2-fold molar excess of LTB$_4$ to bOmCl. bOmCl or RaHBP2 control protein diluted in PBS.
was added last (5 μl each), and reactions were incubated at 37 °C for 30 min. Reactions were centrifuged (12000 × g for 5 s) and hemolysis measured at 412 nm. Percent lysis was calculated using the absorbance value for 100% cell lysis caused by adding 50 μl of water to 50 μl of erythrocyte cells.

**Measurement of LTB4 Binding Using Radioligand** — A 1:2 molar ratio of bOmCI and hC5 were incubated in PBS at room temperature for 10 min to form the bOmCI-hC5 complex. Formation of the complex was confirmed by native PAGE gel shift (see Ref. 8 for example). Equal amounts bOmCI-hC5 or bOmCI alone were serially diluted in 75 μl of PBS before adding 75 μl of PBS containing ~24,000 cpm ³H-LTB₄. Following shaking incubation (3 h, 500 rpm, room temperature), samples were centrifuged at 8000 × g for 2 min, and the radioactivity remaining in solution was measured on a Wallac 1217 Rackbeta liquid scintillation counter after transferring 20 μl of the supernatant to 4 ml of Beckman Ready Value scintillation mixture. PBS only and serial dilutions of RaHBP2 and hC5 in PBS were negative controls.

**Thrombin-mediated Cleavage of C5** — One μg of hC5 and 2.5 NIH units of human thrombin were incubated in PBS at 37 °C for 1.5 h, alone, together, and with or without OmCI or protease inhibitor mixture (Sigma, catalog No. P8340). 1×, 10×, and 100× molar excess of OmCI to hC5 were used. Following incubation, human thrombin was inactivated (65 °C for 4 min), and free C5a was detected using a DRG Diagnostica enzyme immunoassay kit (catalog No. RE59292).

**Immune Complex Acute Lung Injury Model** — All animal experiments were approved by the CNRS institutional animal research committee and complied with the French Government’s ethical and animal experiment regulations. C57/BL6 mice, male, about 8 weeks old and with a body weight of about 25 g, were dosed intranasally with 150 μg of chicken anti-ovalbumin IgG (anti-OVA) together with the drug of interest (bOmCI, bOmCI-LTB₄, RaHBP2 or MK886) in 40 μl of saline. Immediately thereafter, immune complex in the lung was induced by injecting 300 μg of OVA and 0.3% Evans blue into the tail vein. Experiments were performed three times with groups of six animals in each treatment. MK886 was administered at 1 mg/kg by gavage and LTB₄ by intranasal instillation. In the latter experiments, OmCI and RaHBP2 were administered immediately before LTB₄. At 4 h after OVA or LTB₄ administration, mice were killed, BAL was performed, and lungs were perfused with an isotonic solution and then excised. Cells in BAL were counted, and differential staining was performed using Diff-Quik. Vascular leakage was quantified by protein concentration in the bronchoalveolar space, and Evans blue was measured in BAL supernatant by absorbance at 460 nm. The excised lung was fixed in 4% buffered formaldehyde for H&E, and microscopic

![FIGURE 3. Details of ligand binding.](image)

Carbon (yellow) and oxygen (red) surrounded by the OmCI pocket residues (carbon, green; oxygen, red; nitrogen, blue; and sulfur, yellow); this picture was produced with the program PyMOL (44). B, schematic representation of the bOmCI residues forming hydrogen bonds (with distances in Å) and non-bonding hydrophobic contacts to LTB₄ (upper panel) and palmitoleic acid (lower panel). These pictures were produced with the program LigPlot (45).
lesions (endothelial cell damage, inflammatory cell recruitment, and erythrocytes in the alveolar space) were assessed using a semiquantitative score (0–3) by two independent observers. C5a in the BAL supernatant was measured using the C5a micro MTPL EIA kit in accordance with the manufacturer’s instructions (DRG Diagnostics GmbH).

**Statistical Analyses**—The data are presented as the mean ± S.D. or 95% CI as indicated in the figure legends. The significance of differences between two groups was determined by one-way analysis of variance (non-parametric test) using GraphPad Prism software, version 5, or in R using Tukey’s multiple comparison of means. Plots of the residuals were used to confirm the homosce-

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**Figure 4.** C5 and LTB₄ binding activities of OmCI are independent. A, LTB₄ (2-fold molar excess) has no effect on the classical C pathway inhibitory activity of bOmCI assayed by hemolysis. Bars show 95% CI (n = 4 for each data point). B, spectroscopy showing that LTB₄ remains bound (characteristic red-shifted spectrum) to bOmCI in low pH (5.5 and 4), 10 mM sodium acetate buffers. C and D, SPR measurement of binding between bOmCI and human C5 (hC5) in the absence (C) and presence (D) of LTB₄. Curves were fitted using a 1:1 Langmuir model (shown in black). Mean Kᵦ and chi-squared values are shown with their standard deviation calculated from 15 replicates. Replicates were performed over a minimum of two independent experiments. The RU's in D are lower than in C because less bOmCI-LTB₄ than bOmCI coupled to the sensor surface. E, plot showing that ligand binding is saturable. RaHBP2 and hC5 alone do not show saturable binding, and cpm values are equivalent to PBS only (not shown). Raw data shown (n = 2 for each data point) are representative of three independent experiments. The highest concentration of OmCI-hC5 (i.e. 10000 nM) was not assayed, as the amount of hC5 required was prohibitively expensive. F, plot showing interaction of hC5 with bOmCI has no effect on the binding kinetics to LTB₄. The data are representative of two independent experiments (n = 2 for each data point) and show cpm values after subtraction of average cpm (n = 16) of the negative control (RaHBP2). Logarithmic regression line functions are: bOmCI, y = 134.67 ln(x) + 521.6, R² = 0.98; bOmCI-hC5, y = 132.87 ln(x) + 479.2, R² = 0.97.
RESULTS

**OmCI Binds a Variety of Unbranched Fatty Acids, but Its Preferred Ligand Is LTB4**—We reported previously that OmCI expressed in yeast (yOmCI) is bound to ricinoleic acid (C18H34O3) and speculated that the natural host ligand bound by OmCI when secreted into the tick feeding site might be a proinflammatory eicosanoid (6). This was confirmed later using homologues of OmCI derived from other tick species (43). Solvent extraction and GC-MS analysis of the highly purified bOmCI used in this study (supplemental Fig. S1) showed that C16:1 cis-palmitoleic acid (C16H30O2) was the dominant (~64%) fatty acid in the binding pocket of bOmCI, with various other C16–C18 linear fatty acids present in lower proportions (supplemental Fig. S2). Preferential binding to LTB4 was shown using an enzyme immunoassay in which bOmCI sequestered LTB4, preventing the AP-conjugated eicosanoid from interacting with specific capture antibodies of unknown, but presumably high, avidity (Fig. 1A). In the same assay, bOmCI did not show evidence of binding to the cyclic eicosanoid TXB2 (a stable derivative of TXA2) or to LTC4, which has amino acids conjugated to carbon 6 (C6) of the fatty acid chain (Fig. 1A). The positive and negative control tick-derived recombinant lipocalins bound LTC4 only (OmCL1- and cysLT-specific) or none (RaHBP2- and histamine-specific) of the fatty acids tested (Fig. 1A).

The distinctive absorption spectrum of LTB4 was exploited to show that when bound to OmCI, the UV maxima of LTB4 exhibits a +6 nm bathochromic (red) shift to 277, 267, and 287 nm (Fig. 1B). The shift was complete at a 1:1 molar ratio of

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FIGURE 5. **OmCI inhibits IC-ALI.** A, OmCI (100 μg) reduces neutrophils in BALF and lung (myeloperoxidase activity) and prevents hemorrhage, protein and Evan’s Blue (EB) leak, and edema. B, dose-response effect of OmCI (50, 100, and 200 μg/mouse) on neutrophil recruitment. All treatment groups except saline (NaCl) were dosed intranasally with chicken anti-OVA IgG with or without OmCI. All groups then received OVA by injection into the tail vein. Lung inflammation was evaluated 4 h after induction of IC. The mean values ± S.D. of a representative study are given (n = 6 mice/group).
OmCI to LTB₄. Dispersion interactions between the conjugated leukotriene and nearby amino acids cause the shift, which is consistent with the triene chromophore of LTB₄ being completely encompassed by OmCI. Spectral shifts in LTB₄ were not seen when using OmCL1 or RaHBP2 (supplemental Fig. S3A). The absorption intensity and bathochromic shift was not altered by a large molar excess of AA (60×) or 12(S)-HETE (40×), neither of which has triene chromophores, indicating that OmCI is highly specific for LTB₄ (supplemental Fig. S3B).

LTB₄ binding by bOmCI-palmitoleic acid, but not yeast-expressed yOmCI-ricinoleic acid, altered the proteins mobility by native PAGE (Fig. 1C); the shift was complete at a 1:1.5 molar ratio of OmCI to LTB₄. This encouraged us to examine whether LTB₄ induces conformational changes in OmCI that affect its C-inhibitory function.

**Structural Analysis of bOmCI and bOmCI-LTB₄**—We obtained two crystal structures of bOmCI, one in complex with palmitoleic acid and one in complex with LTB₄ (PDB accession codes 3ZUI and 3ZUO). Both show the same overall structure as γOmCI-ricinoleic acid (PDB accession code 2CM4); root mean square deviation C₅₀ = 0.61 and 0.63 Å for the palmitoleic acid and LTB₄ complexes, respectively. Fig. 2, A and B, shows orthogonal views of bOmCI in complex with LTB₄. The main ligand-dependent structural difference with respect to the γOmCI-ricinoleic acid crystal structure is the position of the loop ββ-αC (residues 61–70), which pushes in/out to accommodate a smaller/larger ligand (see Fig. 2C). This movement is mediated by Pro-61, the residue at the bottom of the ligand pocket. The other main locus of structural variability in the bOmCI and γOmCI structures is the conformation of the βH-α3 loop (amino acids 132–142), shown previously to be required for C5 inhibition (43), which in both complexes of bOmCI swings inward, with the concomitant flip of the side chain of His-117 (Fig. 2D). However, the chemical nature of the ligand in the pocket bears no relationship to the conformation of the βH-α3 loop, as the loop was observed in both conformations in different copies of the γOmCI-ricinoleic acid complex (6).

The three ligands all bind with their carboxylic acid head at the solvent-exposed brim of the OmCI pocket, hydrogen bonding to the side chains of Arg-54, Thr-85, and Trp-87 (see Figs. 2, A and B, and 3). All ligands fit into the L-shaped pocket, with the kink at position C12 of LTB₄, ricinoleic acid, and palmitoleic acid (Fig. 3). Hydroxylation at this position is common to ricinoleic acid complexes show a water molecule filling the bottom of the pocket. The other main locus of structural variability in the pocket. The hydrophobic side chains of Phe-36, Tyr-43, Leu-57, Gly-59, Pro-61, Leu-70, Val-72, Met-74, Phe-76, Phe-89, Arg-107, and Trp-133 contact the hydrophobic body of all three ligands (Fig. 3B). The Cα=C=O double bond present in LTB₄, but not in palmitoleic or ricinoleic acid, stacks against the guanidinium head of Arg-107. The same Arg side chain is the source of the selectivity against cyclic eicosanoids such as TXB₂ and TXA₂ (43), as it makes the pocket too narrow to accommodate cyclic ligands. Gln-105 recognizes the -OH group of LTB₄, which -OH at C15 because of the side chains of Phe-36 and Tyr-43, so the anti-inflammatory eicosanoids 15(S)HETE, lipoxin A₄, and lipoxin B₄ are unlikely to be bound by OmCI.

**The LTB₄ and C5 Binding Activities of OmCI Are Independent**—The subtle conformational change induced by LTB₄ binding may have an effect on the binding of OmCI to C5. Conversely, C5 binding may change the binding affinity of OmCI for LTB₄. We tested both possibilities by examining the binding affinity between purified hC5 and bOmCI, with or without LTB₄, and the binding kinetics of OmCI for H-labeled LTB₄ in the presence of excess hC5. We first showed that excess
LTB₄ had no effect on the potency of bOmCI measured by classical hemolytic assay (Fig. 4A). Before undertaking SPR experiments, we confirmed that LTB₄ remains bound to bOmCI at the low pH 4.5 needed to couple the protein to the chip (Fig. 4B), and where applicable, LTB₄ was also included in the running buffer. The SPR data showed no physiologically relevant change in hC₅ affinity with and without LTB₄ (Fig. 4C and D; $K_D = 1 \text{nM} \pm 0.4$ without LTB₄ and $0.3 \text{nM} \pm 0.1 \text{nM}$ with LTB₄). We considered the difference physiologically irrelevant because the concentration of C5 in human serum is $0.4 \text{nM}$, and because both $K_D$ values were more than 40 times tighter than this concentration, OmCI would be present as a complex with C5 whether or not LTB₄ was bound. The $K_D$ calculated here by SPR for the interaction between bOmCI and hC₅ is lower (tighter) than that reported previously (7) for yOmCI ($K_D = 18.5 \text{nM}$) but is characterized by a similar very slow off-rate. yOmCI has two site-directed point mutations that prevent hyperglycosylation when expressed in yeast, which may alter the binding kinetics. Radioligand assays show no alteration of LTB₄ binding in the presence or absence of hC₅ (Fig. 4E and F). OmCI and preformed OmCI-hC₅ complex show equivalent saturable binding to $^3$H-LTB₄, whereas PBS (not shown but equivalent to the following two protein controls), RaHBP2 (specific for histamine), and hC₅ do not (Fig. 4E). The assay measured the ability of OmCI to bind $^3$H-LTB₄ and keep it in solution. Indeed, no more than 20% of the labeled LTB₄ remained in solution in the negative control samples, whereas more than 50% remained in solution at the higher concentrations of OmCI (Fig. 4E), confirming that OmCI binding is needed for solubilization. Because of the low solubility of LTB₄ in this assay, association and dissociation constants could not be accurately derived. However, a comparison of the slope of the logarithmic regression functions for equivalent concentrations of OmCI and OmCI-hC₅ indicate that the binding kinetics between LTB₄ and OmCI are not altered when OmCI is in complex with hC₅ (Fig. 4F). In summary, despite a subtle structural change (Fig. 2C) caused by binding to OmCI, LTB₄ has no physiologically relevant effect on binding between OmCI and C₅, and conversely, C₅ binding by OmCI does not hinder or enhance binding to LTB₄. This result is in agreement with previous suggestions that C₅ binding and entry of LTB₄ to the binding pocket happen on opposite faces of OmCI (6, 46).

OmCI Ameliorates IC-ALI in Mice—Based on our understanding of the binding activities of OmCI, we selected the well characterized mouse model of IC-ALI (33) to test the therapeutic potential of the protein. In this pathology, activation of C and IgG FcγR on effector cells, chiefly alveolar macrophages, are the dominant initial events following immune complex formation (9, 33). C₅a, acting through C₅aR signaling, up-regulates the expression of activating FcγRIII and down-regulates inhibitory FcγRIIB IgG receptors (35, 36). The FcγR imbalance leads to IC-ALI via alveolar macrophage production of proteases, early response cytokines (TNFα and IL-1β), and LTs (predominantly LTB₄ (47)), which with C₅a increase the expression of adhesion molecules on vascular endothelial cells, and induce CXCR1/2 chemokines. These in turn recruit abundant neutrophils that degranulate and release superoxides, causing inflammation and pulmonary microvascular damage (9). We first showed that OmCI itself does not induce an inflammatory response when administered intranasally (Fig.

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**FIGURE 7.** OmCI (100 μg) neutralizes LTB₄-induced inflammation. A, inhibition of leukotriene biosynthesis by MK886 (1 mg/kg by gavage) reduces neutrophil recruitment induced by IC-ALI. B, intranasal instillation of LTB₄ (1 μg) induces neutrophil recruitment in the lung, which is inhibited by prior administration of 2-fold molar excess bOmCI but not by a similar amount of control tick lipocalin histamine-binding protein RaHBP2 (100 μg). Representative data for one of three independent experiments are shown (n = 6 mice/group). Values are shown as mean ± S.D.
5A, compare OmCI and saline). As hoped, bOmCI profoundly inhibited immune complex-induced neutrophil recruitment to lung tissue (measured by myeloperoxidase) and BALF (Fig. 5A). It also decreased pulmonary microvascular damage, with significantly reduced hemorrhage, edema, and protein exudation to the bronchoalveolar space (Fig. 5A). At 100 μg/mouse, bOmCI reached a plateau for near complete inhibition of myeloperoxidase activity and neutrophil recruitment to the alveolar space, with no further decrease at the 200-μg dose (Fig. 5B). Macroscopically a distinct capillary leak of Evans blue from the blood to the lung can be seen, which is attenuated by OmCI (Fig. 6A). Representative microscopic plates and semiquantitative microscopic evaluation show that bOmCI reduced endothelial cell damage, inflammation, and erythrocyte extravasation into the bronchoalveolar space (Fig. 6, B and C).

Inhibition of Both C5 Activation by C and LTB4 Binding Is Needed for Full Inhibitory Effect of OmCI—The proven independence of the two binding activities of OmCI enabled us to investigate the relative importance of LTB4 and C5 activation in IC-ALI. IC-ALI has been investigated intensively by many research groups, but the role of LTB4 in IC-ALI has been overlooked by all but one group (48–50). We therefore first confirmed that LTB4 had a functional role in our model. In agreement with the earlier work (48, 49), we found that 4 h after administration of OVA, LTB4 levels in BAL increase significantly (p < 0.01) in response to immune complex: saline control 125.8 pg/ml LTB4 (95% CI, 94.7–156.9 pg/ml, n = 8), OVA 243.6 pg/ml LTB4 (95% CI, 292.8–194.45 pg/ml, n = 8). Furthermore, administration of MK886, which prevents biosynthesis of LTs, significantly reduced vascular leakage (data not shown) and inflammation, although the inhibitory effect of
**DUAL ACTING INHIBITOR AMELIORATES LUNG DISEASE**

The tick derived bifunctional protein OmCI significantly attenuates the symptoms of experimental IC-ALI. Furthermore, inhibition of C-mediated C5 activation and binding of LTB₄ are both required for full potency of OmCI in this animal model. Our biochemical and structural data show that this is not mediated by an allosteric effect on OmCI, since C5 has no effect on LTB₄ binding, and although LTB₄ increases the binding affinity of OmCI for C5 measured by SPR this is unlikely to have any physiological relevance on the inhibitory activity of the protein in vivo.

The importance of C5a in driving IC-ALI through modulation of FcyR signaling in response to immune complex is well established (33, 35, 36). Current literature on this pathology emphasizes a dominant role for C5 activation within the alveolar space via non-C convertase serine proteases (including thrombin) released from alveolar macrophages in response to immune complex (9, 18, 19, 33). However, the significant inhibitory activity of bOmCI-LTB₄ saturated with LTB₄ (bOmCI-LTB₄), which specifically inhibits C₅ activation by classical and alternative C5 convertases (C₃bC₂C₃b and C₅bBbC₃b, respectively) and has no effect on C₅ activation by non-C proteases, indicates that C₅ activation directly mediated by C₅ convertase, which is present and active in BALF (51, 52), has a significant functional role in IC-ALI. This conclusion is supported by earlier work, which showed that thrombin inhibitors significantly ameliorate IC-ALI in C₃⁻/⁻ mice with supernormal levels of thrombin but not C₃⁺/+ mice with functional C and normal levels of thrombin (18). Thus non-C protease-mediated cleavage of C₅ probably has a secondary role in amplification of C₅a formation in C-sufficient mice.

With the focus on C₅a, the importance of LTB₄ in IC-ALI has largely been overlooked by all but one research group (48–50), although the pivotal role of LTB₄ in other models of lung disease such as LPS-induced ALI- and allergen-induced airway hypersensitivity is well established (13, 53, 54). Our work supports the earlier work showing that LTB₄ plays a significant part in the pathology of IC-ALI (49, 50). Furthermore, we find that LTB₄ inhibition is as important as C₅ convertase C₅ activation for amelioration of symptoms of IC-ALI in the mouse model. This highlights the co-dependent effects of C₅ and LTB₄ on polymorphonuclear leukocyte migration and recruitment and other proinflammatory processes (9, 13–15, 21–24). It seems likely that previous work reporting a dominant role for C₅ (32–37) may well have reduced LTB₄ synthesis, thereby preventing its action on the lung; however, the role of LTB₄ was not examined in these studies.

We find it interesting that natural selection has not excluded binding of a variety of unbranched fatty acids by ectoparasite-
derived OmCI. To date these are known to include palmitoleic acid and related C16-C18 fatty acids and ricinoleic acid (C18) and the preferred ligand LTB4 (C20). The closely related tick lipocalin, TSGP3, shows even greater promiscuity, binding cyclic eicosanoids (here carbocyclic TXA2) as well as LTs (43). Both TSGP homologues and presumably OmCI also show moderately high affinity binding (KD/H11011 60 nM) to AA (43). We speculate that by sequestering AA and possibly LTA4 (the immediate precursor of LTB4), at sites of inflammation such as the tick feeding site, OmCI may decrease transcellular synthesis from AA (14, 55) of LTB4 and other proinflammatory (cysLTs and TXA2) or LT-enhancing eicosanoids such as prostaglandins. Thus, in addition to inhibiting TCC and signaling via the BLT1, BLT2, C5aR, and C5L2 receptors, OmCI may decrease the overall rate of eicosanoid synthesis at inflammatory sites.

Our current hypothesis to explain the potent activity of OmCI in IC-ALI is that intranasally administered OmCI prevents cleavage of C5 by C5 convertases on alveolar epithelial surfaces activated via IC, thereby limiting TCC formation and C5a activation of alveolar macrophages and other cells via FcγR threshold modulation. We propose that C convertase-mediated cleavage of C5 initiates the activation of alveolar macrophages, which subsequently increases the release of proteases that activate C5 independently of C. Separately, sequestration of LTB4 by OmCI limits the action of both convertase and non-C protease (e.g. thrombin)-derived C5a, which recruit leukocytes, and in particular tissue damaging neutrophils, both directly through C5aR and via the LTB4 receptors BLT1 and BLT2 (22, 56). The direct and indirect actions of these co-dependent systems (15, 21–24, 57) are summarized in Fig. 11. At
present we do not know whether intranasally administered OmCI is able to enter tissue and prevent the action of C and LTB₄ within the lung or whether it acts entirely at the epithelial surface. Future studies should examine the effect of OmCI on FcγR and additional pro- and anti-inflammatory mediators and clarify the site at which OmCI acts, as this may have a direct bearing on the optimal route of administration. Investigating the elaboration of the immune response via chemokines and cytokines induced by C5 activation and LTB₄ using OmCI mutants able to bind only C5 or LTB₄ might more fully elucidate the downstream effects of these two mediators.

In conclusion, our data highlight the need to evaluate the functions of LTB₄, and possibly other eicosanoids, more closely in IC-ALI and to reevaluate the relative importance of C5a derived by complement-dependent and -independent pathways in C3-sufficient mice. The data show that dual inhibition of C5 activation and LTB₄ can have a profound inhibitory effect on inflammation and in particular on neutrophil recruitment and activation. Therapeutic indications for OmCI, or other combined inhibitors of C5 and LTB₄, may include acute forms and activation. Therapeutic indications for OmCI, or other combined inhibitors of C5 and LTB₄, may include acute forms of immune complex-mediated diseases with neutrophil-dependent tissue injury, such as transfusion-related ALI, sepsis, rheumatic fever, and some forms of rheumatoid arthritis, peritonitis, and glomerulonephritis (58). The potential therapeutic value of dual acting drugs is widely recognized by the pharmaceutical industry, where there is currently intense interest in bispecific Ab (59). Notably, the functional independence of the two binding activities of OmCI and the detailed structural understanding of fatty acid binding that we have described will enable protein engineering for the selective targeting of eicosanoids most relevant to treatment of specific pathologies.

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