IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Leukotriene B₄ and Its Receptor in Experimental Autoimmune Uveitis and in Human Retinal Tissues

Clinical Severity and LTB₄ Dependence of Retinal Th17 Cells

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Accepted for publication
October 20, 2020.

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Nomacopan, a drug originally derived from tick saliva, has dual functions of sequestering leukotriene B₄ (LTB₄) and inhibiting complement component 5 (C5) activation. Nomacopan has been shown to provide therapeutic benefit in experimental autoimmune uveitis (EAU). Longer acting forms of nomacopan were more efficacious in mouse EAU models, and the long-acting variant that inhibited only LTB₄ was at least as effective as the long-acting variant that inhibited both C5 and LTB₄, preventing structural damage to the retina and a significantly reducing effector T helper 17 cells and inflammatory macrophages. Increased levels of LTB₄ and C5a (produced upon C5 activation) were detected during disease progression. Activated retinal lymphocytes were shown to express LTB₄ receptors (R) *in vitro* and in inflamed draining lymph nodes. Levels of LTB₄R-expressing active/inflammatory retinal macrophages were also increased. Within the draining lymph node CD4⁺ T-cell population, 30% expressed LTB₄R⁺ following activation *in vitro*, whereas retinal infiltrating cells expressed LTB₄R and C5aR. Validation of expression of those receptors in human uveitis and healthy tissues suggests that infiltrating cells could be targeted by inhibitors of the LTB₄-LTB₄ receptor 1 (BLT1) pathway as a novel therapeutic approach. This study provides novel data on intraocular LTB₄ and C5a in EAU, their associated receptor expression by retinal infiltrating cells in mouse and human tissues, and in attenuating EAU via the dual inhibitor nomacopan. (*Am J Pathol* 2021, 191: 320–334; <https://doi.org/10.1016/j.ajpath.2020.10.010>)

Noninfectious posterior uveitis has a predominantly autoimmune etiology, mediated by T helper cell 1 (Th1) and/or T helper cell 17 (Th17) subsets of self-reactive CD4⁺ T lymphocytes.^{1–3} Resident and infiltrating macrophages also play significant roles in the disease progression as effector cells associated with damaging retinal tissues. The disease has a range of heterogeneous clinical presentations and can be further characterized into various clinical spectrums.⁴ Furthermore, uveitis may be associated with systemic diseases.⁵ More than one-half of all patients with uveitis are diagnosed with vision-associated complications such as glaucoma and macular edema, and up to 35% of patients experience severe visual impairment. Uveitis accounts for

approximately 10% to 15% of cases of total blindness and up to 20% of legal blindness cases.^{5,6} Therapies for uveitis are mainly restricted to the use of systemic oral corticosteroids, disease-modifying antirheumatic drugs such as cyclosporin, methotrexate, and mycophenolate, and, more recently, anti-tumor necrosis factor α (TNF α) drugs such as adalimumab. These medications may lead to serious side effects (eg, liver failure, low peripheral lymphocyte counts) when given long term. Local (periocular or intravitreal) corticosteroid

Supported, in part, by a research collaborative grant from Akari Therapeutics (V.L.C.).

Disclosures: M.A.N. and W.W.-D. are employees of Akari Therapeutics.

injections may limit systemic effects; however, they can be associated with potential local adverse effects such as elevated intraocular pressure, glaucoma, and cataracts.⁷ Therefore, development of drugs that more specifically abrogate pathogenic pathways involved in uveitis progression and disease complications is essential, with the hope to transform and improve the management of patients with intraocular inflammatory diseases.

Our knowledge of the contribution of leukotriene B₄ (LTB₄) and complement component 5 (C5) within the retina during uveitis development/progression and in the *in vivo* experimental model of disease (experimental autoimmune uveitis [EAU]) is very limited. There is evidence for C5 activation being involved in later stages of EAU,⁸ and use of an LTB₄ receptor inhibitor blocked EAU progression when given at disease onset. In addition, transferring retinal-specific autoreactive T cells from C57BL/6J mice to 5-LO^{-/-} mice deficient in LTB₄ expression failed to induce uveitis in recipient mice.⁹ Because it is known that gene-deficient adult mice might not have a full complement of immune pathways, the role of these molecules in wild-type mice must be determined.

Nomacopan (previously known as Coversin) is a bifunctional biologic derived from blood-feeding ticks; it specifically sequesters LTB₄ within an internal binding pocket and also prevents activation of C5.^{10,11} Nomacopan's mode of action prevents LTB₄ interacting with its two known G protein-coupled cell surface receptors [BLT1 (high affinity) and BLT2 (low affinity)] and prevents terminal complement activation, thereby preventing formation of the membrane attack complex and the anaphylatoxin C5a, which activates immune cells via its two known G protein-coupled receptors (C5aR1 and C5aR2).

LTB₄ (C₂₀H₃₂O₄) is a potent lipid inflammatory mediator, derived from arachidonic acid by the sequential action of 5-lipoxygenase, 5-lipoxygenase-activating protein, and leukotriene A₄ hydrolase.¹² LTB₄ is an important multifunctional mediator of inflammation; it exerts its effects in an autocrine or paracrine fashion to signal a cascade of kinase reactions, leading to changes in both transcriptional activity and cellular motility.^{12,13} Leukotrienes, including LTB₄, promote the infiltration of monocytes/macrophages and leukocytes into tissues and subsequent cytokine release leading to exacerbations of asthma.^{14,15}

The main cellular sources of LTB₄ in both mice and humans are granulocytes, monocytes, and macrophages. LTB₄ is known to induce secretion of granulocyte-macrophage colony-stimulating factor, TNF α , IL-6, and IL-1 β and the chemokines keratinocyte chemoattractant, monocyte chemoattractant protein-1, CXCL1, and CXCL2 that enhance inflammatory responses.¹⁵ LTB₄ dose dependently increased the secretion of IL-17 and the expression of retinoic acid-related orphan receptor γ t (ROR γ t) mRNA in Th17 polarizing conditions.¹⁶ In addition, cutaneous application of LTB₄ in an *in vivo* model of psoriasis-like skin inflammation resulted in Th17 cells dominating the acute phase of inflammation.¹⁷

The anaphylatoxin C5a, a proteolytic breakdown product of C5 activation, is a chemotactic agent recruiting and activating granulocytes and other immune cells that are essential in innate immunity but which are also linked with adaptive immunity.¹⁸ The C5a receptor (C5aR1) was identified as a marker of differentiating microglial subsets in response to lipopolysaccharide in the inflamed eye.¹⁹ C5a receptor signaling in dendritic cells controls development of Th17 immunity in experimental allergic asthma.²⁰ C5b binds to the cell surface and initiates the formation of the membrane attack complex, which leads to lysis of target cells. C5b is intrinsically labile and becomes inactivated within 2 minutes unless stabilized by binding to another complement component (C6).²¹

In the current study, the role of LTB₄ and C5 pathways was investigated by using nomacopan as a dual inhibitor. Furthermore, to extend the half-life of the parental drug, PASylation technology was used to compare PASylated variants of nomacopan, as therapeutic benefits of these PASylated forms have been reported in other models.^{22,23} The pharmacokinetic variables of nomacopan were improved by N-terminal conjugation with a 600-residue polypeptide composed of Pro, Ala, and Ser (PAS) residues.²² We studied the roles of LTB₄ and C5 activation or LTB₄ alone in EAU progression, and investigated whether targeting C5 and/or LTB₄ activation in a therapeutic approach can suppress disease in mouse models of uveitis. Development of nomacopan variants also allowed us to investigate the therapeutic potential of combined C5 plus LTB₄ inhibition or of LTB₄ inhibition alone in already developing chronic (C57BL/6 mice) and acute (B10.RIII mice) models of EAU. These models are induced by autoreactive CD4⁺ T cells recognizing retinal antigen, mediated by Th17 and/or Th1 cells,^{24,25} and disease is perpetuated by myeloid cells. The models recapitulate some of the clinical phenotypes of noninfectious intraocular inflammatory disease.²⁶ The relevance of the findings to human disease is supported by detection of inflammatory cells expressing LTB₄ receptors and C5a receptors in human uveitis retinal tissue specimens.

Materials and Methods

Human Specimens

Healthy human eye specimens ($n = 3$) from postmortem cases were obtained from the Moorfields Biobank at the UCL Institute of Ophthalmology (London, UK). Three enucleated eyes were provided from noninfectious posterior uveitis patients with end-stage ocular disease ($n = 3$) (Liverpool Clinical Laboratories, Liverpool, UK). All eyes were donated following consent. All specimens were formalin fixed and paraffin embedded, and 4- to 6- μ m tissue sections were prepared from these blocks. In all specimens, the sclera, choroid, and retinal layers were observed intact.

Mice

Female C57BL/6J strain were bought from Charles River Laboratories (Kent, UK) at 5 to 7 weeks of age and female B10.RIII mice (6 to 8 weeks of age) were obtained from a colony gifted from GlaxoSmithKline (Stevenage, UK) and expanded at the Biological Research Service within the UCL Institute of Ophthalmology. All mice were housed in individually vented cages under specific pathogen-free conditions with continuously available water and food for the duration of all experiments, according to UK Home Office Regulations. Treatment of animals conformed to the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research. All animal studies were ethically reviewed and performed in accordance with the Animals (Scientific Procedures) Act 1986 on the Care, Welfare, and Treatment of Animals. All procedures were conducted in concordance with the United Kingdom Home Office license.

Reagents

Nomacopan and its variants are recombinant proteins originally derived from saliva of the soft tick *Ornithodoros moubata*. Nomacopan and its PASylated variants (PAS)²² were diluted in saline for final concentrations: 5 mg/mL of nomacopan or L-nomacopan is the molar equivalent to 20 mg/mL of PAS-nomacopan or PAS-L-nomacopan due to the effect of PASylation. PAS-L-nomacopan is a mutated form in which the C5-binding site has been inactivated and therefore only inhibits LTB₄.²³

Synthetic LTB₄ solutions (Tocris Bio-Techne, Abingdon, UK) were diluted in saline and used on the same day. Dexamethasone powder (Sigma-Aldrich, St. Louis, MO) was dissolved in absolute ethanol and saline for intravitreal injections (1 to 2 μ L of 1×10^{-3} mol/L). In some experiments, commercial preparations of dexamethasone (Maxidex 0.1%; Alcon, Camberley, UK) were topically applied as a treatment control.

Experimental Autoimmune Uveitis

EAU was induced as previously described. Mice^{24,27} (6 to 8 weeks old) were immunized s.c. with 300 μ g of interphotoreceptor retinoid-binding protein (IRBP)_{161–180} (for B10.RIII) or 400 μ g IRBP_{1–20} (for C57BL/6J) (peptides SGIPYII-SYLHPGNTILHVD and GPTHLFQPSLVLDMAKVLLD, respectively; Cambridge Peptides, Cambridge, UK) in phosphate-buffered saline emulsified with Complete Freund's Adjuvant (CFA) (Sigma-Aldrich, Gillingham, UK) supplemented with *Mycobacterium tuberculosis* (Difco, Voigt Global Distribution, Lawrence, KS). Mice also received 0.4 μ g of pertussis (Sigma-Aldrich) i.p. They were randomized postimmunization when signs of disease appeared and were grouped according to specific treatments. The C57BL/6J EAU model was used for most studies because the window of

treatment is longer. The more acute B10.RIII model was used for some small-scale experiments.

Drug Administration

Intravitreal injections were performed under a surgical microscope. Animals were anesthetized with ketamine (50 mg/mL) and Medetomidine (Domitor, 1 mg/mL) in sterile injectable water. A 30-gauge needle was used to make a hole in the nasal sclera (approximately 1 mm posterior to the limbus) and a 33-gauge blunt-end needle (VWR International Ltd., Leicestershire, UK) was inserted with a 45 degree injection angle into the vitreous through the hole. One or 2 μ L of drugs or saline was then injected gently into the vitreous of one eye per mouse.

T-Cell Activation

Cells isolated from the draining lymph nodes (dLNs) of primed B10.RIII mice, 7 days after IRBP immunization, were cultured and stimulated with 2.5 μ g/mL IRBP_{161–180}, phytohemagglutinin (10 μ g/mL), cell stimulation cocktail (phorbol 12-myristate 13-acetate and ionomycin, 1:500; eBioscience, Hatfield, UK), or plate-bound anti-CD3 (25 μ g/mL) with soluble anti-CD28 (2 μ g/mL) *in vitro* and stained for T-cell markers.

Isolated Retinal Cell Suspension

Individual eyes were investigated for immunophenotyping of T-cell subsets and for myeloid markers. Ocular tissues were extracted from eyes *in situ*, applying scalpel and forceps under an inverted microscope. Each eye was cut behind the corneal edge while the whole globe was held in place by forceps. After cutting, the globes were everted by applying force using forceps to obtain the whole retina plus vitreous from the globe but leaving the choroid behind. Tissue extracts were kept in X-VIVO 20 medium (Lonza, Basel, Switzerland) in 10% fetal calf serum until preparing cell suspensions by pipetting with 1000 μ L pipette tips and passing through a cell strainer (polystyrene Falcon conical tube). After one wash with phosphate-buffered saline, the cells were used for immunophenotyping.

Clinical Scoring

Clinical disease was scored by using retinal fundus examination, via bright-field live imaging Micron III and visualized by Discover V2.2 software (Phoenix Technology Group, Pleasanton, CA). All mice were under general anesthesia, and pupils were dilated applying topical tropicamide 1% (Sandoz Inc., Princeton, NJ). Clinical assessment was made based on the severity of the optic disc inflammation, retinal vessel cuffing, retinal tissue infiltrate, and structural damage. Each of these parameters was given a score from 0 to 5, and the collective total was representative of clinical disease for the whole eye, with a maximum score of 20 obtainable per eye.²⁸ Scores for individual eyes were compared pretreatment and post-treatment as a paired study.

Histology Scoring

Eyes were enucleated, fixed in 4% glutaraldehyde for up to 1 hour, and transferred to 10% formaldehyde for at least 24 hours; they were then embedded, processed for hematoxylin and eosin staining, and examined histologically. Scores were assigned by a masked observer, according to the criteria for EAU scoring, based on the level of immune cell infiltration and the degree of retinal damage, as previously described.²⁹

Antibodies and Flow Cytometry

For flow cytometry, the following anti-mouse antibodies (Abs) were used: ROR γ T-PE, -PerCP, Cy5.5 and -FITC, IL-17A-BV421 and -PE, CD3-AF488 and -PE-Cy7, CD4-BV395, CD8-PE-Cy7, T-bet-PECy7 and -AF647, CD25-BV711, IFN- γ -PE-Cy7 and -BV421, IFN- γ -BV605, FOXP3-AF647 and -PE. Abs for detecting myeloid markers were CD45-BV605, Ly-6G-AF700, CD11b-PE, CD11c-BV786, and Ly-6c-PerCP, and -PE-Cy7; all were purchased from eBioscience, BD Biosciences (Oxford, UK), and BioLegend (London, UK). LTB₄ receptors (BLT1) were identified with a primary unconjugated monoclonal Ab (EPR7113; Abcam, Cambridge, UK), for 1 hour at room temperature and then washed and stained with anti-mouse AF488 and visualized by flow cytometry.

Cell suspensions were stained for cell surface markers before staining for intracellular molecules. For intracellular cytokine staining, the cells were restimulated with a cell stimulation cocktail (containing *phorbol* 12-*myristate* 13-*acetate* and ionomycin) with the addition of a protein transport inhibitor cocktail (brefeldin A; 1:500; eBioscience) for 4 hours.

Viable cells were identified by using a Live/Dead fixable viability marker (Zombie; BioLegend) according to the manufacturer's instructions. Single-stained OneComp beads (eBioscience) were used to generate compensation matrices. Isotype and fluorescence minus one controls were used to identify gating boundaries. CountBright beads were applied to calculate the absolute numbers of cells (Thermo Fischer Scientific, Waltham, MA). Flow cytometric data were acquired by using an LSR Fortessa (BD Biosciences, San Jose, CA) and BD FACSDiva version 6.1.3 software, and data analysis was performed by using FlowJo version 10.0.7 (FlowJo LLC, Ashland, OR).

Immunofluorescence Microscopy

The formalin-fixed and paraffin-embedded blocks were sectioned (4 μ m), dewaxed, retrieved in citrate buffer (10 mmol/L, pH 6), permeabilized in 0.1% Triton X-100, blocked (5% bovine serum albumin), and stained with primary Abs at room temperature for 1 to 2 hours. Primary Abs were rat anti-mouse CD4 monoclonal Ab (mAb), rabbit anti-human CD4, rabbit anti-human/mouse BLT1 mAb, rabbit anti-human C5aR1 mAb, and rabbit anti-mouse

C5aR1 mAb (all, Abcam) and anti-human/mouse BLT1 polyclonal Ab (2654; Bioss Antibodies, Seattle, WA), used at 1:100 concentrations. Secondary Abs used in this study are described in the figure legends.

Images were captured on an upright Zeiss LSM710 spectral confocal microscope and analyzed by using ZEN 2.1 software (Carl Zeiss, Oberkochen, Germany).

Enzyme-Linked Immunosorbent Assay for LTB₄ and C5a

Immunoassays were performed for the quantitative determination of LTB₄ and C5a levels. Approximately 5 μ L of vitreous fluid from each eye was transferred to 200 μ L tubes containing 50 μ L of phosphate-buffered saline. After gentle pipetting, the fluids were centrifuged for 5 minutes at 250 $\times$ g and supernatants transferred into new tubes and stored at -80°C until assay. LTB₄ (R&D Systems, Minneapolis, MN) and C5a (Thermo Fisher Scientific) kits were used and assays performed according to the manufacturers' instructions.

Statistical Analysis

Statistical analysis was conducted by using GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA) or Excel (Microsoft Office, Microsoft Corporation, Redmond, WA). Differences between groups were determined by *t*-test (for mouse data) or one-way analysis of variance (with a Tukey post hoc analysis) as appropriate for the experiment. *P* < 0.05 was considered significant.

Results

Targeting LTB₄/C5 Pathways Attenuates EAU

EAU was induced in C57BL/6J mice.^{24,27} When signs of disease appeared (day 14 to 15), mice were treated intravitreally with equimolar amounts of nomacopan (5 mg/mL in 1 to 2 μ L), PAS-nomacopan (20 mg/mL), PAS-L-nomacopan (20 mg/mL), dexamethasone (1×10^{-3} mol/L), or saline. Repeat injections of drugs or saline were administered 3 days after the first injection (day 18 post-immunization) (Supplemental Figure S1A), and disease progression was monitored by fundoscopy (before treatment and at harvesting) and scored based on clinical/pathologic criteria such as optic disc and tissue inflammation, vessel cuffing, and structural damage³⁰ compared with a healthy eye. The scorers (M.E. and V.L.C.) were masked to treatment.

A moderate suppression of EAU was detected in the nomacopan-treated group. Significant disease suppression was found in the group treated with PAS-L-nomacopan (*P* = 0.001) at the peak of disease, as shown on day 21 (Figure 1, A and B and Supplemental Figure S1). Clinical disease suppression was confirmed by using histopathologic grading, in which significant reductions in infiltrating immune

cells and in structural damage, including retinal folding, were observed (Figure 1A and Supplemental Figure S1B). In a preliminary study, twice-daily topical administration of L-nomacopan for 7 days (days 14 to 21) showed mild suppression of EAU in C57BL/6J mice ($P < 0.05$) (data not shown), and topically applied nomacopan or PAS-nomacopan failed to show an effect (data not shown).

Investigation of infiltrating immune cells by retinal flow cytometry revealed a significant reduction in Th17 cells expressing IL-17 (Figure 1, C and D), and CD4⁺ T cells expressing transcription factors ROR γ t/T-bet (double-positive Th1/17 cells) (Supplemental Figure S1, C and D), in C57BL/6J EAU mice when treated with PAS-nomacopan or PAS-L-nomacopan (1 to 2 μ L of each; 20 mg/mL) and compared with the saline controls. In addition, isolated cells from inflamed eyes were stained for myeloid markers and studied for the impact of the treatments on their active status. Applying a gating strategy^{31,32} (Supplemental Figure S2, A and B) to detect CD4⁺ T cells and inflammatory and resident macrophages, we observed a significant reduction in the numbers of the “inflammatory (active)” CD45⁺Ly-6G⁻CD11b⁺CD11c⁻Ly-6c^{high} macrophage population with no significant reduction in resident CD45⁺Ly-6G⁻CD11b⁺CD11c⁻Ly-6c^{low} macrophages when EAU eyes were treated with PAS-nomacopan or PAS-L-nomacopan compared with the saline-treated mice (Figure 1, E and F). However, we observed an increased proportion of resident cells in the sample shown, and confirmed it for all samples. This could reflect a change in infiltrated Ly6C⁺ macrophages adopting a less activated phenotype in response to treatment.

EAU disease development in C57BL/6J mice is relatively slow, with a peak at days 20 to 22 postimmunization with a chronic clinical manifestation. We found that treating C57BL/6J mice with PAS-nomacopan or PAS-L-nomacopan intravitreally on days 16 and 18 postimmunization could still suppress disease when mice were left to develop EAU over an extended time (26 days postimmunization). Investigating the clinical criteria in EAU on day 26, we observed that PAS-nomacopan could significantly reduce retinal tissue inflammation, and PAS-L-nomacopan could suppress retinal

vessel inflammation. Both drugs prevented structural damage but neither caused a significant reduction in optic disc inflammation (Figure 1G and Supplemental Figure S2C).

EAU disease in B10.RIII mice develops relatively faster than in C57BL/6J mice and, as an acute form of EAU, peak disease occurs on days 13 to 15. Disease manifestations are similar to those in C57BL/6J mice but are clinically more severe, with enhanced retinal structural damage and hemorrhage developing at peak disease with more evidence of edema. The impact of sequestering LTB₄ and inhibiting C5 was investigated by applying nomacopan over a shorter 2-day treatment period. A suppression of clinical signs 2 days after intravitreal administration of nomacopan was detected (Figure 1H), accompanied by a reduction in percentages of effector CD4⁺ Th17 and Th1/17 (ROR γ t/T-bet) cells (Supplemental Figure S3, A–E).

Vitreous Levels of LTB₄/C5a at Different Stages of EAU

Fundoscopy was performed just before harvesting vitreoretinal fluids. In the C57BL/6J mice, levels of LTB₄ and C5a, used as a marker of C5 activation within the vitreoretinal space of control and EAU mice not treated with drugs were investigated (Figure 2, A–C). The levels of LTB₄ and C5a were markedly increased in vitreoretinal fluids extracted from EAU eyes during disease progression compared with the CFA or naive controls (Figure 2, B and C). LTB₄ levels correlated with disease progression in C57BL/6J mice from day 12 onward (Figure 2D) as well as in B10.RIII mice at different stages of disease (data not shown).

Intra-experimental variations in the levels of LTB₄ were detected, especially between days 5 and 12 (50 to 500 pg/mL). In all experiments (with fluids collected from days 2 to 21), the levels of LTB₄ were consistently increased compared with the CFA controls. The level of C5a in the vitreoretinal fluids increased in the early stages of disease and was substantially higher again during the peak of the disease on day 21 postimmunization (Figure 2B). However, in all experiments, the level of LTB₄ increased significantly up to day 21 post-EAU (Figure 2C).

Figure 1 Targeting leukotriene B₄ (LTB₄) attenuates experimental autoimmune uveitis (EAU) progression in the mouse model of uveitis. **A:** Representative fundus images of EAU treated with nomacopan (Nom), PAS-nomacopan (PAS-Nom), PAS-L-nomacopan (PAS-L-Nom), or saline intravitreally, and histopathology images corresponding to the disease status. Histological score of 5, 4, 3, 2, and 3 for saline, Nom, PAS-Nom, PAS-L-Nom, and dexamethasone (DEX) treatments, respectively, on a scale of 0 to 5. **B:** Clinical score on individual eyes based on funduscopy validation, just before the first intravitreal injection (day 15) and harvesting on day 21 (following a second injection on day 18). Flow cytometry on retinal cell suspensions from mice treated with Nom, PAS-Nom, PAS-L-Nom, DEX, or saline. Equimolar amounts of Nom were applied (5 mg/mL). Plots have been gated on live, single lymphocytes, and then with specific staining as follows. **C and D:** CD4 and IL-17 and gated for CD4⁺ cells and then IL-17⁺ cells. The summary graph of the data is also shown. **E and F:** Plots were gated for myeloid markers. The cells were gated as CD45⁺ Ly-6G⁻ CD11b⁺ CD11c⁻ Ly-6c^{hi} for detecting infiltrating cells and ⁻Ly-6c^{lo} for resident cells. Summary graph for infiltrating and resident macrophages. **G:** Stack bar graph showing day 15 (just before treatment) and day 26 (after two intravitreal injections of Nom, PAS-Nom, PAS-L-Nom, or saline, on days 15 and 18). The clinical criteria are described in the legend. Each bar was drawn based on the mean value $\pm$ SD of each score ($n = 6$ to 8 mice). The preclinical data are representative from four to six independent experiments ($n = 6$ to 8 mice per group). **H:** Graphical summary showing clinical score on days 11 (pretreatment) and 13 (posttreatment) from B10.RIII mice ($n = 3$ in each group). Masked scores, one-way analysis of variance (clinical scores) and unpaired t -test P values compared with vehicle. **White arrows** indicate infiltrating cells; **black arrows** indicate retinal folds. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bar = 200 μ m. C, choroid; I, infiltrating macrophage; iO, inflamed optic disk; iR, inflamed retinal tissue; iV, inflamed vessel; L, lens; M ϕ , macrophage; R, resident macrophage; Re, retina; V, vitreous.

Figure 2 Leukotriene B_4 (LTB_4) and C5a levels in different stages of experimental autoimmune uveitis (EAU) development in C57BL/6 mice. **A:** Representative fundus images from healthy mice and EAU mice on days 12, 16, and 21 of disease and their corresponding data from enzyme-linked immunosorbent assay assays detecting C5a (**B**) and LTB_4 (**C**) levels in vitreous fluids. Data were compared with the amount of LTB_4 and C5a in control mice [complete Freund's adjuvant (CFA) alone] and healthy mice (with no immunization). Representative data from four to five independent experiments in which vitreous fluids were collected at early stages of disease and three independent experiments at late stages of disease ($n = 3$ to 6 mice per group). Unpaired t -test P values compared with vehicle, $\pm$ SD shown. **D:** Data obtained from enzyme-linked immunosorbent assay were plotted to clinical readouts (vessel cuffing and tissue infiltration scores) for any correlation and relationship between disease scores and LTB_4 levels. **Dashed gray line** indicates the trendline. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. ctrl, control; fiL, focal inflammatory lesion; iO, inflamed optic disk; iR, inflamed retinal tissue; iV, inflamed vessel; O, optic disk; V, vessel.

The amounts of LTB_4 positively correlated specifically with two clinical criteria: retinal vessel cuffing (mild, moderate, or severe; maximum score of 5) and retinal tissue infiltration (small, large, or linear lesions; maximum score of 5) (Figure 2D). Intraocular LTB_4 levels in nomacopan-treated EAU mice were not determined by enzyme-linked immunosorbent assay due to the effect of nomacopan, which, by sequestering LTB_4 , could potentially mask its detection. This issue has also been a challenge in another study in which nomacopan was applied in a mouse model of inflammatory skin disease.²³

LTB_4 Recruits Immune Cells to the Vitreoretinal Space

To study LTB_4 function within the posterior chamber, synthetic LTB_4 (1 to 2 μ L, 0.1 μ g/mL) was injected into the vitreous in eyes from naive mice. We titrated the synthetic LTB_4 (100 pg/mL, 1 ng/mL, and 1 μ g/mL) in naive B10RIII mice and examined eyes by using funduscopy. The introduction of LTB_4 into the vitreous induced focal inflammatory lesions as detected by funduscopy (Figure 3A) and recruited immune cells into the vitreoretinal space (Figure 3B). Neither $CD4^+$ T cells nor a significant number of neutrophils were detected in the vitreoretinal space postinjection at various time points (Supplemental

Figure S4, A and D). In contrast, immunoreactivity to the infiltrating macrophage/myeloid markers was increased after intravitreal injection of synthetic LTB_4 (Figure 3B). $BLT1^+$ cells predominantly expressing high levels of Ly-6c in combination with CD45, Ly6G, CD11b, and CD11c markers indicating inflammatory (active or infiltrating) macrophages were detected. However, some of those cells expressed a low level of Ly-6c, which was used as an indicator of resident macrophages. Using these markers, an increase in inflammatory macrophages was observed at day 5 posttreatment (Figure 3C).

LTB_4R (BLT1) Expressing Cells in EAU

As described in Figure 1, C–F, both PAS-nomacopan and PAS–L-nomacopan were able to suppress a significant number of Th17 cells as well as inflammatory macrophages during EAU. We next determined if the receptors for LTB_4 and C5a were expressed within retinal tissues.

Immunofluorescence staining was performed on tissue sections from EAU eyes to identify LTB_4R (BLT1) and C5aR (C5aR1); a significant increase of infiltrating cells was observed in the vitreous cavity expressing one or both receptors in EAU compared with healthy eyes (Figure 4, A and B). The levels of these receptors provided evidence for

Figure 3 Leukotriene B₄ (LTB₄) recruits myeloid cells to the vitreoretinal space. **A:** Synthetic LTB₄ was injected intravitreally into healthy eyes in mice (B10.RIII), and eyes were monitored by funduscopy for any disease-like phenotypes. Representative fundus images at indicated time points. Eyes were harvested at different time points and retinal flow cytometry performed for T-cell (Supplemental Figure S4) and myeloid markers. A significant level of macrophages was detected in the retina after 5 days following the LTB₄ injection. Most of those cells were positive for BLT1 expression. In overlaying plots, blue indicates BLT1⁺ macrophages in a population of red, which indicates BLT1⁻ cells. **B:** The gating strategy was based on CD45⁺BLT1⁺Ly-6G⁻CD11b⁺CD11c⁻Ly-6c low and high. The maximum level of BLT1⁺ macrophages was detected after 5 days of LTB₄ injections. **C:** Graphical summary showing means $\pm$ SD from at least three independent experiments ($n = 3$ to 6). Unpaired t -test P values compared with resident myeloid cells. * $P < 0.05$, ** $P < 0.01$. d, day; fIL, focal inflammatory lesion; I, infiltrating macrophage; M ϕ , macrophage; O, optic disk; R, resident macrophage.

infiltrating retinal cells having the capacity to respond to their ligands (LTB₄ and C5a).

To investigate which immune cell types were BLT1⁺ and involved in EAU progression, retinal cell suspensions derived from IRBP-primed B10.RIII mice were stained for CD3/CD4 and myeloid markers and assessed by flow cytometry. Despite very low cell numbers obtained at such an early stage of EAU (day 7 postimmunization), we detected a significant proportion of CD3⁺ cells, with the majority of CD3⁺CD4⁻ cells (CD8⁺ T cells) co-expressing BLT1 while a few CD11b⁺ cells positive for BLT1 in the early stages of disease. Using the same protocol for staining retinal immune cells from EAU B10.RIII mice on day 15, at the peak of the disease, data showed a significant increase in CD4⁺ and CD11b⁺ cells expressing LTB₄ receptors (Figure 4, C–E).

Activated Lymphocytes Expressing LTB₄ (BLT1) and C5a Receptors

Increased levels of BLT1⁺ and C5aR1⁺ cells in dLNs of EAU were detected compared with no-disease control dLNs (Figure 5, A and B). The relative levels of BLT1⁺ cells and

C5aR1⁺ cells in dLNs were different in each EAU mouse (two examples are given). However, there was no correlation with disease outcome or the immune profiles of retinal infiltrating cells.

To investigate if expression of BLT1 is associated with T-cell activation, dLNs from primed EAU B10.RIII mice (day 7 postimmunization) were used. Single-cell suspensions were treated *in vitro* with plate-bound anti-CD3/CD28 (for 3 days), phytohemagglutinin (data not shown), *phorbol* 12-*myristate* 13-*acetate*/ionomycin (PMA/I), IRBP₁₆₁₋₁₈₀, or synthetic LTB₄ (1 μ mol/L), to investigate autocrine activity, for 18 hours and LTB₄R expression was assayed on CD4⁺T cell subsets. With the exception of LTB₄, we detected a marked increase in CD4⁺ T cells expressing BLT1 relative to unstimulated controls in all stimulating conditions tested (Figure 5, C and D). By gating on CD4⁺BLT1R⁺ cells, we found increases in both Th17 (IL-17⁺ROR γ t⁺) and Th1 cells (IFN γ ⁺T-bet⁺) in response to IRBP relative to unstimulated controls (Figure 5, E and F). Furthermore, analyzing CD3⁺CD4⁻ cells for BLT1 expression revealed a population of CD8⁺ cells that co-expressed BLT1 (Supplemental Figure S5, A and B).

LTB₄ and C5a Receptor Expression in Healthy and Uveitic Eye Specimens

To investigate the expression pattern of BLT1 and C5aR1 in human eyes, retinal sections were stained for BLT1 and C5aR1 and assessed by using immunofluorescence microscopy. Only minimal immunoreactivity in healthy eyes was observed (posterior chamber) (Figure 6A). However, in uveitic retinal tissue sections, significant levels of infiltrating immune cells were observed within the posterior segment, with reasonable numbers of cells expressing LTB₄ and/or C5a receptors (Figure 6, B and C).

Uveitic retinal tissue sections had local ectopic lymphoid-like structures containing infiltrating immune cells based on previous studies.³³ Although there were only a few BLT1⁺ cells or C5aR1⁺ cells within the retinal layers, those numbers increased considerably within these lymphoid-like structures or inflammatory foci and throughout the areas of cellular infiltration. Only a few cells expressed both receptors in the damaged retinal layers (Figure 6, B and C); however, the majority of cells in the inflammatory foci expressed both BLT1 and C5aR1 (Figure 6D).

Discussion

The current study provides novel data showing that the LTB₄ pathway is important during initiation and later stages of EAU, LTB₄ can be detected within the vitreous, and receptors for both LTB₄ and C5a (BLT1 and C5aR1, respectively) are expressed by infiltrating cells and can be up-regulated on activation. In addition, infiltrating leukocytes in uveitic retinal tissues, but not tissue-resident cells in human noninflamed retinal tissues, express significant levels of both the LTB₄ and C5a receptors.

Despite increasing evidence for the role of LTB₄ in promoting inflammatory diseases and showing that targeting the receptor has a suppressive effect in inflamed tissues, specifically targeting the LTB₄ pathway is not the current standard of care for managing inflammatory disease. There is only one drug (zileuton, a 5-lipoxygenase inhibitor) marketed for the inhibition of LTB₄, which was approved for the treatment of steroid-resistant asthma. LTB₄ receptor inhibitors such as CPI05696 and U75302 used in laser-induced age-related macular degeneration and lipopolysaccharide-induced cardiac dysfunction mouse models, respectively, were successful but are not clinically approved.^{34,35} The involvement of LTB₄ in EAU and other inflammatory diseases is clearly evident and fulfills an unmet need to generate safer and more potent LTB₄ pathway-blocking drugs. A recent study

supports the efficacy of nomacopan in a phase 2 clinical study and in a mouse model of pemphigoid skin disease,²³ and the current study shows the clinical benefits of locally administered nomacopan variants in two different mouse models of uveitis.

The impact of nomacopan and its variants after two intravitreal administrations (days 15 and 18) was studied and efficacy monitored at the disease peak (day 21) and at a later time point (day 26). Despite comparable levels of disease severity in EAU observed between peak and a later time point, tissue damage was persistent when monitored at day 26, with no obvious repair of tissue in saline-treated controls. From these preclinical studies, PAS-L-nomacopan was more effective in suppressing EAU and had less retinal tissue damage. These findings suggest that inhibiting LTB₄ at inflamed tissue sites could prevent the progression of EAU and also suppress late-stage disease complications, when structural damage is irreversible and leads to vision loss. Therefore, the role of LTB₄ became the focus of the study, although the role of C5 in EAU progression cannot be excluded as we did not investigate it at different stages of EAU or at different therapeutic time points. The C5 pathway has already been shown to be involved in late-stage EAU.³⁶

Previous reports have found that LTB₄ can promote the generation of Th17 cells, an effector CD4⁺ T-cell subset, through its specific transcription factor, RORγt, while suppressing the differentiation of regulatory T cells.^{16,37} The regulatory T-cell levels did not vary from baseline in response to targeting LTB₄ in our mouse model, whereas a significant reduction of Th17 cells and of T cells co-expressing RORγt/T-bet was observed ($P = 0.01$). In addition to effector CD4⁺ T cells, myeloid cells (specifically macrophages) are also involved in EAU progression. Macrophages are important not only for tissue damage but also as active participants in shaping the effector T-cell response that leads to inflammatory diseases.^{38–40} In addition to functioning as proinflammatory cells, monocytic cells can differentiate into a regulatory phenotype, known as myeloid-derived suppressor cells, which inhibit autoaggressive T cells.⁴¹

Due to the extremely low retinal cell numbers, we faced challenges in profiling retinal macrophages and for investigating inflammatory macrophages during inflammatory contexts, primarily because distinguishing them from resident microglia is unreliable due to shared surface markers. The gating strategy was modified from previously published protocols.^{31,32,42} CD45 expression at an intermediate level, which has been reported as a microglia marker,¹⁹ was not detectable in all EAU samples and thus was not included in

Figure 4 Infiltrating immune cells in experimental autoimmune uveitis (EAU) express BLT1 and C5aR1. Immunofluorescence staining for BLT1 (green), C5aR1 (red), and DAPI (blue) on eye sections obtained from a healthy eye (A) and EAU B10RIII mice (B). Activated CD4⁺ cells express BLT1. C–E: Isolated retinal immune cells obtained from primed B10RIII (day 7 postimmunization) or at the peak of the disease (day 15) stained for T cells (CD3 and CD4) and myeloid marker (CD11b) in addition to BLT1. There are significant numbers of CD3⁺CD4⁺ cells expressing BLT1; some CD4⁺ cells and CD11b⁺ cells are positive for BLT1 on day 7, and these cells increased at the peak of the disease (day 15). Green arrow (BLT1), red arrow (C5aR1), and yellow arrow (BLT1 and C5aR1). Scale bars: 100 μm (A); 20 μm (B, left panel); 10 μm (B, right panel). d, day; R, retina; S, sclera; V, vitreous.

Figure 5 Leukotriene B₄ (LTB₄) and C5a receptors in inflamed lymph nodes (LNs). **A:** Immunofluorescence staining for BLT1 (green), C5aR1 (red), and nucleus by DAPI (blue) on sections obtained from healthy, uninfamed LNs from B10.RIII mice. **B:** Compared with the inflamed draining LNs (dLNs) of mice with experimental autoimmune uveitis (EAU). **Yellow arrows** indicate cells expressing both receptors. **C:** Gating strategy for CD4 and BLT1. **D:** Lymphocytes from primed B10.RIII mice (7 days' postimmunization) were isolated and activated overnight with different stimuli, here showing interphotoreceptor retinoid-binding protein (IRBP)₁₆₁₋₁₈₀ and *phorbol 12-myristate 13-acetate*/ionomycin (PMA/I). The cells were stained first with monoclonal primary antibody against BLT1 and then corresponding secondary conjugated with AF488. **E–G:** The antibodies for T-cell markers were CD4, IL-17, retinoic acid–related orphan receptor γt (RORγt), interferon-γ (IFN-γ), and T-bet, for detecting Th17 (**E**), Th1/17 (**F**) and Th1 (**G**). Data were compared with the level of positive cells in unstimulated lymphocytes (**D**). CD4⁺ cells detected as Th17 and Th1 cells where expressed RORγt/IL-17, IFN-γ/T-bet, respectively, whereas Th1/17 cells were positive for T-bet and RORγt expression in IRBP-treated primed CD4⁺ T cells. Scale bars = 50 μm.

the analysis. Therefore, using retinal flow cytometry, only resident and inflammatory macrophages could be identified in EAU, and the level of inflammatory macrophages was found to be reduced after treating mice intravitreally with PAS–L-nomacopan and PAS-nomacopan. The relative proportion of resident macrophages increased in response to treatments, which might reflect changes in the phenotype of

infiltrating Ly6c⁺ cells. It has been shown that macrophages expressing Ly6C^{Lo}, which are detected next to endothelial cells lining the blood vessels, are thought to be derived from Ly6C^{hi} macrophages in response to signaling, including delta-like 1 (DLL1) and upon up-regulation of transcription factor NR4A3.⁴³ Studies of traumatic muscle injury showed recruitment of Ly6C^{hi} macrophages at early stages; at later

Figure 6 BLT1 and C5aR1 expression in healthy human eyes and patients with uveitis. **A:** Immunofluorescence staining for BLT1 (green), C5aR1 (red), and nucleus by DAPI (blue) on formalin-fixed and paraffin-embedded sections from healthy eye donors (**A**) and on uveitis patient sections (**B–D**). Hematoxylin and eosin staining included as a guide for retinal structure for immunofluorescence images. **Green, red, and yellow arrows** highlight BLT1, C5aR1, and double-positive BLT1 and C5aR1 cells, respectively. **Yellow box** refers to the area which has been captured. Scale bars: 100 μm (**A**); 50 μm, **B** and **C** (left panels), **D** (right panel); 20 μm, **B** and **C** (right panels), **D** (left panel). C, choroid; R, retina; RPE, retinal pigment epithelium; S, sclera; V, vitreous.

stages, those Ly6C^{hi} macrophages switch to an Ly6C^{lo} phenotype and produce a high level of growth factors to promote muscle regeneration.^{44,45} This phenotypic change in expression of Ly6C might also occur within the central nervous system.

The detection of significant levels of intraocular LTB₄ and C5a in the vitreoretinal space in EAU and not in healthy eyes suggests their involvement in disease progression. The level of LTB₄ was positively correlated with the clinical scoring criteria associated with vessel and tissue

inflammation or tissue edema. However, the correlation between LTB₄ levels and overall disease severity was not linear, reflecting the changes during EAU progression. Similarly, it has been shown that high-dose injection of LTB₄ into mouse lungs leads to tissue damage.^{46,47} However, the impact of nomacopan variants on LTB₄ levels is technically challenging, and the effect on C5a levels remains unclear.

Because LTB₄ has been shown to affect vascular permeability,⁴⁸ detecting LTB₄ in the vitreoretinal space at such an early stage postimmunization may facilitate subsequent cell infiltration into the immune privileged eye, including CD4⁺ T cells. To determine if a similar mechanism occurs in the retina, we introduced LTB₄ into the vitreous cavity of healthy eyes. Investigating infiltrating leukocytes and myeloid cells at different time points following LTB₄ injection revealed a significant level ($P = 0.02$) of activated macrophages recruited into the vitreoretinal space; however, it had no effect on other immune cells, including CD4, CD8 T cells, and neutrophils. The majority of those infiltrating macrophages expressed BLT1 ($P = 0.024$), whereas there was also a population of BLT1⁺ resident macrophages. We have shown that LTB₄ functions as a recruiter for infiltrating macrophages but whether it induces, directly or indirectly, BLT1 expression on resident macrophages or infiltrating macrophages needs further investigation.

It has previously been reported that both C5aR and C3aR up-regulate pathogenic T-cell development, and those receptors are required in the pathogenesis of EAU.⁸ C5aR was also identified as a marker of differentiating microglial subsets during an lipopolysaccharide response in C57BL/6J mice,¹⁹ and microglia are known to be part of EAU disease progression. The presence of BLT1- and C5aR1-expressing retinal infiltrating cells in EAU identified candidate target cells for nomacopan therapy via inhibition of the ligands (LTB₄ and C5) and, subsequently, their signaling pathways.

In the current study, a significant number of cells co-expressing both receptors in the eye were detected in contrast to dLNs, in which only rare BLT1⁺C5aR1⁺ cells were observed, suggesting that BLT1⁺C5aR1⁺ cells were activated in inflamed eyes. Investigating the BLT1⁺ cell types in early EAU-primed mice (B10.RIII), in which disease had been initiated but signs of disease were not always detectable by funduscopy (day 7), very low numbers of infiltrating cells were found with some retinal CD3⁺ and CD11b⁺ cells expressing BLT1⁺. Because only very few CD4⁺ T cells expressed BLT1, it was concluded that the majority of BLT1⁺ T cells were CD8 cells. However, the activation status of retinal macrophages in the early stages of disease was not determined. As previously discussed, at the peak of EAU disease, infiltrating CD4⁺ and CD8⁺ T cells as well as myeloid cells expressed significant levels of BLT1⁺. We hypothesized that the BLT1⁺CD3⁺CD4[−] cells function as effector T cells and showed that activation, either via the T-cell receptor (IRBP) or nonspecifically via

mitogen, induced CD4⁺ T-cell expression BLT1⁺. LTB₄ treatment itself did not up-regulate receptor expression in these experiments. The finding that CD8⁺ T cells expressed BLT1 is in agreement with findings identifying LTB₄ as a potent non-chemokine mediator of CD8⁺ effector T-cell migration *in vitro* and *in vivo* and showed that the LTB₄-BLT1 pathway is a link by which cells of the innate immune response modulate the effector arm of adaptive immunity.⁴⁹ Relative levels of BLT1 expression among retinal infiltrating cells requires further investigation. Nevertheless, intravitreal BLT1⁺ T cells might respond to the LTB₄ detected within the vitreous fluid and could be targeted indirectly by treatment with nomacopan.

Macrophages, like effector CD4⁺ T cells, exhibit functional flexibility and plasticity dependent on signals in the microenvironment. As a result, their functions can be altered by external stimuli that modify their transcriptional programs and lead to an activated state.⁵⁰

The impact of TNF α on macrophage differentiation is well established,^{32,51} and the ability of LTB₄ to induce TNF α expression^{52,53} supports a connection between those pro-inflammatory molecules. Neutralization of TNF α activity, which suppresses macrophage activities during ocular inflammation, protects the retina.⁵⁴ According to the literature, there is no evidence of macrophage infiltration into the retina or uvea until day 11 to 12 postimmunization in EAU.^{55,56} However, a marked up-regulation of inflammatory cytokines such as TNF α , inducible nitric oxide synthase, and IL-1 α in early stages of disease has been reported, and these cytokines could be generated by the innate immune response in the retina.^{56,57} Because macrophages are key producers of TNF α , anti-TNF α agents are efficacious as treatments for some patients with uveitis.⁷ The increased level of LTB₄ detected at early stages of disease (day 2) might up-regulate TNF α secretion by tissue-resident macrophages before the infiltration of macrophages, supporting the hypothesis that innate immunity occurs early in EAU.⁵⁸ By adding LTB₄ into the posterior chamber, it was found that both resident and infiltrating macrophages expressed LTB₄ receptors, which could mimic the early stage of EAU. Determining whether resident microglia express BLT1 will be an objective of future studies.

Investigating expression of those receptors in human retinal tissue sections showed that very few tissue resident cells expressed BLT1 or C5aR1 in healthy retinal sections, whereas BLT1⁺ or C5aR1⁺ cells were readily detected in the choroid, throughout the retinal layers, and within the vitreous cavity in uveitis retinal sections. There were also cells that co-expressed both receptors, especially within inflammatory foci where these co-expressing cells were densely localized. Activation of both signaling pathways has been described during neutrophil chemotaxis and in some arthritis animal models,^{14,59} but, to our knowledge, no study has shown both receptors expressed by one individual cell. Unfortunately, it was not possible to obtain retinal tissue sections from donors with early-stage disease, and we therefore do not know the effects of therapy on the

BLT1/C5aR1 signaling pathways by retinal cells; this topic requires further investigation.

Although LTB₄ is a well-characterized inflammatory mediator, only a few studies on its role in ocular diseases have been performed. In an animal model of age-related macular degeneration, LTB₄ promoted neovascularization and macrophage recruitment,³⁴ and a high level of LTB₄ was detected in a model of diabetic retinopathy in which a BLT1 antagonist inhibited disease and induced endothelial cell death.⁶⁰ In addition, a BLT1 antagonist (CP105696), given at disease onset in the induced model and adoptive transfer of activated CD4⁺T cells, inhibited EAU development.⁹ Those retinal diseases are leading causes of blindness worldwide, which involve inflammation during disease progression.⁴ These findings provide further evidence supporting LTB₄ as a specific target for intraocular diseases.

Further research is needed in areas of inflammation, regulation under homeostatic conditions, and understanding how inflammatory mediators such as LTB₄ alter immune responses within the eye. This information will allow for the development of customized immunomodulatory therapies. The current study described the efficacy of nomacopan in suppressing EAU disease and revealed the involvement of LTB₄ in disease development/progression. The basis of the drug efficacy was investigated by using nomacopan to examine the therapeutic potential of dual inhibition of C5 and LTB₄ and PAS-L-nomacopan to specifically inhibit LTB₄ in EAU. We showed that both PAS-nomacopan and PAS-L-nomacopan suppressed inflammation in EAU, but because PAS-L-nomacopan did so more effectively than PAS-nomacopan, it suggests that the LTB₄ pathway was dominant in driving the inflammation and disease at the time points selected for this study.

In conclusion, this study provides evidence to support a role for LTB₄ in the pathogenesis of EAU, the presence of receptor-expressing target cells in uveitis, and candidate nomacopan variants as novel biologics with the potential to suppress intraocular inflammatory diseases.

Author Contributions

M.E. performed, conducted, and analyzed the experiments and wrote the manuscript. Y.-H.C. assisted in experimental studies. M.A.N. and W.W.-D. contributed to the preclinical study designs and reviewed the manuscript. S.E.C. reviewed the manuscript and provided histopathologic expertise and human materials. V.L.C. initiated, supervised, and conceived the project; reviewed the manuscript; and secured funding for the study.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2020.10.010>.

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