Journal Pre-proof

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PII: S0002-9440(20)30491-0

DOI: https://doi.org/10.1016/j.ajpath.2020.10.010

Reference: AJPA 3462

To appear in: *The American Journal of Pathology*

Received Date: 23 June 2020

Revised Date: 15 October 2020

Accepted Date: 20 October 2020

Please cite this article as: Eskandarpour M, Chen Y-H, Nunn MA, Coupland SE, Weston-Davies W, Calder VL, Leukotriene B4 (LTB4) and its receptor in Experimental Autoimmune Uveitis (EAU) and in human retinal tissues: clinical severity and LTB4-dependence of retinal Th17 cells., *The American Journal of Pathology* (2020), doi: https://doi.org/10.1016/j.ajpath.2020.10.010.

Leukotriene B4 (LTB4) and its receptor in Experimental Autoimmune Uveitis (EAU) and in human retinal tissues: clinical severity and LTB4-dependence of retinal Th17 cells.

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Running Title: Blocking LTB4 in EAU; Th17 and macrophages

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This work was supported, in part, by a research collaborative grant from Akari Therapeutics (VLC).

The authors have the following conflicts of interest to declare. MAN and WW-D. are employees of Akari Therapeutics.

Abstract

Nomacopan, a drug originally derived from tick saliva, has dual functions of sequestering leukotriene B4 (LTB4) and inhibiting complement component 5 (C5) activation. It was demonstrated that nomacopan provides therapeutic benefit in experimental autoimmune uveitis (EAU). The 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 significant reduction of effector Th17 cells and inflammatory macrophages.

Increased levels of LTB4 and C5a (produced upon C5 activation) were detected during disease progression. Retinal activated lymphocytes were shown to express LTB4 receptors (R) *in vitro* and in inflamed draining lymph nodes (dLN). Levels of LTB4R-expressing retinal active/inflammatory macrophages were also increased. Within the dLN $CD4^{+}$T cell population, 30% expressed $LTB4R^{+}$ following activation *in vitro*, while 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-BLT1 pathway as a novel therapeutic approach.

In conclusion, 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.

keywords:

uveitis, EAU, Leukotrienes, LTB4, BLT1, C5, C5aR1, nomacopan

Abbreviations

AMD: Age-related Macular Degeneration

C5: complement C5

CFA: Freund's Complete Adjuvant

DEX: dexamethasone

dLN : draining lymph node

EAU: Experimental Autoimmune Uveitis

FFPE: Formalin-Fixed Paraffin-Embedded

IRBP: interphotoreceptor retinoid binding

LTB4: leukotriene B4

PAS: PASylation

PHA: Phytohemagglutinin

PMA/I: Phorbol Myristate Acetate and Ionomycin

Introduction

Non-infectious posterior uveitis has a predominantly autoimmune etiology, mediated by Th1 and/or 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 different clinical spectrums.[4] Furthermore, uveitis may be associated with systemic diseases[5]. More than half of all patients with uveitis end up with vision associated complications, such as glaucoma and macular edema, and up to 35% of patients suffer severe visual impairment. Uveitis accounts for about 10–15% of cases of total blindness and up to 20% of legal blindness.[5, 6] Therapies for uveitis are mainly restricted to the use of systemic oral corticosteroids, and disease-modifying anti-rheumatic drugs (DMARDs), such as cyclosporin, methotrexate, mycophenolate and, more recently, anti-TNFα drugs, e.g. adalimumab. These medications may lead to serious side effects when given long term e.g. liver failure, low peripheral lymphocyte counts. Local (periocular or intravitreal) corticosteroid injections may limit systemic effects; however, they may be associated potential local adverse effects such as elevated intraocular pressure, glaucoma and cataracts.[7] Therefore, the development of drugs that more specifically abrogate pathogenic pathways involved in uveitis progression and disease complications is essential, with a hope to transform and improve the management of patients with intraocular inflammatory diseases.

Our knowledge of the contribution of LTB4 and C5 within the retina during uveitis development/progression and in the *in vivo* experimental model of disease (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 the 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]. Since it is known that gene-deficient adult mice might not have a full complement of immune pathways, there is a need to determine the role of these molecules in wild-type mice.

Nomacopan, previously known as Coversin, is a bifunctional biologic derived from blood-feeding ticks that specifically sequesters leukotriene B4 (LTB4) within an internal binding pocket and also prevents complement factor 5 (C5) activation.[10, 11] Nomacopan's mode of action prevents LTB4 interacting with its two known G protein coupled cell surface receptors (GPCR 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 GPCRs C5aR1 and C5aR2.

LTB4 ($C_{20}H_{32}O_4$) is a potent lipid inflammatory mediator, derived from arachidonic acid by the sequential action of 5-lipoxygenase (5-LOX), 5-lipoxygenase-activating protein (FLAP) and LTA4 hydrolase (LTA4H).[12] LTB4 is an important multifunctional mediator of inflammation which 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 GM-CSF, TNFα, IL-6 and IL-1β and chemokines KC, MCP-1, CXCL1, and CXCL2 that enhance inflammatory responses.[15] LTB4 dose-dependently increased the secretion of IL-17 and the expression of RORγt 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 is a proteolytic breakdown product of C5 activation, that 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 LPS 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 (MAC), 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 this study the role of LTB4 and C5 pathways was investigated using nomacopan as a dual inhibitor. Furthermore, in order to extend the half-life of the parental drug, PASylation technology was used to compare PASylated variants of nomacopan, since therapeutic benefits of these PAsylated forms have been reported in other models.[22, 23] The pharmacokinetics of nomacopan were improved by N-terminal conjugation with a 600 residue polypeptide composed of Pro, Ala, and Ser (PAS) residues.[22] The roles of LTB4 and C5 activation or LTB4 alone were studied in EAU progression and we investigated if 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 non-infectious intraocular inflammatory disease.[26] The relevance of the findings to human disease was 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 post-mortem cases were obtained from the Moorfields Biobank, at the UCL Institute of Ophthalmology. Three enucleated eyes were provided from non-infectious posterior uveitis patients with end-stage ocular disease (n=3) (Liverpool Clinical Laboratories). All eyes were donated following consent. All specimens were formalin fixed and paraffin embedded (FFPE), and 4-6 µm tissue sections prepared from these blocks. In all specimens, the sclera, choroid and retinal layers were observed intact.

Mice

Female C57BL/6J strain bought from Charles River, UK at 5-7 weeks of age and female B10.RIII mice (6-8 weeks) were obtained from a colony gifted from GlaxoSmithKline (Stevenage, UK) and expanded at the Biological Research Service within 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*[11]. 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 20mg/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) was dissolved in absolute ethanol and saline for intravitreal injections (1-2µl of $1x10^{-3}$M). In some experiments commercial preparations of dexamethasone (Maxidex 0.1%; Alcon, Camberley, U.K.) were topically applied as a treatment control.

Experimental Autoimmune Uveitis (EAU)

EAU was induced as previously described. Mice[24, 27] (6-8 weeks old) were immunized s.c. with 300 µg of interphotoreceptor retinoid-binding protein $(IRBP)_{161–180}$ (for B10.RIII) or 400 µg $IRBP_{1-20}$ (for C57BL/6J) (peptides SGIPYIISYLHPGNTILHVD and GPTHLFQPSLVLDMAKVLLD respectively, Cambridge Peptides, Cambridge, U.K.) in PBS emulsified with CFA (Sigma-Aldrich, Gillingham, UK) supplemented with *Mycobacterium tuberculosis* (Difco, Voigt Global Distribution, Lawrence, KS). Mice also received 0.4 µg of pertussis (Sigma-Aldrich) i.p. Mice were randomized post-immunization when signs of disease appeared and were grouped as to specific treatments. The C57BL/6J EAU model was used for most studies since the window of treatment is longer. The more acute B10RIII model was used for some small scale experiments.

Drug Administration: Intravitreal injections were performed under a surgical microscope. Animals were anaesthetized with Ketamine (50mg/ml) and Domitor (1mg/ml) in sterile injectable water. A 30G needle was used to make a hole in the nasal sclera (approximately 1 mm posterior to the limbus) and A 33G blunt-end needle (Hamilton) was inserted with a 45° injection angle into the vitreous through the hole. 1 or 2 µL of drugs or saline was injected gently into the vitreous of one eye per mouse.

T cell activation: Cells isolated from the draining lymph nodes (dLN) of primed B10.RIII mice, 7 days following IRBP immunization, were cultured and stimulated with 2.5 µg/ml $IRBP_{161–180}$, PHA (10µg/ml), cell stimulation cocktail (PMA and ionomycin, 1:500, eBioscience) or plate-bound anti-CD3 (25 µg/ml) with soluble anti-CD28 (2 µg/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 whilst the whole globe was held in place by forceps. After cutting, the globes were everted by applying force using forceps to obtain whole retina plus vitreous from the globe but leaving the choroid behind. Tissue extracts were kept in *x-vivo*-20 medium (Lonza) in 10% FCS until preparing cell suspensions by pipetting with 1000µl pipette tips and passing through a cell strainer (polystyrene tube, Falcon). After one wash with PBS, the cells were used for immunophenotyping.

Clinical scoring: Clinical disease was scored using retinal fundus examination, via bright-field live imaging (Micron III and visualized by Discover software (Phoenix Technology, USA). All mice were under general anesthesia and pupils were dilated applying topical tropicamide (1% SANDOZ). 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 were 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 pre- and post-treatment as a paired study.

Histology scoring: Eyes were enucleated, fixed in 4% glutaraldehyde for up to 1h and transferred to 10% formaldehyde for at least 24h, embedded, processed for H&E 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 immunoassays

For flow cytometry, the following anti-mouse antibodies (Abs) were used: RORγt-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 (Hatfield, UK), BD Biosciences (Oxford, UK) and BioLegend (London, UK). LTB4 receptors (BLT1) were identified with a primary unconjugated monoclonal antibody (EPR7113, Abcam), 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 prior to staining for intracellular molecules. For intracellular cytokine staining, the cells were restimulated with a cell stimulation cocktail (contains PMA and ionomycin) with the addition of protein transport inhibitor cocktail (brefeldin A; 1:500; eBioscience) for 4h.

Viable cells were identified using a Live/Dead fixable viability marker (Zombie, BioLegend, UK) 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 (ThermoFischer). Flow cytometric data were acquired using an LSR Fortessa (BD Biosciences, San Jose, CA) and BD FACSDiva version 6.1.3 software and data analysis performed using FlowJo (version 10.0.7).

Immunofluorescence microscopy

The FFPE blocks were sectioned (4 µm), dewaxed, retrieved in citrate buffer (10 mM, pH 6), permeabilized in 0.1% Triton X-100, blocked (5% BSA), and stained with primary Abs at room temperature for 1–2 h. Primary antibodies were rat anti-mouse CD4 mAb, rabbit anti-human CD4, rabbit anti-human/mouse BLT1 mAb, rabbit anti-human C5aR1 mAb, and rabbit anti-mouse C5aR1 mAb (all from Abcam) and anti-human/mouse BLT1 pAb (2654; Bioss, WA, USA) were 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 using ZEN 2.1 software (Carl Zeiss, Oberkochen, Germany).

ELISA for LTB4 and C5a

For the quantitative determination of LTB4 and C5a levels, immunoassays were performed. Approximately 5µl of vitreous fluids from each eye were transferred to 200µl tubes containing 50µl PBS. After gentle pipetting, the fluids were centrifuged for 5 min at 250g and supernatants transferred into new tubes and stored at -80^{o}C until assay. LTB4 (R&D

systems) and C5a (ThermoFisher) kits were used and assays performed according to the manufacturers' instructions.

Statistical analysis

Statistical analysis was done with GraphPad Prism v.6.0 (GraphPad Software) or Excel (Microsoft Office). Differences between groups were determined by Student *t* test (for mouse data) or one-way ANOVA (with a Tukey post hoc analysis) as appropriate for the experiment. The values $p < 0.05$ were considered significant.

Results

Targeting LTB4/C5 pathways attenuates EAU

EAU was induced in C57BL/6J mice[24, 27]. When signs of disease appeared (day 14-15), mice were treated intravitreally with equimolar amounts of nomacopan (5mg/ml in 1-2µl), PAS-nomacopan (20mg/ml), PAS-L-nomacopan (20mg/ml), dexamethasone (DEX, $1x10^{-3}$ M) or saline. Repeat injections of drugs or saline were administered 3 days after the first injection (day 18 post immunization) (schematic treatment; Supplemental Figure S1A) and disease progression was monitored by fundoscopy (before treatment and at harvesting) and scored based on clinical/pathological criteria such as optic disc and tissue inflammation, vessel cuffing and structural damage[30] as compared with a healthy eye. The scorer was masked to treatment. A moderate suppression of EAU was detected in the nomacopan treated group. A 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 1A, B and Supplemental Figure S1). Clinical disease suppression was confirmed by histopathological grading, where 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 (day 14-21) showed a 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, D) and $CD4^+$ T cells expressing transcription factors RORγt/T-bet (double positive Th1/17 cells; Supplemental Figure S1C, D), in C57BL/6J EAU mice, when treated with PAS-nomacopan or PAS-L-nomacopan (1-2 µl of each; 20mg/ml) and compared to 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 S2A and B) to detect $CD4^+$ T cells and inflammatory and resident macrophages, we demonstrated 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 to the saline treated mice (Figure 1E, F). However, we observed an increased proportion of resident cells in those sample shown however this was confirmed for all samples. 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 day 20-22 post-immunization 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 post-immunization could still suppress disease when mice were left to develop EAU over an extended time (26 days post-immunization). 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 is on days 13-15. 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' following intravitreal administration of nomacopan was detected (Figure 1H), accompanied by a reduction in percentages of effector $CD4^+$ Th17 and Th1/17 (RORγt/T-bet) cells (Supplemental Figure S3A-E).

Vitreous levels of LTB4/C5a at different stages of EAU

Fundoscopy was performed just prior to 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 2A-C). The levels of LTB4 and C5a were markedly increased in vitreoretinal fluids extracted from EAU eyes during disease progression compared to the CFA or naïve controls (Figure 2B and C). LTB4 levels correlated with disease progression in C57BL/6J mice from day 12 onwards (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 to 12 (50-500 pg/ml). In all experiments (collecting fluids from day 2 to 21) the levels of LTB4

were consistently increased compared to the CFA controls. The amount 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 post immunization (Figure 2B). However, in all experiments, the level of LTB4 increased significantly up to day 21 post EAU (Figure 2C). The amounts of LTB4 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 LTB4 levels in nomacopan-treated EAU mice were not determined by ELISA due to the effect of nomacopan which, by sequestering LTB4, could potentially masks its detection. This issue has also been a challenge in another study where nomacopan was applied in a mouse model of inflammatory skin disease. [23]

LTB4 recruits immune cells to the vitreoretinal space

To study LTB4 function within the posterior chamber, synthetic LTB4 (1-2 µl, 0.1 µg/ml) was injected into the vitreous in eyes from naïve mice. We titrated the synthetic LTB4 (100pg/ml, 1ng/ml and 1µg/ml) in naïve B10RIII mice and examined eyes by fundoscopy. The introduction of LTB4 into the vitreous induced focal inflammatory lesions as detected by fundoscopy (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 post injection at different time points (Supplemental Figure S4A, D). In contrast, immunoreactivity to the infiltrating macrophage/myeloid markers was increased after intravitreal injection of synthetic LTB4 (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 post treatment (Figure 3C).

LTB4R (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 LTB4 and C5a were expressed within retinal tissues.

Performing immunofluorescence staining on tissue sections from EAU eyes, to identify LTB4R (BLT1) and C5aR (C5aR1), showed a significant increase of infiltrating cells in the vitreous cavity expressing one or both receptors in EAU as compared to healthy eyes (Figure 4A, B). The levels of these receptors provided evidence for 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 post immunization) 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 were 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 4C-E).

Activated lymphocytes expressing LTB4 (BLT1) and C5a receptors

Increased levels of $BLT1^+$ and $C5aR1^+$ cells in dLNs of EAU were detected as compared to no-disease control dLNs. (Figure 5A, B). The relative levels of $BLT1^+$ cells and $C5aR1^+$ cells in dLNs were different in each EAU mouse (here showing two examples). 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 post immunization) were used. Single cell suspensions were treated *in vitro* with plate bound anti-CD3/CD28 (for 3 days), PHA (data not shown), PMA/ionomycin (PMA/I), $IRBP_{161-180}$ or synthetic LTB4 (1µM), to investigate autocrine activity, for 18h and assayed LTB4R expression on $CD4^+$T cell subsets. Under all stimulating conditions tested, with the exception of LTB4, we detected a marked increase in $CD4^+$ T cells expressing BLT1 relative to unstimulated controls (Figure 5C, D). By gating on those $CD4^+BLT1R^+$ cells, we found increases in both Th17 ($IL\text{-}17^+ROR\gamma t^+$) and Th1 cells ($IFN\gamma^+T\text{-}bet^+$) in response to IRBP relative to unstimulated controls (Figure 5E, F). Furthermore, analyzing $CD3^+CD4^-$ cells for BLT1 expression showed a population of $CD8^+$ cells which coexpressed BLT1 (Supplemental Figure S5A, 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 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 6B, 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. Whilst there were only a few cells expressing both receptors in the damaged retinal layers (Figure 6B and C), the majority of cells in the inflammatory foci expressed both BLT1 and C5aR1 (Figure 6D).

Discussion

In this study, we provide novel data demonstrating 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 upregulated upon activation. In addition, tissue-resident cells in human non-inflamed retinal tissues do not express the receptors but infiltrating leukocytes in uveitic 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 and there exists only a single marketed drug (Zileuton; 5-lipoxygenase inhibitor) for inhibition of LTB4 which was approved for treatment of steroid-resistant asthma. LTB4 receptor inhibitors such as CP105696 and U75302 used in laser-induced-AMD and LPS-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 fulfils 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 here we show 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 (on day 15 and 18) was studied and efficacy monitored at the disease peak (day 21) and at a later timepoint (day 26). Despite comparable levels of disease severity in EAU observed between peak and a later timepoint, 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, suggesting that inhibiting LTB4 at inflamed tissue sites could prevent the progression of EAU and also suppress the late stage disease complications, where structural damage is irreversible and leads to visual 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. It has already been shown that the C5 pathway is involved in late stage EAU.[36]

Previous reports have shown that LTB4 can promote the generation of Th17 cells, an effector $CD4^+$ T cell subset, through its specific transcription factor, RORγt, whilst suppressing the differentiation of Treg cells.[16, 37] The Treg 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 coexpressing 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 also differentiate into a regulatory phenotype, known as myeloid-derived suppressor cells (MDSC), which inhibit auto-

aggressive 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 since distinguishing them from resident microglia is unreliable because of 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 these EAU samples and so were not includes in the analysis. Therefore, by retinal flow cytometry, only resident and inflammatory macrophages could be identified in EAU and it was found that the level of inflammatory macrophages was reduced after treating mice intravitreally with PAS-L-nomacopan and PAS-nomacopan, while 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 $Ly\text{-}6C^{Lo}$ which are detected next to endothelial cells lining the blood vessels which are thought to be derived from $Ly6C^{hi}$ macrophages in response to signaling, including delta-like 1 (DLL1) and upon upregulation of transcription factor NR4A3.[43] Studies of traumatic muscle injury showed recruitment of $Ly6C^{hi}$ macrophages at early stages while in later stages those $Ly6C^{hi}$ macrophages switch into $Ly6C^{lo}$ 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 CNS.

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.

Since LTB4 has been shown to affect vascular permeability,[48] detecting LTB4 in the vitreoretinal space at such an early stage post immunization 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 post LTB4 injection, revealed a significant level (p=0.02) of activated macrophages recruited into the vitreoretinal space but 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) while there was also a population of $BLT1^+$ resident macrophages. We have shown that LTB4 functions as a recruiter for infiltrating macrophages but whether or not 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 upregulate 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 LPS response in C57BL/6J mice,[19] and microglia are known to be part of EAU disease progression. The presence of BLT1-expressing 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 this study a significant number of cells co-expressing both receptors in the eye were detected in contrast to dLN where 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 fundoscopy (day 7), very low numbers of infiltrating cells were found with some retinal $CD3^+$ and $CD11b^+$ cells expressing $BLT1^+$. Since 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 non-specifically via mitogen, induced $CD4^+$ T cell expression $BLT1^+$. LTB4 treatment itself did not upregulate receptor expression in our 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 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, demonstrate 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 TNFα on macrophage differentiation is well-established[32, 51] and the ability of LTB4 to induce TNFα expression[52 53] supports a connection between those pro-inflammatory molecules. It has been shown that the retina is protected when TNFα activity is neutralized

which suppresses macrophage activities during ocular inflammation.[54] According to the literature, there is no evidence of macrophage infiltration into the retina or uvea until days 11 to 12 post-immunization in EAU.[55, 56] However, a marked upregulation of inflammatory cytokines such as TNFα, iNOS and IL-1α in early stages of disease has been reported, and these cytokines could be generated by the innate immune response in the retina.[56, 57] Since macrophages are key producers of TNFα, anti-TNFα 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 upregulate TNFα 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. Whether or not resident microglia express BLT1 will be an objective of future studies.

Investigating expression of those receptors in human retinal tissue sections showed that in healthy retinal sections, very few tissue resident cells expressed BLT1 or C5aR1, while in uveitis retinal sections, $BLT1^+$ or $C5aR1^+$ cells were readily detected in the choroid, throughout the retinal layers and within the vitreous cavity. There were also cells which co-expressed both receptors, especially within inflammatory foci where these co-expressing cells were densely localised. Activation of both signaling pathways has been shown during neutrophil chemotaxis and in some arthritis animal models,[14,59] but no study has shown both receptors expressed by one individual cell to our knowledge. Unfortunately, it wasn't possible to obtain retinal tissue sections from donors with early stage disease, and therefore we don't know the effects of therapy on the BLT1/C5aR1 signaling pathways by retinal cells, which needs 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 (AMD), LTB4 promoted neovascularization and macrophage recruitment,[34] and a high level of LTB4 has been detected in a model of diabetic retinopathy where 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 adoptively 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.

There is a great need for further research in areas of inflammation, regulation under homeostatic conditions and understanding how inflammatory mediators such as LTB4 alter immune responses within the eye. This will allow for the development of customized immunomodulatory therapies. Here the efficacy of nomacopan in suppressing EAU disease and revealing the involvement of LTB4 in disease development/progression was demonstrated. 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, the basis of the drug efficacy was investigated. We showed both PAS-nomacopan and PAS-L-nomacopan suppressed inflammation in EAU, but since 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 timepoints selected for this study.

In summary, evidence is provided 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 potential to suppress intraocular inflammatory diseases.

Authorship contributions

ME performed, conducted and analyzed the experiments and wrote the manuscript. YC assisted in experimental studies. MN, WD contributed to the preclinical study designs and reviewed the manuscript. SEC reviewed the manuscript and provided her histopathological expertise and human materials. VLC initiated, supervised and conceived the project, reviewed the manuscript and secured funding for this study.

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Figure legends

Figure 1 Targeting LTB4 attenuates EAU progression in the mouse model of uveitis.

A: Representative fundus images of EAU treated with Nom, PAS-Nom, 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 DEX treatments, respectively on a scale from 0 to 5. **B:** Clinical score on individual eyes based on fundoscopy 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 nomacopan were applied (5mg/ml). Plots have been gated on live, single lymphocytes, and then with specific staining as follows: **C, D** for CD4 and IL-17 and gated for $CD4^+$ cells and then $IL\text{-}17^+$ cells. The summary graph of the data. **E, F:** Plots were gated for myeloid markers. The cells were gated as $CD45^+$ $Ly\text{-}6G^-$ $CD11b^+$ $CD11c^-$ $Ly\text{-}6c^{hi}$ for detecting infiltrating cells and - $Ly\text{-}6c^{lo}$ 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 intravitreally injections of Nom, PAS-Nom, PAS-L-Nom or saline, on days 15 and 18). The clinical criteria have been indicated as legends. Each bar was drawn based on the mean value $\pm$ SD of each score (n=6-8 mice). The preclinical data is representative from 4-6 independent experiments (n=6-8 mice per group). **H)** Graphical summary showing clinical score on day 11 (pre-treatment) and 13 (post treatment) from B10.RIII mice (n=3 in each group).

Masked scores, one-way ANOVA (clinical scores) and unpaired t test P values compared to vehicle. *P < 0.05, **P < 0.01, ***P < 0.001, **(A)** iO= inflamed optic disk, iV= inflamed vessel,

iR= inflamed retinal tissue, V= vitreous, Re= retina, C= choroid, L=lens, white arrow = infiltrating cells, black arrow= retinal folds. scale bar = 200µm **(E)** R=resident macrophage, I= infiltrating macrophage.

Figure 2 LTB4 and C5a levels in different stages of 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 ELISA assays detecting C5a **(B)** and LTB4 **(C)** levels in vitreous fluids. Data were compared to the amount of LTB4 and C5a in control mice (CFA alone) and healthy mice (with no immunization). Representative data from 4-5 independent experiments where vitreous fluids were collected at early stages of diseases and 3 independent experiments at late stages of disease (*n* = 3-6 mice per group. unpaired t test P values compared to vehicle, ±SD shown. **D:** data obtained from ELISA were plotted to clinical readouts (vessel cuffing and tissue infiltration scores) for any correlation and relationship between disease scores and LTB4 levels. $p>0.01$, $y=1.6846\ln(x)-1.3116$, $R^2 = 0.7042$. Dashed gray line indicates the trendline. *P < 0.05, **P < 0.01, ***P < 0.001, O= optic disk, V= vessel, iO= inflamed optic disk, iV= inflamed vessel, iR= inflamed retinal tissue, fiL= focal inflammatory lesion.

Figure 3 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 fundoscopy 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 post 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**: The graphical summary showing means ± SD from at least three independent experiments (*n*=3-6). unpaired t test P values compared to resident myeloid cells. *P < 0.05, **P < 0.01. O= optic disk, fiL= focal inflammatory lesion, R=resident macrophage, I= infiltrating macrophage.

Figure 4 Infiltrating immune cells in EAU express BLT1 and C5aR1.

A: Immunofluorescence staining for BLT1 (green), C5aR1 (red), and DAPI (blue) on eye sections obtained from a healthy eye and **B:** EAU B10RIII mice. V= vitreous, R= retina, S= sclera. Activated CD4$^+$ cells express BLT1. **C-E:** Isolated retinal immune cells obtained from primed B10RIII (day 7 post immunization) 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 whereas some CD4$^+$ cells and CD11b$^+$ cells are positive for BLT1 on day 7, these cells increased at the peak of the disease (Day 15). Green arrow (BLT1), red arrow (C5aR) and yellow arrow (BLT1 and C5aR). Scale bars (A) 100µm, (B, left panel) 20 µm, (B, right panel) 10 µm.

Figure 5 LTB4 and C5a receptors in inflamed lymph nodes.

A: Immunofluorescence staining for BLT1 (green), C5aR1 (red), and nucleus by DAPI (blue) on sections obtained from healthy, uninflamed lymph nodes from B10.RIII mice, Scale bar= 50µm **B:** compared to the inflamed dLNs of EAU mice. Yellow arrows indicate cells expressing both receptors **C:** Gating strategy for CD4 and BLT1. **D:** Lymphocytes from primed B10.RIII mice (7 days post-immunization) were isolated and activated overnight with different stimuli, here showing $IRBP_{161\text{-}180}$ and 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, RORγt, IFNγ and T-bet. Data were compared to the level of positive cells in unstimulated lymphocytes (**D**). $CD4^+$ cells detected as Th17 and Th1 cells where expressed RORγt/IL-17, IFNγ/T-bet respectively while Th1/17 cells were positive for T-Bet and RORγt expression in IRBP treated primed $CD4^+$ T cells.

Figure 6 BLT1 and C5aR1 expression in healthy human eyes and uveitis patients.

A: Immunofluorescence staining for BLT1 (green), C5aR1 (red), and nucleus by DAPI (blue) on FFPE sections from healthy eye donors and **B-D:** on uveitis patient sections. H&E staining included as a guide for retinal structure for immunofluorescence images. Scale bars: (A) 100µm, B,C (left panels), D(right panel) 50 µm, B,C, (right panels), D(left panel) 20 µm. Green, red and yellow arrows highlight BLT1, C5aR1 and double positive BLT1 and C5aR1 cells respectively. RPE= retinal pigment epithelium, V=vitreous, C=choroid, S=sclera and R=retina. Yellow box refers to the area which has been captured.

Figure 1.

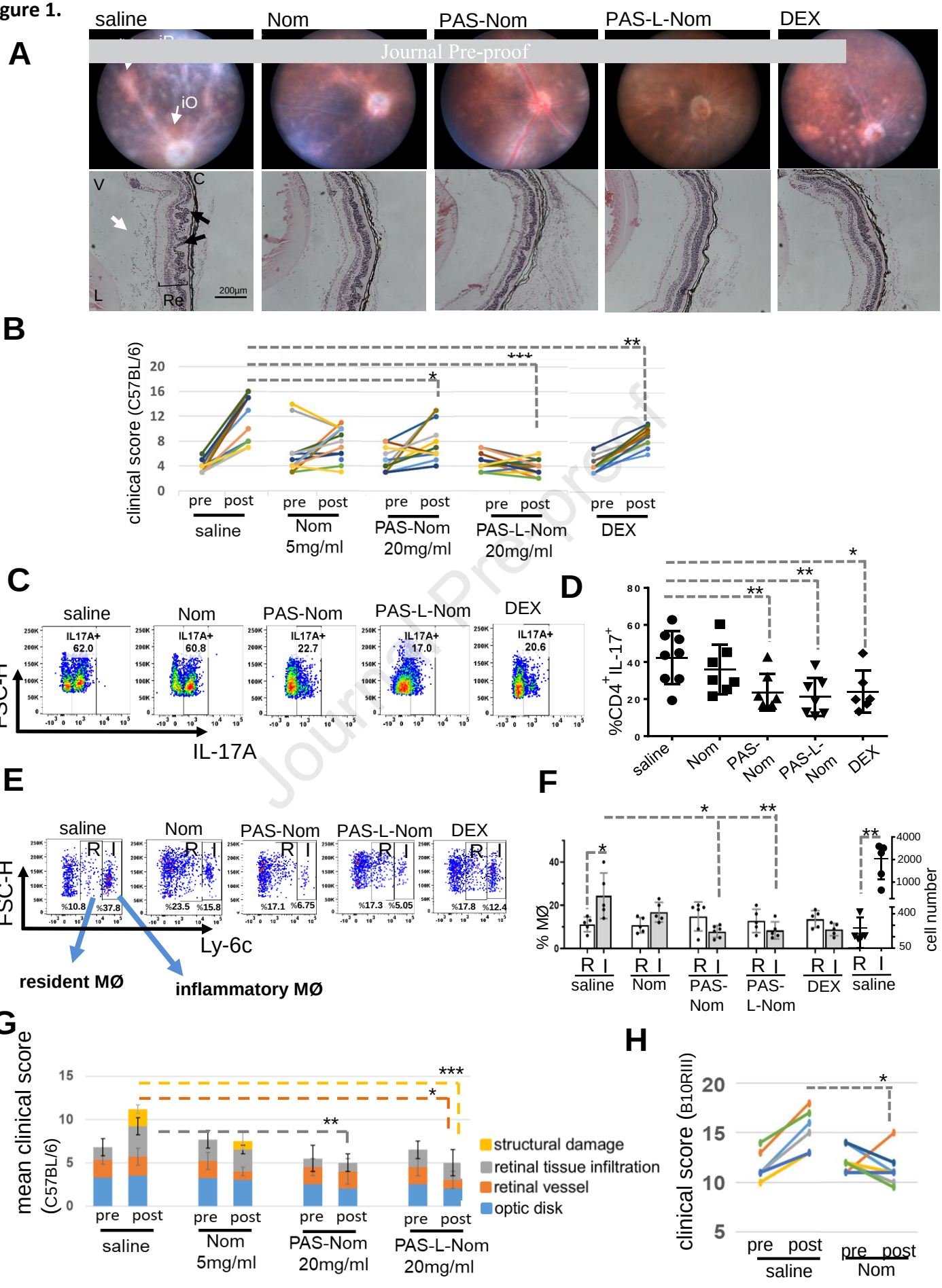


Figure 2.

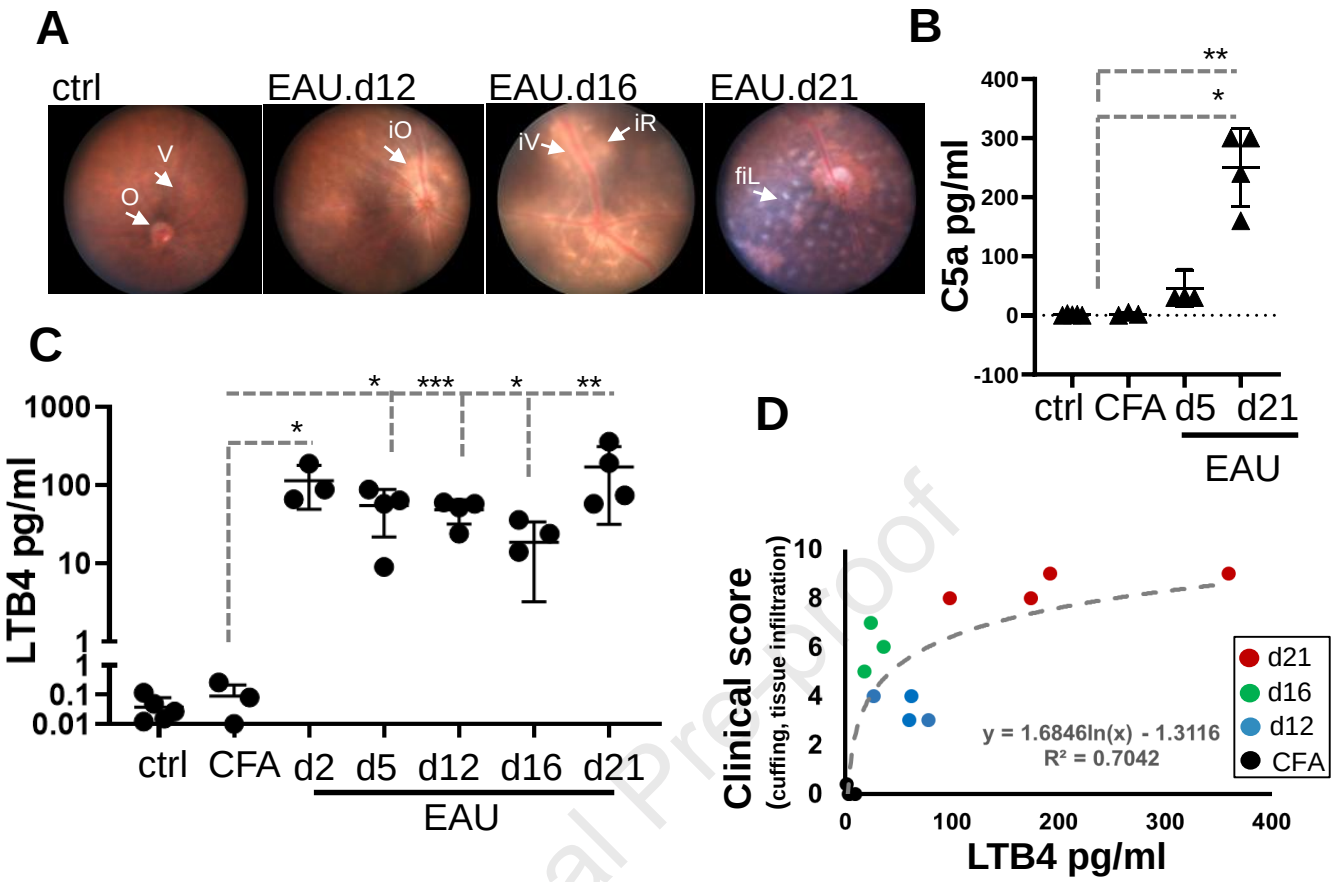
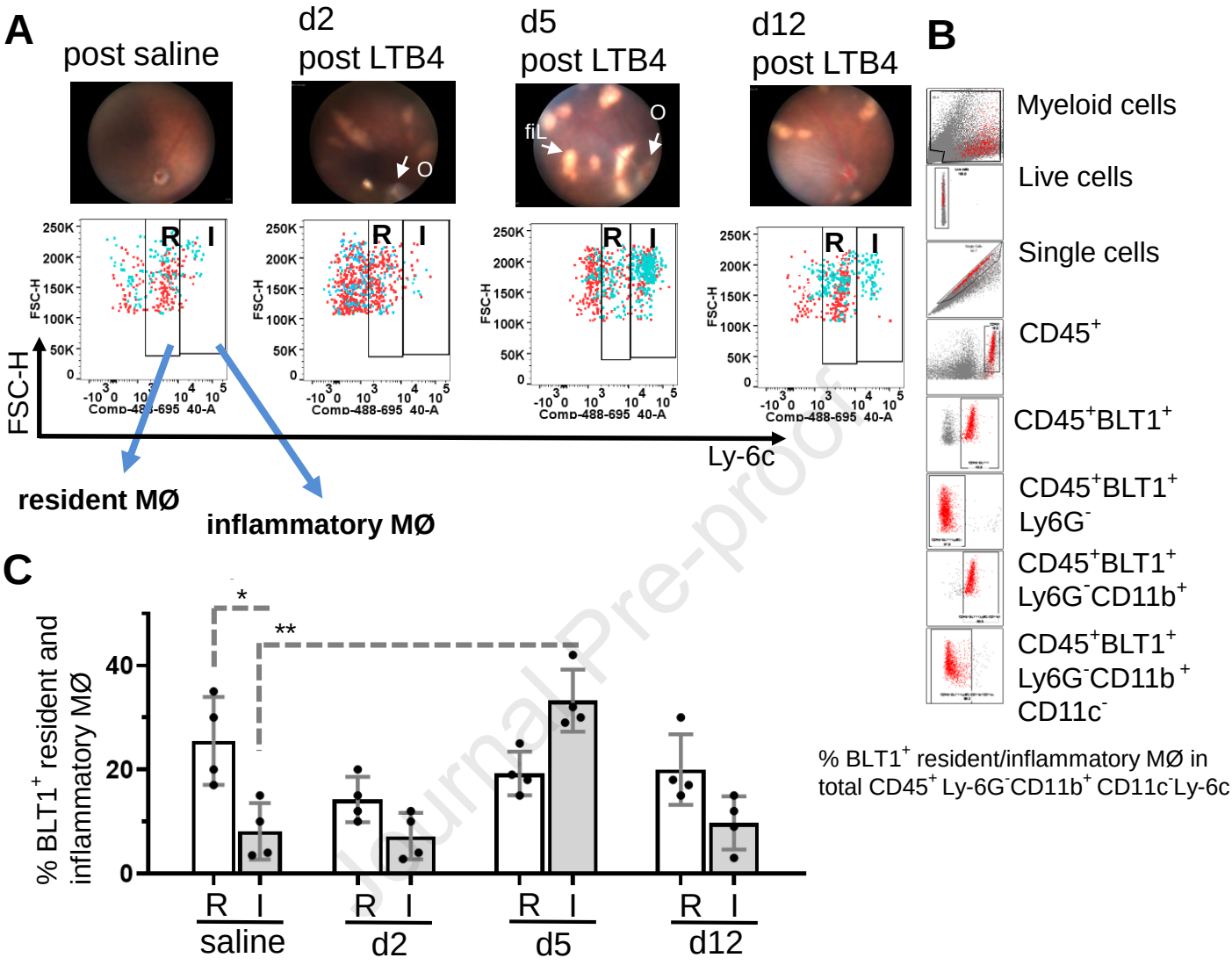
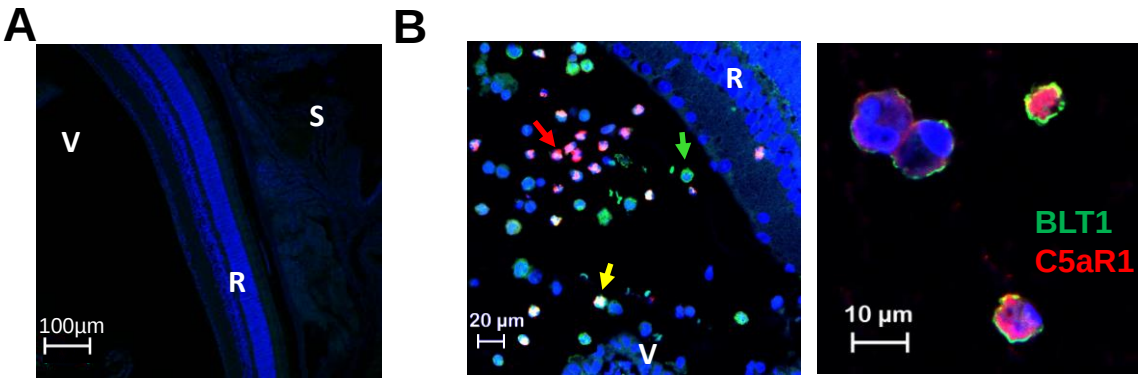


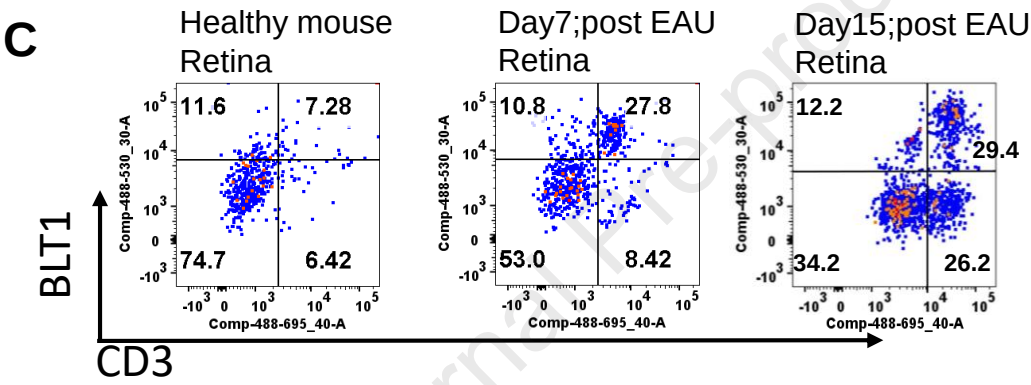
Figure 3.

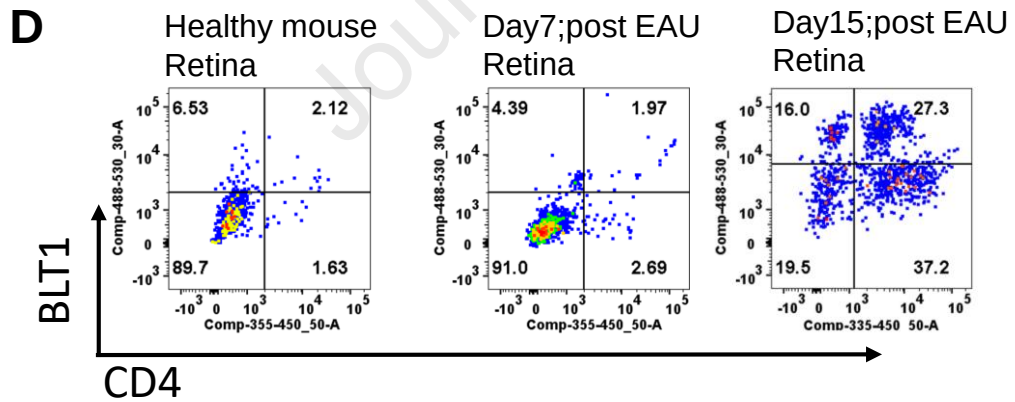


% $BLT1^+$ resident/inflammatory MØ in total $CD45^+$ $Ly\text{-}6G^-CD11b^+$ $CD11c^-Ly\text{-}6c$

Figure 4.







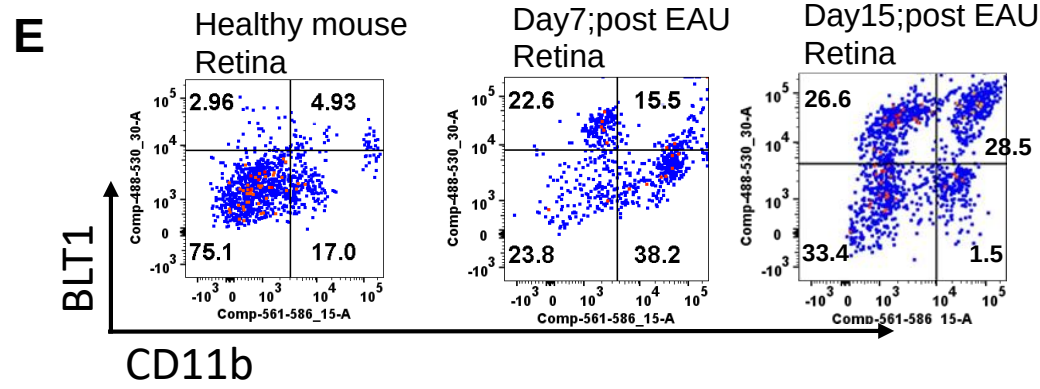


Figure 5.

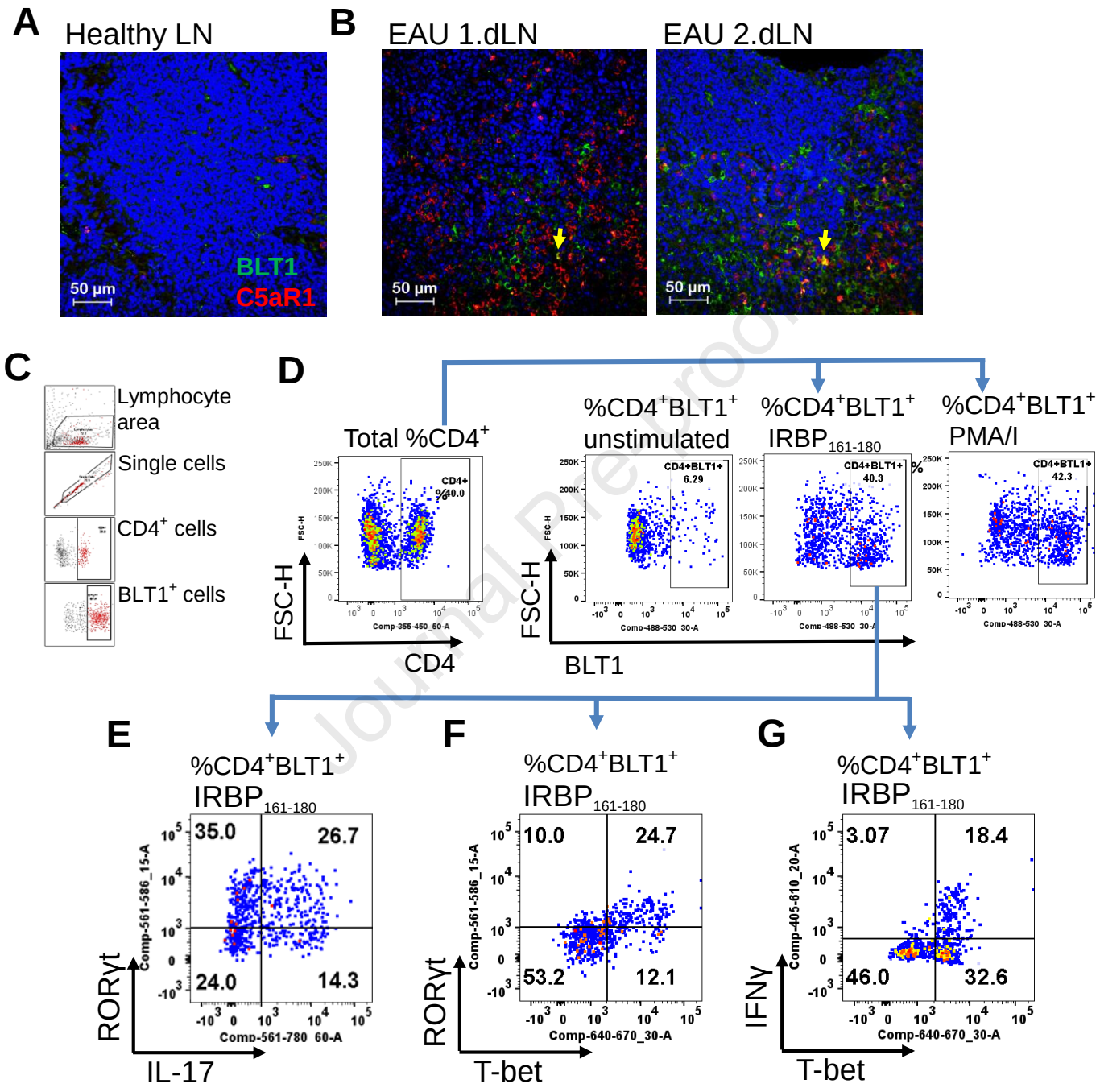


Figure 6.