

LETTER

Allergic eye disease: Blocking LTB4/C5 in vivo suppressed disease and Th2 & Th9 cells

To the Editor

Vernal keratoconjunctivitis (VKC) affects children and can impair vision if the cornea becomes involved. Immunosuppressives (steroids and cyclosporin A) are required but can have side effects, and novel immunotherapeutic approaches are needed.¹ The aim of this study was to investigate the contributions of LTB4 and complement C5 in a model of allergic eye disease (experimental allergy conjunctivitis, EAC).² Previous studies have demonstrated that nomacopan, a bifunctional recombinant biologic derived from blood-feeding ticks, has anti-inflammatory properties by capturing LTB4 and preventing it from interacting with its two known G protein-coupled cell surface receptors (GPCR BLT1 and BLT2). Simultaneously, nomacopan inhibited C5 end terminal complement activation, thereby preventing formation of C5b-9 and C5a.³ These two pathways have evolutionary connections as phylogenetic analysis showed that tick saliva started as an LTB4 inhibitor, and subsequently acquired the ability to inhibit C5. It has been shown in a mouse model of inflammatory arthritis that C5a production resulted in the release of LTB4 to promote further neutrophil migration to the interstitium.⁴

EAC is a model of allergic eye disease mainly driven by effector Th2 cells and mast cells (MC). During EAC, conjunctival inflammation can be detected after 5 days, scored non-invasively² (Figure S1A–C), with elevated levels of conjunctival IL-9-expressing CD4⁺T cells and MC detected in tissues and cells expanded from conjunctival explants (Figure S1D–J). Significantly increased levels of tryptase⁺ conjunctival MC were observed, co-expressing intracellular IL-9 in the sub-epithelial area within the fornix of conjunctival EAC tissues (Figure S1G).

In this study, we investigated the effects of topically administered nomacopan in EAC and observed a significant suppression of disease in treated mice, and a decrease in IL-9-expressing CD4⁺T cells (Figure 1A–C; Figure S2A). Recent studies have demonstrated that IL-9 can be produced by Th9 and Th2 cells.^{5,6} To determine which CD4⁺T cell subset was producing IL-9, transcription factor expression was investigated. IL-9-producing Th2 cells express GATA3, but not PU-1, whereas IL-9-producing Th9 cells express PU-1, but not GATA3. Within the infiltrating CD4⁺T cells in EAC, Th9 cells (IL-9⁺PU.1⁺) and IL-9-expressing Th2 cells (GATA3⁺IL-4⁺IL-9⁺) cells were increased in OVA-challenged mice compared with controls (Figure S1H–J).

During early stages of EAC (Days 1–6), no effect by nomacopan was observed due to low inflammatory scores overall. However, at days 7 and 10, a significant decrease in IL-9⁺CD4⁺T cells (Figure 1C–F) following nomacopan treatment was observed. The levels of Th2 (IL-4⁺ PU.1⁺ GATA3⁺) cells and IL-9-expressing Th2 cells were also significantly reduced (Figure S2B–D). Interestingly, although there was an overall reduction in IL-9 expression levels, there were significant changes in Th9 (IL-9⁺PU.1⁺IL-4⁺) levels only at the higher concentration of nomacopan (Figure 1D).

This *in vivo* model shares many features with VKC in man since there is a predominant CD4⁺T cell infiltration of the conjunctival tissues and evidence of fibrosis.² Hence, we used VKC tissue specimens and tear fluids to compare with EAC tissues. Tears collected from VKC patients during active disease showed a significantly higher level of IL-9 (clinically uncontrolled) as compared with inactive VKC (controlled; Figure 2A). VKC conjunctival tissue sections were examined for expression of CD4, IL-9, IL-9 receptor (IL-9R), and tryptase (Figure 2B–E). IL-9-expressing CD4⁺T cells and MC were both significantly up-regulated in all active VKC specimens (*n* = 7) as compared with controls. We observed CD4⁺T cells co-localized with IL-9 in the sub-epithelial and stromal areas of VKC sections (Figure 2B) and IL-9R expression (Figure 2C). Infiltrating MC (Tryptase⁺) and other cell types also expressed IL-9R (Figure 2D) within stromal areas, with the frequency of IL-9R-expressing MC significantly higher than IL-9R-expressing CD4⁺T cells (Figure 2E).

To further investigate whether the receptors for those ligands targeted by nomacopan were expressed during EAC, conjunctival tissue expression and localization of C5aR and BLT1 (LTB4 receptors) were investigated (Figure S2E) as well as in VKC and healthy tissue controls (Figure 2F,G). Relative expression of BLT1 and C5aR was analysed by counting positively stained cells in at least 5 non-overlapping areas (ImageJ). Interestingly, increases in infiltrating immune cells within the stromal areas correlated with increased expression of BLT1 (means ± SD; 34.98 ± 15) and C5aR (56.67 ± 12) in EAC, and comparable levels were also observed in VKC: BLT1 (40.48 ± 10); C5aR (44.90 ± 20). Very few cells co-expressed both receptors while only rarely were any BLT1⁺ or C5aR⁺ cells detected in healthy mouse and human tissues, suggesting that these receptors are exclusively expressed during disease, potentially by infiltrating CD4⁺T cells and MC.

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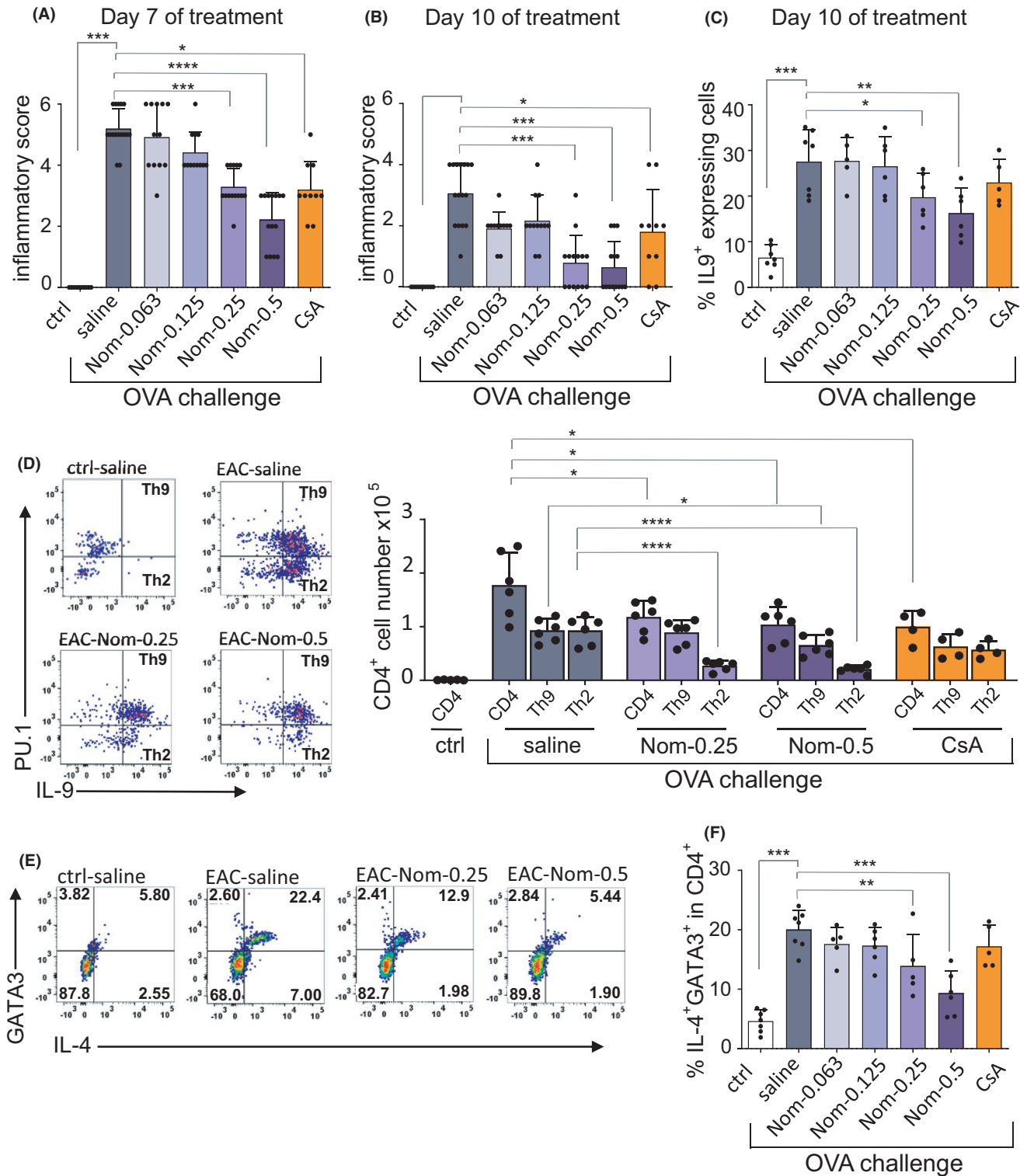


FIGURE 1 Topical nomacopan attenuated conjunctivitis in EAC and decreased IL-9 in CD4⁺T cells. (A, B) Clinical scores of eyes were compared with saline-treated controls. (C–F) Conjunctival explant cells stained for T-cell markers. Flow cytometry plots represent staining for: (C) IL-9, (D) IL-9 and PU.1 (Th9 cells) and a summary graph of percentages for IL-9⁺PU.1⁺ (Th9) or IL-9⁺PU.1⁺ (Th2) cells. (E, F) Representative plots were gated for Th2 cells (IL-4⁺GATA3⁺PU.1⁺) in treatment groups and a summary graph. Four independent experiments, ANOVA and unpaired *t*-tests; mean ± SD (*n* = 6–8 mice per group). **p* < .05, ***p* < .01. Nom = nomacopan, CsA = Cyclosporine A

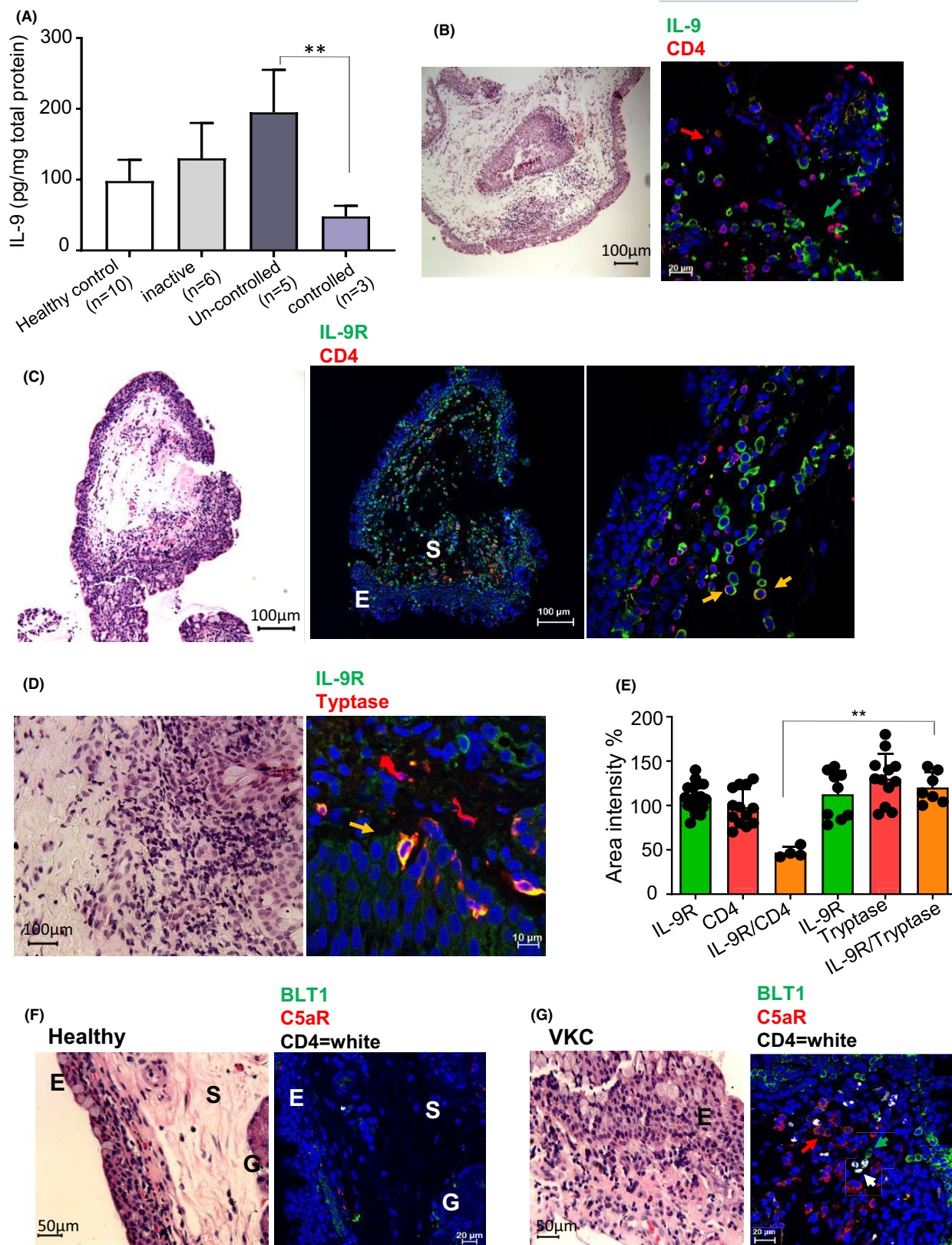


FIGURE 2 (A) IL-9 levels in tear fluids, demonstrating an increase in clinically uncontrolled (i.e. active) VKC as compared with those with clinically controlled disease; (B) Histology/immunofluorescence staining of VKC conjunctival sections for CD4 and IL-9; (C) CD4 and IL-9R; (D) Tryptase and IL-9R. (E) Scatterplots based on immunoreactivity and intensity for IL-9R, tryptase or IL-9R and CD4 as percentages of the immunostained areas. Representative summary graph of one of 7 biopsies. Values were added from 3 fields. (F, G) Representative staining for C5aR1, BLT1, CD4 on healthy, 1 of 3 (F) and VKC 1 of 7 (G) conjunctival tissues. Mean $\pm$ SD, ANOVA, ** p < .01, *** p < .001. E = epithelial layer; S = stroma; G = gland (Lacrimal)

We conclude that IL-9 was up-regulated during VKC and EAC. Nomacopan significantly suppressed EAC severity, accompanied by a decrease in IL-9-producing Th2 cells, Th2 cells and to a lesser extent, Th9 cells, suggesting a key pro-inflammatory role for IL-9-secreting CD4⁺T cells in allergic eye disease.

Our findings support nomacopan as a potential treatment for allergic eye disease due to its ability to down-regulate LTB4/C5 pathways in EAC.

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CONFLICT OF INTEREST

No authors have any Conflicts of Interest with the exception of Drs. Nunn and Weston-Davies, who are both employees of Akari Therapeutics Inc.

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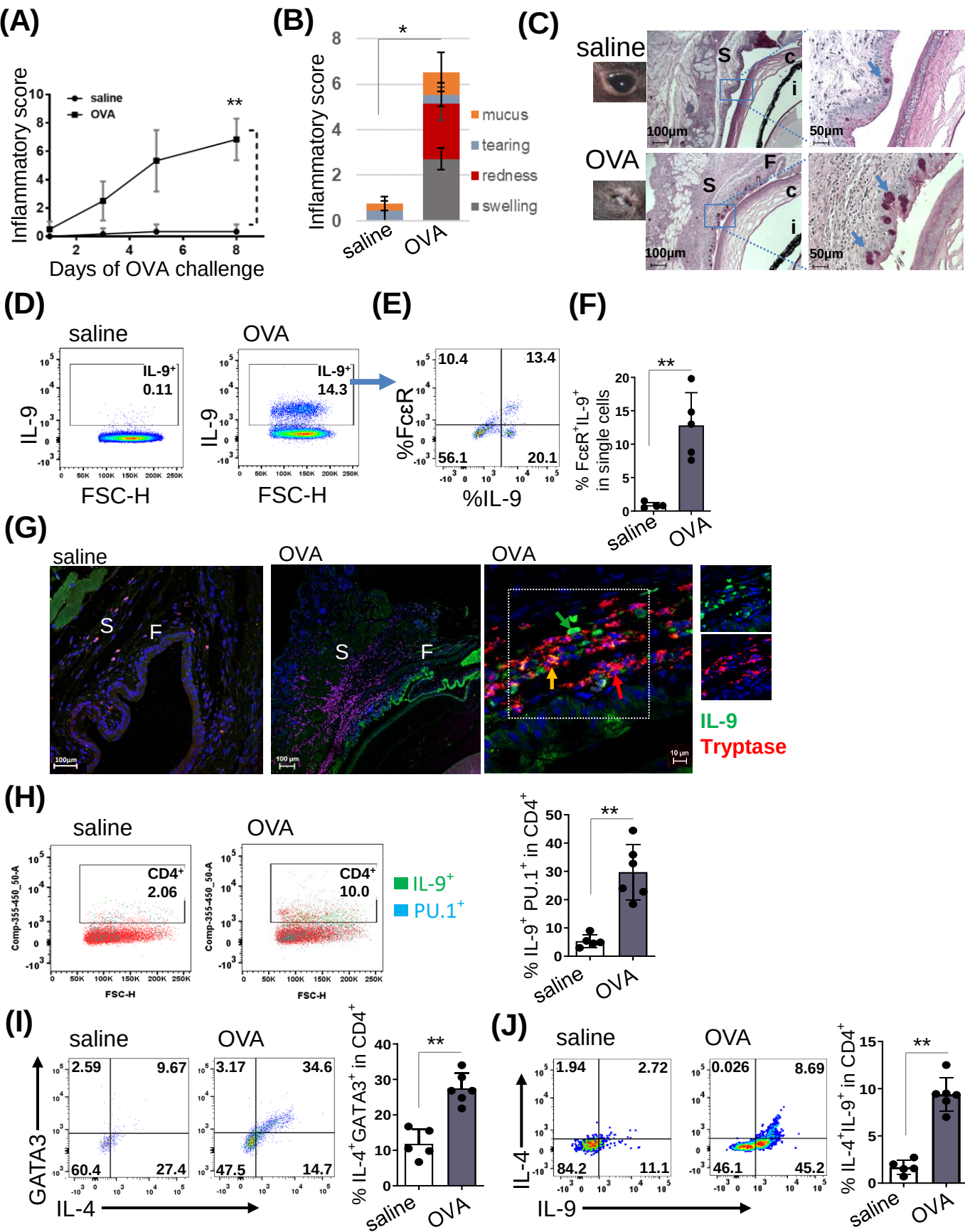
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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Supplemental figure 1.

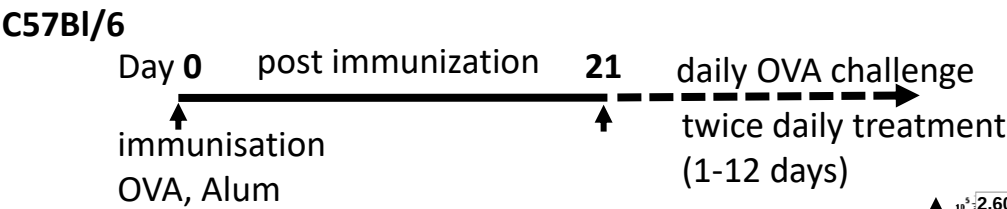


Supplemental Figure 1

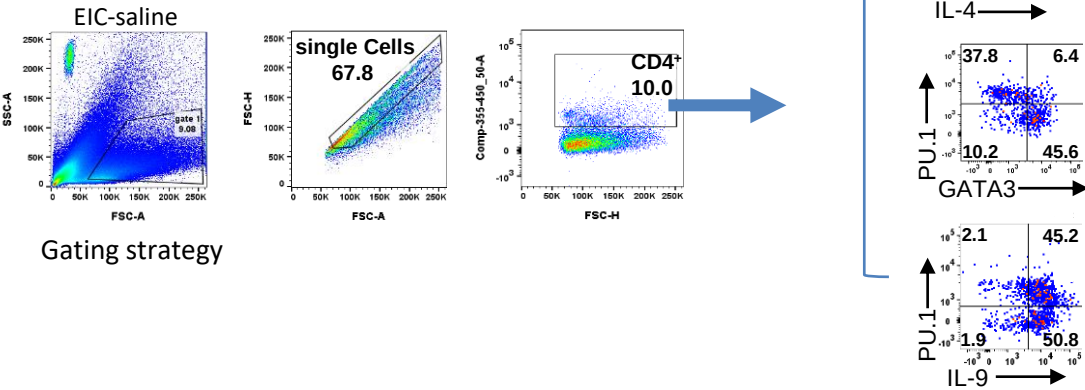
(A) Clinical scores in EAC. (B) Scoring of mice challenged with OVA (EAC) vs. saline. (C) Representative of saline control (upper) and EAC (lower) in parallel with Periodic acid–Schiff (PAS) staining. Arrows indicate PAS-stained goblet cells. (D-E) Conjunctival explant cells from EAC or controls, stained for IL-9 and FcεR (indicating MC). (F) Summary graph. (G) Immunofluorescence staining for IL-9-expressing MC in fornix (F). (H) EAC conjunctival cells stained for CD4 and overlaid for IL-9 and PU.1 staining. (I, J) Conjunctival explant cells gated for CD4 with IL-4 and GATA3 (I) or IL-4 and IL-9 (J). Mean±SD (n=5-6 mice, data points from two eyes). *P<0.01, **P<0.001. 3-4 independent experiments. S= stroma; C= cornea.

Supplemental Figure 2.

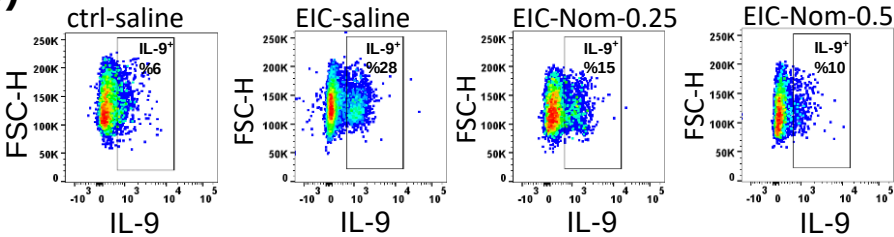
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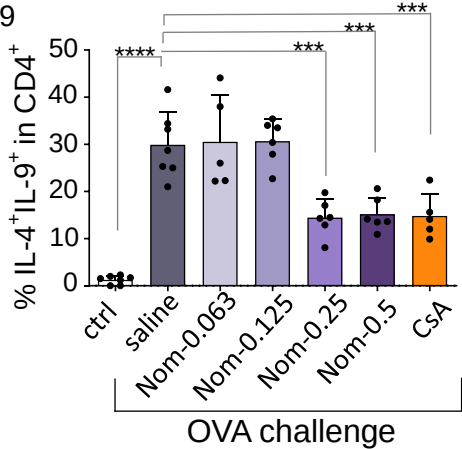
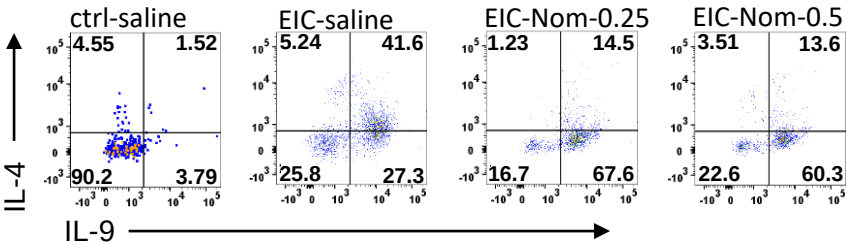
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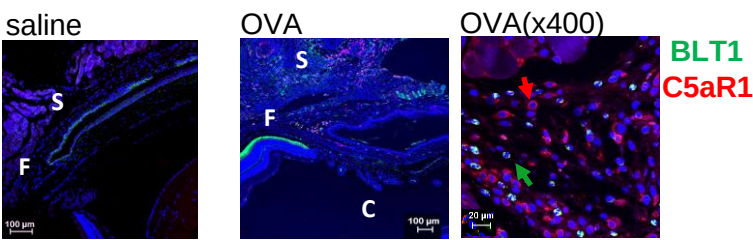
(C)



(D)



(E)



Supplemental Figure 2.

(A) Schematic diagram of disease model. (B) Gating strategy for CD4⁺ T cell subsets from conjunctival explant for CD4, GATA3, IL-4, PU.1 and IL-9. (C-D) EAC conjunctival explant cells were stained for T cell markers from EAC mice. (C) Stained for IL-9 and (D) Gated for IL-4 and IL-9 and the corresponding summary graph. Mean $\pm$ SD, (n=6-8 mice). *P<0.01,**P<0.001 from 4 independent experiments. (E) Representative immunofluorescence staining for BLT1 and C5aR in EAC indicating accumulation of positive cells in fornix and stromal areas (n=6 control, n=8 EAC). markers F= fornix; C=ciliary body; S=stroma.

Supplemental data

Experimental Allergy Conjunctivitis (EAC) mouse model

We used a clinically severe model of EAC where female C57Bl/6 mice (aged 6-8 weeks; Charles River) were immunized as previously described, with a 100 μ L suspension containing 1mg of OVA with 1mg Imject alum and 300ng pertussis toxin (all Sigma-Aldrich). From day 21, mice were challenged once daily with OVA (250 μ g/5 μ l) applied topically to both eyes for 12 days and also treated topically (twice daily) for up to 12 days. Topical challenge with saline was applied as a vehicle control.

Disease progression was clinically scored daily during the 12 days of challenge and treatments based on a scoring which evaluate the degree of conjunctival inflammation (lid swelling, redness, tearing and mucus deposition). Each parameter was graded on a scale range from 0 (absent) to 3+ (maximal) by two independent masked observers. All procedures were performed in accordance with the revised Animals (Scientific Procedures) Act (1986) and the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and in accordance with UK Home Office Licences.

Human conjunctival tissue sections

Fresh conjunctival biopsies were donated from patients with active conjunctival allergy associated in all cases with corneal macroerosion (n=7; males; age 9-16 years) after obtaining informed parental consent. The study was approved by the Westminster NRES Committee. London- (HEALTH RESEARCH AUTHORITY) the Research Ethics Service. Normal, uninflamed conjunctival tissues were provided via the Moorfields Eye Hospital Biobank, from consenting donors (n=3, males; age: 40-65 years). Prior ethics approval was obtained.