The Ocular Surface 23 (2022) 131–139

Contents lists available at ScienceDirect

The Ocular Surface

journal homepage: www.elsevier.com/locate/jtos

Topical application of cannabinoid-ligands ameliorates experimental dry-eye disease

Bao N. Tran [a], Martina Maass [a], Gwen Musial [a], Michael E. Stern [a,b], Uta Gehlsen [a,1], Philipp Steven [a,*,1]

[a] Division of Dry-Eye and Ocular GVHD, Department of Ophthalmology, Faculty of Medicine and University Hospital Cologne, University of Cologne, Cologne, Germany
[b] ImmunEyez LLC, Irvine, CA, USA

ARTICLE INFO

Keywords:
Dry eye disease
Cannabinoid receptor
Cannabinoids
THC
Cannabinoid selective antagonist
Gene expression
Inflammation
Corneal nerve morphology

ABSTRACT

Purpose: Dry eye disease (DED) is a multifactorial disease, with limitations regarding efficacy and tolerability of applied substances. Among several candidates, the endocannabinoid system with its receptors (CB1R and CB2R) were reported to modulate inflammation, wound healing and pain, which are also core DED pathomechanisms. This study is to investigate the therapeutic responses of Δ-9 tetrahydrocannabinol (a non-selective agonist) and two selective antagonists, SR141716A (CB1R antagonist) and SR144528 (CB2R antagonist), as a topical application using a DED mouse model.

Method: Experimental DED was induced in naïve C57BL/6 mice. Expression of CBR at the ocular surface of naïve and DED mice was determined by qPCR and in-situ hybridization. Either THC or CBR antagonists were compounded in an aqueous solution and dosed during the induction of DED. Tear production, cornea sensitivity, and cornea fluorescence staining were tested. At the end of each experiment, corneas were stained with β3-tubulin for analysis of corneal nerve morphology. Conjunctiva was analyzed for $CD4^+$ and $CD8^+$ infiltration.

Results: CB1R and CB2R are present at the ocular surface, and desiccating stress increased CBR expressions ($p < 0.05$). After 10 days of DED induction, treated groups demonstrated a reduced CBR expression in the cornea, which was concurrent with improvements in the DED phenotype including fluorescence staining & inflammation. Applying THC protected corneal nerve morphology, thus maintained corneal sensitivity and reduced $CD4^+$ T-cell infiltration. The CB1R antagonist maintained cornea sensitivity without changing nerve morphology.

Conclusions: Endocannabinoid receptor modulation presents a potential multi-functional therapeutic approach for DED.

1. Introduction

Dry eye disease (DED), a high-prevalence and multifactorial disease [1], consists of complex interplay among pathomechanisms such as tear film instability, evaporation, dysfunction of meibomian glands, secretion of pro-inflammatory mediators (such as TNF-α, IL-1β, or IL-17), damage of epithelial cells, and attenuation of corneal nerve function [2]. These pathomechanisms cause dysfunction of the lacrimal functional unit, in which, further external and internal triggers such as environmental stress, infections, surgery, or drugs exacerbate the disease [3]. DED therapy aims at restoring a normal ocular supportive tear composition, volume and stability, reducing inflammation, restoring meibum secretion and improving impaired wound healing. Topical therapeutic regimens typically consist of anti-inflammatory formulations and artificial tears (no active compound). In addition, mechanical warming of the lid margins (meibomian glands), topical autologous serum and vaulted contact lenses can also be utilized [2,4]. Although multiple therapeutic options are available to physicians and patients, treatment failures are common. Besides the limited efficacy of selected drugs and procedures, the most common cause of these failures is lack of compliance related to overload of different products and occurring side-effects [2,4,5].

Therefore, there is a demand for new therapies with higher efficacy, ideally targeting multiple pathomechanisms in the DED vicious circle simultaneously to reduce the number of different products. Recently, the cannabinoid system (ECS) has been shown to have potential therapeutic

* Corresponding author.
E-mail address: philipp.steven@uk-koeln.de (P. Steven).
[1] The authors contributed equally.

https://doi.org/10.1016/j.jtos.2021.12.008
Received 20 July 2021; Received in revised form 30 November 2021; Accepted 13 December 2021
Available online 16 December 2021

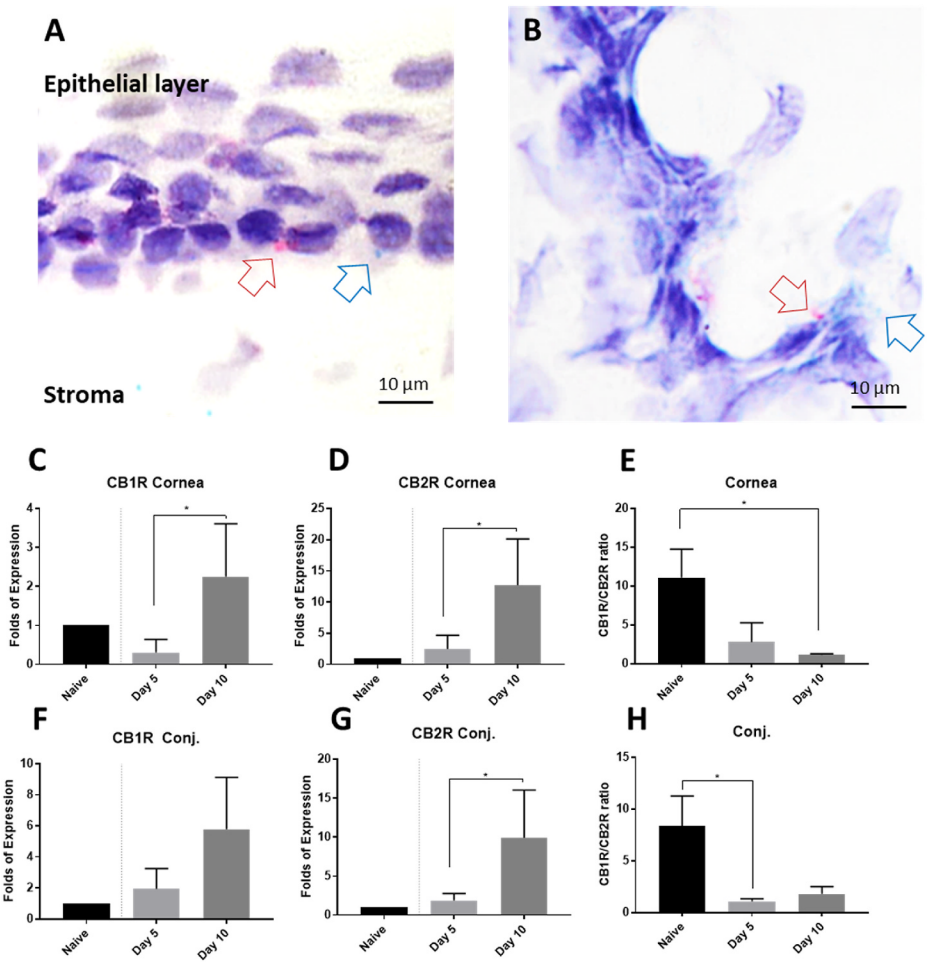

Fig. 1. Expression of CB1R and CB2R in ocular tissue in naïve and DED mice: In-situ hybridization confirmed the expression of CB1R (blue) and CB2R (red) in the cornea (A) and conjunctiva (B). RT-qPCR results of the expression of CB1R (C and F) and CB2R (D and G) in cornea and conjunctiva (Conj.) during 10 days of DS, the control group (naïve) is separated with a vertical line. CB1/CB2 ratios significantly decreased in the cornea (E) and conjunctiva (H) during 10 days of DS. *: $p < 0.05$.

effects on neurotransmission [6], inflammatory symptoms [7], and epithelial injures [8] in the ocular surface and related tissues. ECS is a biological system including cannabinoid receptors (CBRs), endogenous ligands, and relating enzymes. CBRs which are ubiquitously present in the human body and involved in various physiological processes such as pain regulation, inflammation reduction, and wound-healing improvement [9,10].

The cannabinoid receptor 1 (CB1R) is expressed on axons and synaptic terminals of sensory neurons [11,12]. Activation of CB1R decreases the release of neurotransmitters such as GABA [13], noradrenaline [14], substance P, and other mediators [15], which then prevents neuro-toxicity and neuronal damage [13,16]. CB1R-related pathways were described to be involved with maintaining neuroprotection, synaptic integrity, and plasticity in central nervous system or retina [16,17]. Besides, CB2R is present on various immune cells such as macrophages, dendritic cells, and T-cells [18]. Additionally, changes in the local milieu of inflammatory mediators regulate CB2R expression [19]. Activating CB2R leads to inhibiting these immune cells' activities and reducing production of IFN-γ, TNFα, and other cytokines [18,20]. Apart from potential therapeutic effects on pain and inflammation, evidence for the role of cannabinoids in corneal epithelium injuries and re-epithelialization was published [8,21].

This study was set up to analyze the role of the ECS in DED and to investigate the topical use of cannabinoid ligands as a potential multifunctional therapy. Using a DED mouse model, we tested the hypotheses that (i) CB1R and CB2R expression is altered in experimental dry-eye disease and (ii) the *in-vivo* use of cannabinoid ligands (formulated as eye drops) would attenuate the dry-eye phenotypes of the model used.

2. Materials & methods

2.1. Desiccating stress (DS) mouse model (DED)

All experiments were performed using C57BL/6 N mice (female, 8–12 weeks, Charles River Laboratories, Germany), mice were housing in the normal conditions for 2 weeks before experiments. Husbandry and all experimental procedures followed approved protocols based on the State Agency for Nature, Environment, and Consumer Protection of the State of North Rhine-Westphalia, Germany (LANUV).

Experimental dry-eye was induced as published previously with minor modifications [22–24].

Specifically, each mouse received scopolamine hydrobromide (5 mg/kg/day) through an implanted osmotic pump (Model 1002, Alzet, CA, USA), and were placed under desiccating conditions for 14 consecutive days (humidity: 25 ± 5%, temperature: 25 ± 5 °C, constant airflow for 19 h/day, following regulations of the animal ethics committee).

For substance testing, mice (n = 5/each) were treated with either drug formulation of Δ9-tetrahydrocannabinol (THC, non-specific agonist), SR-141716A (CB1R selective antagonist), or SR144528 (CB2R selective antagonist) (3 times/d, 5 µl/eye). Each experiment was conducted with one group of the carrier, and another group of mice served as untreated DED control. Two independent sets of experiments

were performed for each formulation, data was presented from a representative set of experiment, 5 mice (10 eyes) per group. Mice were screened for a dry eye phenotype before DED-induction (baseline) (average of FL score of in each mouse is up to 1). If a group contained one mouse with one eye of FL grade 2, this was defined as being acceptable to meet 3R-principles (Replacement, Reduction, Refinement). Mice with a significant higher baseline or signs of ocular surface disease (both eyes FL grade 2 or higher) were excluded. All readouts were performed in a blinded fashion.

To quantify corneal epitheliopathy, 3 μL of 1% fluorescein sodium (Fluorescein Alcon, TX, USA) was topically applied onto mouse eyes, then the eye was rinsed once with sodium chloride before measurement. Corneal staining was observed using a binomicroscope and a cobalt blue light source (Illuminator Intensilight C-HGFI, Nikon, Tokyo, Japan). Fluorescein score (FL score) was graded (from 0 to 5) using a modified Oxford scheme on both eyes of each mouse [25]. Representative images of FL staining are provided as suppl Figure 1. Data was presented for 5 mice (10 eyes) per group.

To measure tear production (TP), a cotton phenol red thread (Zone Quick, Sigma Pharmaceuticals, IA, USA) was placed into the lateral canthus for 10 s. Wetting of the phenol red thread was measured in mm using a ruler. Phenotyping was performed 12 h after last topical application of any eye drop to prevent interference.

To measure corneal mechanical sensitivity (sensitivity), serialized filaments with different thicknesses (von Frey filament, Bioseb, Chaville, France) were carefully applied to the central corneal without anesthesia. Blinking response was noted as a positive mechanical threshold. Corneal sensitivity was tested according to the "simplified up-down" method published by Bonin [26,27] (For details, see supplement).

2.2. Drug formulation

A license for handling Tetrahydrocannabinol was obtained from the Federal Institute for Drugs and Medical Devices (Bundesopiumstelle des Bundesinstituts für Arzneimittel und Medizinprodukte, Lincence Nr: 463 1128). Δ9-tetrahydrocannabinol (THC) was purchased from Bionorica SE, Neumark, Germany. THC was weighed and processed according to the guidance of the producer. Two antagonists, SR-141716A (Anta CB1) for CB1R and SR144528 (Anta CB2) for CB2R, Cremophore EL, and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich, Darmstadt, Germany.

Briefly, cannabinoid substances were dissolved in DMSO to form a stock solution (20 mg/mL). Then the stock solution was diluted with DMSO, Cremophore and isotonic sterile saline to get required concentrations. Carrier for THC was Cremophore EL (10%) and DMSO (15%) in isotonic sterile saline. Carrier for Anta CB1 or Anta CB2 was Cremophor EL (5%) and DMSO (7.5%) in isotonic sterile saline (0.9%).

2.3. Immunohistochemistry

Corneal nerve fiber staining and quantification: Whole corneas were fixed with paraformaldehyde (4% in PBS) for 30 min and incubated with rabbit anti-mouse β3-tubulin (dilution 1:1000) (ab18207, Abcam, Cambridge, UK) at 4 °C overnight followed by a second antibody (diluted 1:150 in PBS), goat anti-rabbit IgG (HL, Alexa Fluor 488, A11034, Thermo Fisher, CA, USA), for 60 min at room temperature, protected from light [28]. Images from the central cornea (830 × 590 μm) were taken by using a BX53 Olympus (Hamburg, Germany) microscope and then analyzed using a semiautomatic algorithm established using the freeware imaging program MeVisLab (version 2.8.1., Germany). For this, fluorescein images were loaded into Mevislab. Depending on image quality (brightness, sharpness, and fluorescein signals), up to 10 seed points and Region growing parameters were manually set. Area covered by nerve were detected by the software (binary images) with excluding the background [29,30]. Then the binary images were skeletonized (1pixel width). The axon length per area (mm/mm^2) was quantified from the skeletonized images. Experiment was conducted with 5 mice per group, in which, both eyes of each animal were used (n = 10).

Conjunctival $CD4^+$ or $CD8^+$ cell staining: Mouse eyes with attached lids (n = 4/group) were excised and flash frozen in liquid nitrogen. Tissue cryosections (8 μm) were fixed with acetone (−20 °C, 10 min), and then stained with the primary antibody at dilution 1:150, CD4 Monoclonal Antibody (GK1.5, 14-0041-85, eBioscience, Waltham, MA, USA) or CD8a Monoclonal Antibody (14-0081-82, eBioscience). The secondary antibody staining, Goat anti-Rat IgG (HL, Alexa Fluor 555, A21434, eBioscience, Waltham, MA, USA) was performed at room temperature for 1 h (1:150 dilution), and followed by nuclear counterstaining with Hoechst (Sigma- Aldrich, St. Louis, MO, USA). Two sections from each eye were examined and photographed with the BX53 Olympus (Hamburg, Germany) microscope. $CD4^+$ or $CD8^+$ density was measured as numbers per 100 μm in the conjunctival epithelium.

2.4. RNA extraction, reverse transcription, and quantitative real-time PCR

Entire cornea and conjunctival tissues (from 2 eyes) are added to a tube containing the lysis buffer, and snap frozen in liquid nitrogen. QIAGEN RNeasy Plus Mini Kit (Venlo, Netherland) was used according to the manufacturer's instructions to isolate RNA. RNA concentration was measured using NanoDrop 2000 (Thermo Fisher, DE, USA). First-strand cDNA was synthesized from 400 ng of total RNA with Thermo Scientific RevertAid Synthesis Kit (DE, USA). Real-time PCR (qPCR) was performed by using SsoFast EvaGreen Supermix (Biorad Laboratories, CA, USA). The primers for qPCR amplification of CB1R and CB2R were designed by Primer 3 [31,32]. Primer pair for CB1R were TTGCTCAGACATCTTCCCACTC and CTGTGAGCCTTCCAGAGAATGT (1369–1488), and the primer pair for CB2R were GTGAAGACAAGGGACCTGTTCT and AGGATGAAGCAGGAACCAGAAG (1696–1852). The HPRT gene was used as an endogenous reference for each qPCR run. Gene expression of mRNA of CB1R and CB2R were analyzed by the comparative CT method ($2^{-\Delta\Delta Ct}$). Each 96-well reaction plate contained the following groups: DED untreated group, 3 groups treated with different drug formulation, and the carrier-treated group (n = 5 sample per group). The results were normalized by the Ct of the reference gene, and the mean Ct of mRNA level in the control group was used as the calibrator [33,34].

Real-time qPCR was also performed to detect the expression of IL-1β in the cornea, IL-1β and IFN-γ in the conjunctiva. Primer pair for IL-1β were GTCCTGTGTAATGAAAGACGGC and CTGCTTGTGAGGTGCTGATGTA, primer for IFN-γ were CTTTGCAGCTCTTCCTCAT and GTCACCATCCTTTTGCCAGT.

2.5. In situ hybridization

To detect the distribution of CB1R and CB2R within the ocular tissues, whole eye samples were snap-frozen in liquid nitrogen and stored in −80 °C before cryostat sectioning. Tissue sections (10 μm) were treated with pretreatment solutions of proprietary compositions (Advanced Cell Diagnosis, CA, USA). RNA hybridization was performed using the RNAscope® 2.5 HD Duplex Reagent Kit (322430, Advanced Cell Diagnosis) with probes for CB1R and CB2R, RNAscope Probe - Mm-Cnr1 (420721) and RNAscope Probe - Mm-Cnr2-C2 (407351-C2) (Advanced Cell Diagnosis, CA, USA). After hybridization, sections were counterstained with hematoxylin Gills and mounted with Vectamount (Vector Labs, CA, USA). Samples were analyzed using a standard bright-field microscope (BX53 Olympus, Hamburg, Germany).

2.6. Data analysis

The results are reported as mean ± standard deviation. Statistical analyses were carried out using Graph-pad Prism 7.04 (GraphPad, CA,

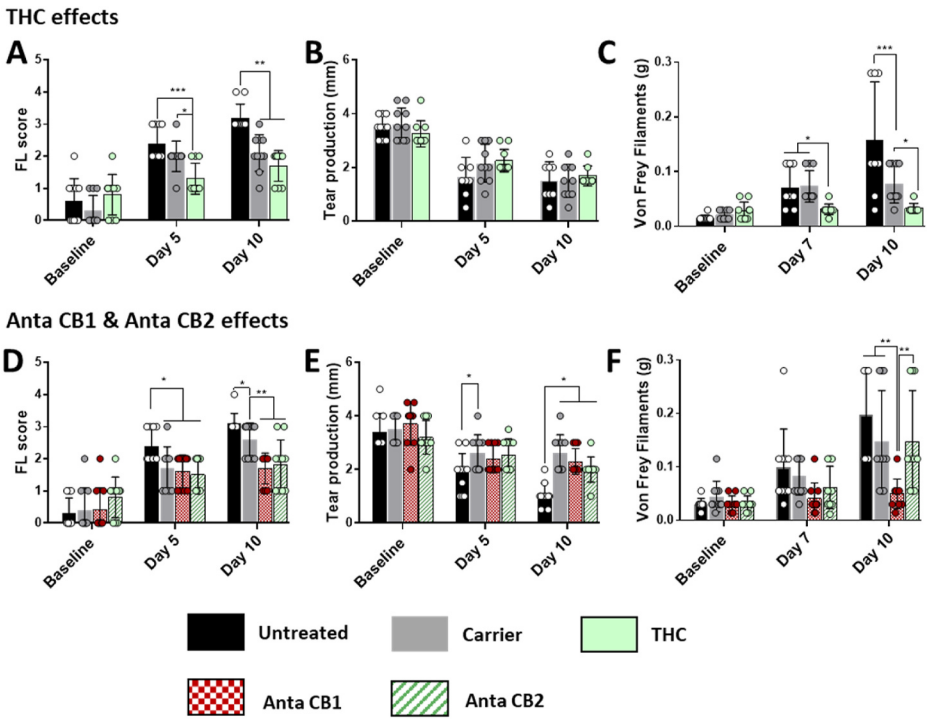

Fig. 2. Effects of THC (A–C) and antagonists (D–F) on in-vivo phenotypes including FL score (A, D), tear production (B, E), and sensitivity by von-Frey filaments (C, F), (n = 5 mice/10 eyes, each circle represents one eye). Topical application of either THC, Anta CB1 or Anta CB2 lead to distinct alterations of dry-eye phenotype. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. Data are representative of two sets of independent experiments.

USA). Statistical differences among groups in readouts were compared by non-parametric ANOVA with Tukey post hoc. A p-value of less than $p < 0.05$ was considered statistically significant.

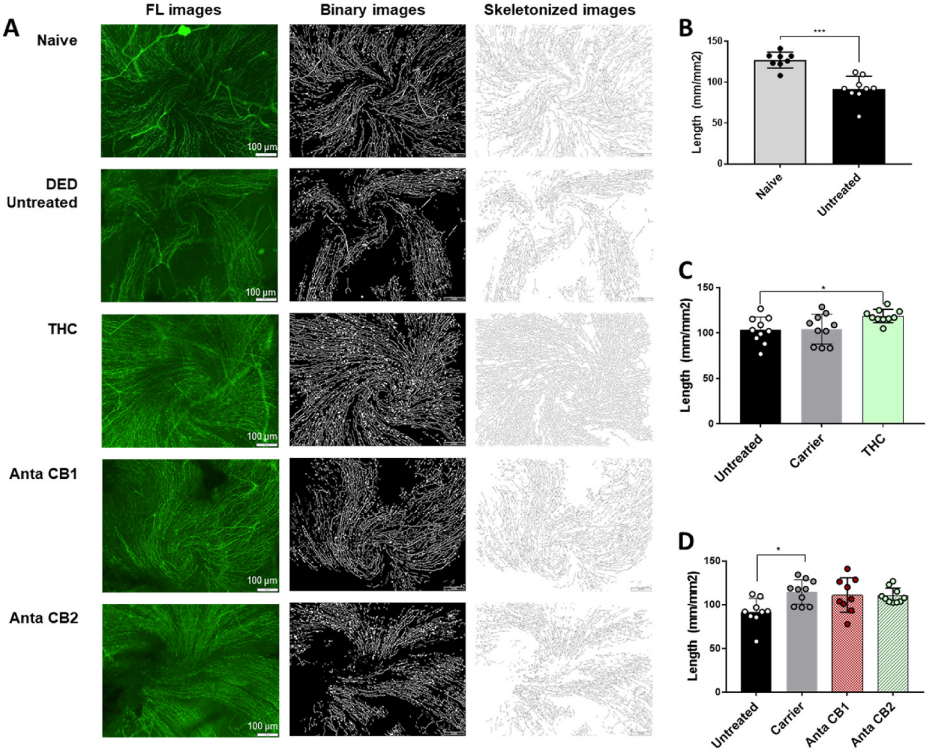

Fig. 3. A) Representative immunohistology images (left, green: β3-tubulin) with related binary and skeletonized images (right) (bar = 100 µm) are presented (center areas of the cornea at day 10 of DS). DS reduced nerve morphology, while THC maintained nerve morphology after 10 days of DS. (B) Nerve length per mm^2 (in mm/mm2) in the central cornea of naïve and untreated DED (B), and cannabinoid treatment groups (C, D) at day 10 (n = 10 corneas per group). *: $p < 0.05$.

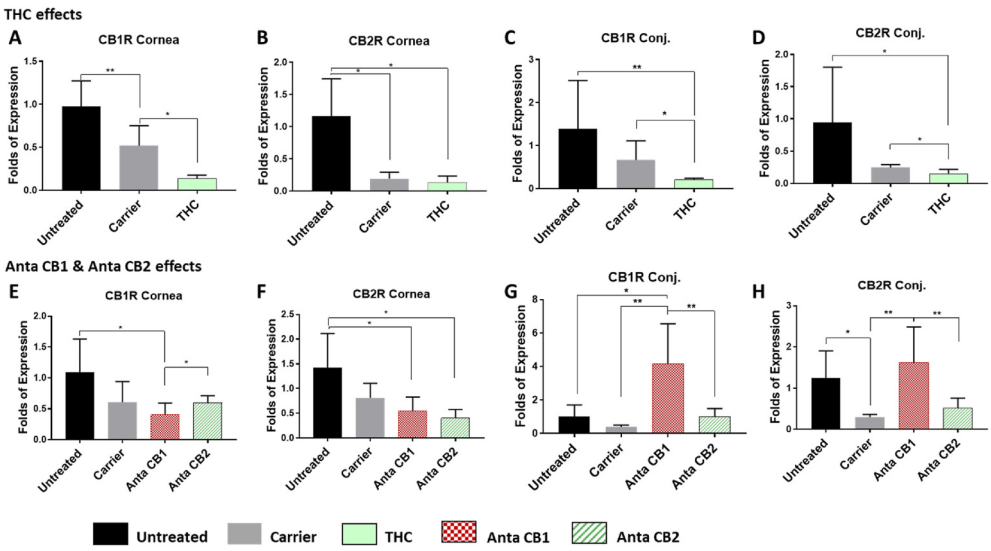

Fig. 4. Effects of THC (A–D) and antagonists (E–H) on expressions of CB1R and CB2R in the cornea and conjunctiva at day 10 of DS. (n = 5 mice (10 eyes)/group). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. RT-qPCR data showed the relative folds of CBR expressions in the cornea and conjunctiva on day 10 of DS. In the cornea, THC, Anta CB1, and Anta CB2 reduced the expression of CB1R and CB2R. In the conjunctiva, treatment with different ligands modified the CBR expression.

3. Results

3.1. CB1R and CB2R expression

3.1.1. Expression of CB1R and CB2R in naïve mice

In-situ hybridization experiments showed that CB1R and CB2R are expressing in naïve cornea and conjunctiva (Fig. 1A and B). In detail, CB1R and CB2R signals are present mainly in the epithelial layer of the cornea and conjunctiva. In the corneal stroma and conjunctiva substantia layer, both CB1R and CB2R were detected, however, in a less frequent and more scattered pattern (Fig. 1A and B).

3.1.2. Effects of desiccating stress on CBR expression

To quantify the amount of CB1R and CB2R, RT-qPCR of the cornea and conjunctiva of naïve and DED mice was performed (Fig. 1C–H). During DED-induction, the mRNA level of CB1R and CB2R in the cornea and conjunctiva increased significantly (Fig. 1 C– H). In the cornea, CB1R and CB2R expressions increased by 2.64 and 12.74 folds respectively compared to baseline levels (Fig. 1C and D), the ratio of CB1R/CB2R in the cornea was significantly decreased at day 10 compared to baseline (Fig. 1E). A similar trend was also found in the conjunctiva (Fig. 1F–H).

3.2. Topical application of cannabinoid ligands

3.2.1. Effects on dry-eye phenotype in-vivo

Topical application of THC (Fig. 2A–C) or Anta CB1 or Anta CB2 (Fig. 2D–F) lead to distinct alterations of dry-eye phenotype.

Corneal FL scores in mice treated with THC were significantly lower than the untreated ($p < 0.001$) and the carrier group ($p < 0.05$) on day 5. On day 10 the THC group has less corneal staining but demonstrated no statistical difference to the carrier group (Fig. 2A). Corneal FL scores in mice treated topically with Anta CB1, Anta CB2 and carrier were significantly lower compared to untreated mice at day 5 ($p < 0.05$). After 10 days of treatment, a significantly lower FL score was observed in the Anta CB1 and Anta CB2 group compared to both the carrier group ($p < 0.01$) and untreated group ($p < 0.01$) (Fig. 2D).

Tear production (TP) was reduced in the untreated group, from 3.5 ± 0.7 (baseline) to 1.0 ± 0.4 (day 10, $p < 0.05$) (Fig. 2B). Treatments with THC, Anta CB1, and Anta CB2 showed significantly higher TP values than the untreated group on day 10 ($p < 0.05$) (Fig. 2B and E). There was no statistical difference between cannabinoid formulations and relating carriers.

DS reduced corneal sensitivity of the untreated group (the von-Frey filament force increased significantly, $p < 0.01$, Fig. 2C &F). After THC treatment, corneal sensitivity remained at a baseline level during 10 days of DS. THC-treated mice responded to filaments with less thickness compared to the carrier-treated ($p < 0.05$) and untreated mice ($p < 0.001$) at Day 7 and 10 (Fig. 2C). Interestingly, Fig. 2F showed that only the Anta CB1 group exhibited a normal corneal sensitivity. Anta CB1 responded to lower filament force compared with the Anta CB2 and the carrier group ($p < 0.01$). No statistical difference was detected among Anta CB2, carrier, and untreated groups at day 7 or 10.

Over the entire application period, mice did not show any behavioral alterations.

3.2.2. Corneal nerve morphology

Representative images of corneal nerve morphology are shown in Fig. 3A. DS for 10 days induced a significant reduction of nerve morphology in DED mice (compared to naïve mice), in which nerve-free areas were observed in the center of the cornea (Fig. 3A, DED mice). Fig. 3A also showed that the THC group had higher density than DED mice and other Anta CB1 or Anta CB2 groups.

Semi-automatic quantification of the nerve length per mm^2 (in mm/mm^2) demonstrated a significant decrease of corneal nerve. The value of DED untreated mice was 91.81 ± 15.61 mm/mm^2, which is lower than that of naïve (133.24 ± 19.17 mm/mm2), ($p < 0.01$, Fig. 3B).

THC treatment (Fig. 3C) lead to a significantly higher nerve length per mm^2 in comparison with untreated DED mice ($p < 0.05$). There was no significant difference between the carrier and untreated groups.

Fig. 3D presents the effect of antagonist treatments on corneal nerve length per mm^2. After 10 days of treatment, only carrier (114.72 ± 13.91mm/mm2) showed an increased effect on corneal nerve length per mm^2 compared to the untreated DED mice. In contrast, Anta CB1 and Anta CB2 did not significantly affect the corneal nerve morphology compared to the untreated group.

3.2.3. Effect of topical cannabinoid treatment on CBR expression

Fig. 4 depicts the expression of CBRs in DED-mice treated with THC, Anta CB1, or Anta CB2. In the cornea, topical application of THC (Fig. 4A–D) lead to a reduction of CB1R and CB2R expression compared to the untreated control. The THC group demonstrated a lower CB1R

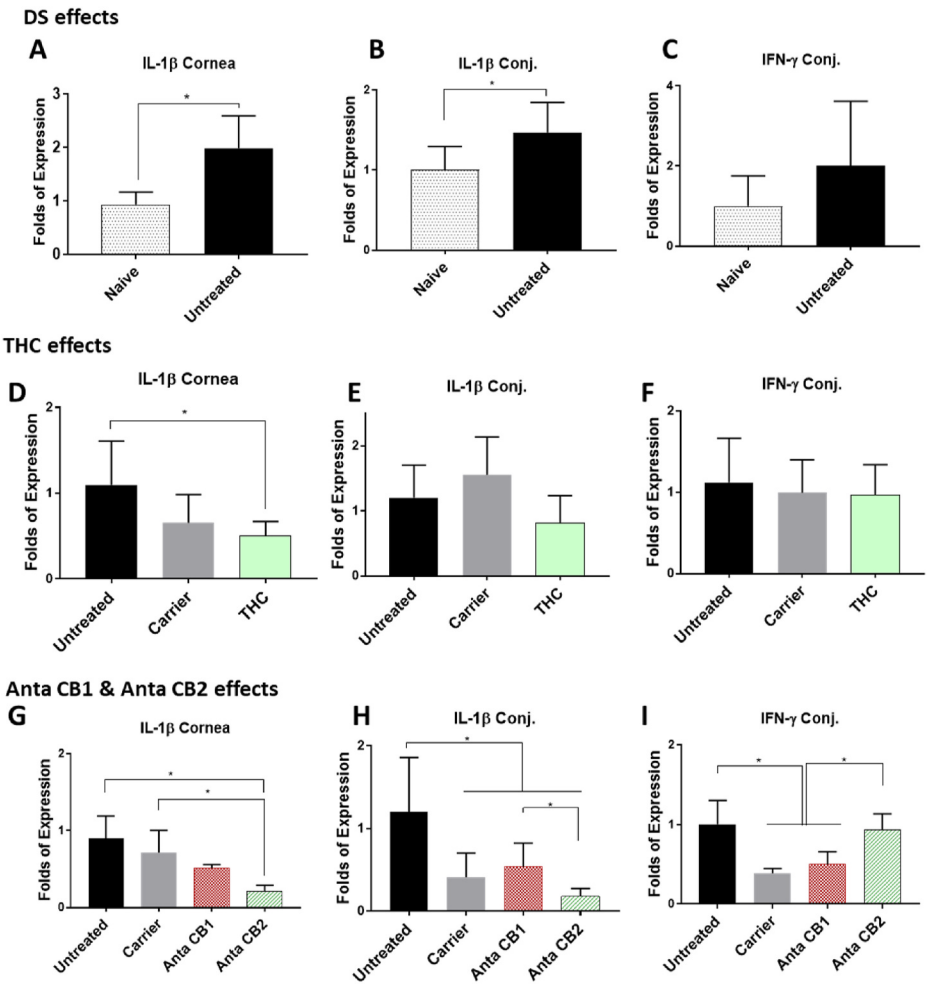

Fig. 5. Effects of DS (A–C), THC (D–F) and antagonists (G–I) on cytokines in the cornea and conjunctiva after 10 days. (A–C) DS induced an increase of IL-1β in the cornea and conjunctiva. In the cornea, THC, Anta CB1, and Anta CB reduced IL-1β (D, G). In the conjunctiva, while THC showed no significant effects, Anta CB2 treatment significantly changed the expression of IL-1β and IFN-γ (F, I). Data are expressed as mean ± SD (n = 5 samples/group). *p < 0.05.

expression in the cornea and conjunctiva than the carrier group (Fig. 4A, $p < 0.05$). In the conjunctiva, expression of CB1R and CB2R in the THC treated group was lower than in both the carrier ($p < 0.05$) and the untreated group.

Fig. 4E–F shows that Anta CB1 and Anta CB2 treatments reduced the expression of CB1R and CB2R compared to the untreated control. In the cornea, there were statistic differences in CB1R expression between Anta CB1 and Anta CB2 treatments (Fig. 4E). Anta CB2 treatment significantly reduced the expression of CB2R in the cornea compared to the carrier (Fig. 4F).

Fig. 4G and H showed that Anta CB1 significantly increased the expression of both CB1R and CB2R in the conjunctiva ($p < 0.01$) compared to other groups. There was no statistical difference between Anta CB2 and the according carrier.

3.2.4. *Effect of cannabinoid treatment on inflammation factors*

IL-1β and INF-γ expression: DS significantly increased IL-1β in cornea and conjunctiva (Fig. 5A and B). IFN-γ was not detected in the cornea at an acceptable level (Cq values was over 35). Topically applying cannabinoids showed effects on the cytokine expression.

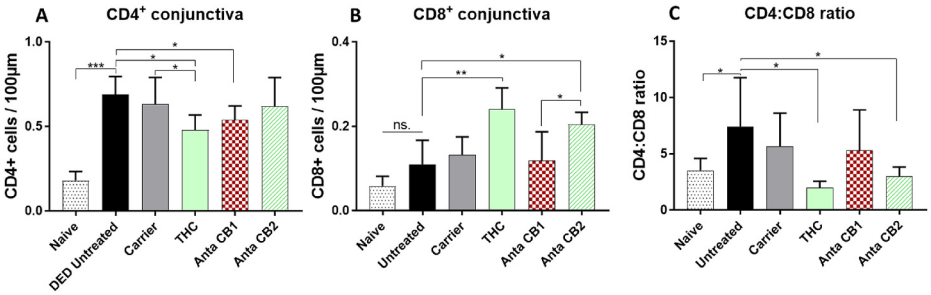

Fig. 6. (A) $CD4^+$, (B) $CD8^+$ cell density, and (C) CD4:CD8 ratio in conjunctiva of naïve and DED-induced mice after 10 days: (A) THC or Anta CB1 treatment reduced $CD4^+$ density compared to the DED untreated group. (B, C) Treatment with THC or Anta CB2 increased $CD8^+$ density and reduced CD4:CD8 ratio compared to the DED untreated group. Data are expressed as mean ± SD (n = 4 eyes/group). *$p < 0.05$,**$p < 0.01$, ***: $p < 0.001$.

Table 1
Summarizing the cannabinoid effects on DED phenotypes and other readouts.

Effects of CBR ligands		DED phenotypes and readouts						
		FL score	Sensitivity	Corneal nerve	CBR in cornea	IL-1β in cornea	$CD4^+$ conj.	$CD8^+$ conj.
1	Activates CBR non-selectively	↓	Maintained	Maintained	↓	↓	↓	↑
2	Inhibits CBR 1 selectively	↓	Maintained	*Ns.*	↓	↓	↓	Ns.
3	Inhibits CBR 2 selectively	↓	*Ns.*	*Ns.*	↓	↓	Ns.	↑

(*Ns.: not significant, ↑: increase, ↓: decrease).

Applying THC for 10 days reduced the expression of IL-1β in the cornea ($p < 0.05$, Fig. 5D) but not in the conjunctiva compared to the untreated group (Fig. 5E and F). Treatment with Anta CB1 and Anta CB2 reduced the expression of IL-1β in the cornea (Fig. 5G) and conjunctiva (Fig. 5B) compared to DED untreated group. Only Anta CB2 treatment increased the expression of IFN-γ compared to Anta CB1 and carrier (Fig. 5I, $p < 0.05$).

Conjunctival $CD4^+$ and $CD8^+$ T-cell infiltration: DS increased $CD4^+$ density in the conjunctival epithelium ($p < 0.05$, Fig. 6A), whereas THC treatment decreased the number of $CD4^+$ cells significantly compared to the carrier and the DED untreated group. Treatment with Anta CB1 also reduced the $CD4^+$ cells in the conjunctiva compared to the DED untreated group ($p < 0.05$). In contrast, Anta CB2 did not influence the number $CD4^+$ cells in the conjunctiva.

THC or Anta CB2 treatment showed a higher $CD8^+$ density than untreated DED mice (Fig. 6B). Consecutively, a significant decrease in CD4:CD8 ratio is present in the THC and Anta CB2 treated groups compared to the untreated group ($p < 0.05$, Fig. 6D).

The main effects of cannabinoids on DED phenotype are summarized in Table 1.

4. Discussion

This study describes for the first time a change of expression of CB1R and CB2R in cornea and conjunctiva during the induction of experimental DED. Topical application of cannabinoid ligands led to a consecutive reduction of receptor expression accompanied with amelioration of the induced DED phenotype. The expression of CBR in cornea and conjunctiva under healthy conditions has been published previously [35–37], however, this study shows that desiccating stress and consecutive development of a DED phenotypes upregulated CBRs expression, implicating a functional role in the pathophysiology of the disease. CB2R (mainly expressed on immune cells) was increased at a higher rate compared to CB1R during 10 days of DS. The increase of CB2R expression is most likely related to the well-known infiltration of immune cells into the ocular surface in the early phases of experimental DED [38,39]. An overexpression within resident immune cells, such as dendritic cells, could be another reason and should be investigated in future studies.

DED was shown to be accompanied with a significant increase in IL-1β and IFN-γ levels at the ocular surface [34,40,41], together with an infiltration of CD4 + T cells in the conjunctival epithelium [24,34]. Activating CB2R down-regulates inflammatory mediators and reduces immune cell activity (such as macrophages, T-cells, or neutrophils) [7,20,42]. Here, we observed a significant reduction of $CD4^+$ T cells and IL-1β in the cornea after topical application of THC (a CBR non-selective agonist). Although THC and CB2R-antagonist hereby both demonstrated similar results regarding IL-1β in the cornea, the CB2R-antagonist lead to an additional increase of INF-y expression in the conjunctiva. In an autoimmune mouse model, increasing IFN-γ was reported to decrease IL-1β and subsequently IL-17 as an immune-regulatory response [43]. In this study, THC and the selective CB2R-antagonist also increased $CD8^+$ density. Whether these $CD8^+$ T cells may act as suppressor cells to prevent the pathogenic infiltration of $CD4^+$cells [44,45] remains to be investigated, but could explain the improvement of the ocular surface phenotype in our study. This potential anti-inflammatory pathway of CB2R-antagonist should also be investigated further, as Th17 cells and subsequently IL-17 have been demonstrated in mouse models of DED as key effector cells/cytokine involved in destruction of epithelial barrier function [46,47]. Treatment with CB1R antagonist reduced IL-1β (in cornea) and $CD4^+$ cells (in conjunctiva), which can be explained by the fact that CB1R antagonists exhibited a similar or partial effect as CB2R agonists by decreasing inflammatory cytokines [48,49] or attenuating inflammation [50]. Overall, these findings indicate a functional role of CB2R in the pathophysiology of DED and its potential significance as a therapeutic target to treat the according inflammatory response.

The reduction of corneal epithelial staining, as the central macroscopic pathology in experimental DED, resulted from both treatment with THC and CBR antagonists. This is an interesting finding, as we expected only the non-selective agonist to be efficient. CB1R, expressed on corneal epithelial cells, co-localizes and interacts with transient receptor potential vanilloid (TRPV) proteins, hereby promoting epithelial wound healing [51]. In further studies using this mouse model this functional interaction should be analyzed, particularly, as TRPV proteins are involved in processes of neurosensation and inflammation and TRPV1-antagonists have been proposed and tested in first clinical trials to treat dry-eye related pain [52].

In this study, we also detected effects on neuronal function after topical cannabinoid application, suggesting a role of the ECS in corneal sensation. (i) CB1R selective antagonist and non-selective agonist (THC) displayed significant beneficial effects on mechanical sensitivity; (ii) THC maintained structure and density of the corneal subbasal nerve plexus.

Experimental DS in our setup reduced corneal sensitivity and decreased corneal nerve morphology, which is in concordance to previous findings [28]. Topical THC application preserved corneal nerve length and maintained corneal sensitivity. In contrast, the CB1 antagonist maintained only corneal sensitivity without preventing loss of corneal nerves, whereas the CB2 antagonist revealed no function in this respect. The pathway, through which THC facilitates its proposed neuroprotectivec function, is not investigated in the cornea, however it is likely that activation of CB1R by THC in DS leads to reduced neurotransmission and suppression of glutamate-mediated neurotoxicity (excitotoxicity) as previously published [13,16]. Treatment with THC, therefore, could open up the possibility to protect corneal nerves in potential neurodestructive conditions such as dry-eye or other neurodegenerative corneal diseases.

Interestingly, the CB1R antagonist also maintained corneal sensitivity, despite the loss of corneal nerves. This might be related to altered nerve transmission, in which inhibiting CB1R increases nerve transmission [53–55], hereby increasing sensitization levels and therefore compensating reduced numbers of nerves. However, increased nerve transmission is an abnormality which also lead to neuropathic cascades [56,57]. The latter, considering the recent investigation of neuropathic pain as part of dry-eye would be unfavourable and prohibit a longterm therapeutic use of CB1R antagonists.

In summary, the effects of topical cannabinoids on corneal nerves are complex. As it has been described that DED patients may present with both increased and decreased corneal sensitivity [58,59] there is a potential link of the ECS and corneal nerve function within different phases of the disease. THC more likely than the CBR1 antagonist could be used in order to protect corneal nerves and restore homeostasis of nerve

Table 2
Predicted cannabinoid effects on DED phenotype.

Ligands	CB1R		CB2R	
	Agonist	Antagonist	Agonist	Antagonist
Function	(−) neurotransmitter release (prevent hypertoxicity): GABA [13], noradrenaline [14], substance P [15]	(+) neurotransmitter release: substance P [54], acetylcholin [55]	(−) immune cells' activities (T-cell, macrophage, neutrophil, dendritic cells) [7,62] (−) secretion of cytokines (IL-1β, IL-6 TNF-α) [20]	(+) IFN- γ [63] (+) IFN- γ decreased IL-1 β and subsequently IL-17 [43]
General effect	Neuroprotection [13,16] Maintain neuroplasticity [17]	Increasing neurotransmission [53]	Anti-inflammation [18]	Anti-inflammation through Th17/IL-17 axis [43]
Potential application in DED	Pain management [7] Maintain nerve morphology [17] Maintaining nociception [64]	Enhancing corneal nerve function (e.g. short-term application) [64]	Slow the inflammation progression of DED vicious circle [8,63] Regulating inflammation [7,18]	

(+): increase, (−): decrease.

function.

There are several limitations in this study. Corneal nerve function was measured by mechanical stimuli, only. It is well known, that corneal nerves carry different nociceptors (mechanical, cold, polymodal) and future investigations should take this into account and include further read-outs such as the use of capsaicin [60]. Also, we did not test potential effects on the intraocular pressure. Historically, the first use of topical cannabinoids formulated in eye drops was performed to treat glaucoma [61]. Finally, follow-up studies should investigate the role of CBRs in dry-eye disease by using, e.g. knock-out lines, and by examining in detail the proposed immune-regulatory mechanisms of topical cannabinoids.

Overall our data demonstrates evidence for the use of cannabinoid ligands as topical eye drops for the treatment of DED. Although the use of a combined CB1/CB2 agonist seems to be the most promising candidate, there are beneficial effects by selective ligands, that can be used short-term or to target specific pathomechanisms (Table 2). Ongoing studies will need to investigate the proposed modes-of-action in depth, address formulation issues and pharmacokinetics to elucidate potential side-effects and to proceed into clinical trial applications.

Declaration of competing interest

Bao Tran, Martina Maass, Gwen Musial, and Uta Gehlsen have no conflict of interests. Michael E. Stern is the member of ImmunEyez LLC, Irvine, CA, USA. Philipp Steven is named as co-inventor in a patent WO 2018/060282 A1

Acknowledgement

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska Curie grant agreement IT-DED^3 No.765608.

We thank Denis Hos, MD PhD for supporting qPCR experiments, and Thomas Langman, PhD, and Mona Tabel for supporting in in-situ hybridization experiments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtos.2021.12.008.

References

[1] Stapleton F, Alves M, Bunya VY, Jalbert I, Lekhanont K, Malet F, et al. TFOS DEWS II epidemiology report. Ocul Surf 2017;15:334–65. https://doi.org/10.1016/j.jtos.2017.05.003.

[2] Bron AJ, de Paiva CS, Chauhan SK, Bonini S, Gabison EE, Jain S, et al. TFOS DEWS II pathophysiology report. Ocul Surf 2017;15:438–510. https://doi.org/10.1016/j.jtos.2017.05.011.

[3] Baudouin C, Messmer EM, Aragona P, Geerling G, Akova YA, Benítez-Del-Castillo J, et al. Revisiting the vicious circle of dry eye disease: a focus on the pathophysiology of meibomian gland dysfunction. Br J Ophthalmol 2016;100:300–6. https://doi.org/10.1136/bjophthalmol-2015-307415.

[4] Jones L, Downie LE, Korb D, Benitez-del-castillo JM, Dana R, Deng SX, et al. The ocular surface TFOS DEWS II management and therapy report. Ocul Surf 2017;15:575–628. https://doi.org/10.1016/j.jtos.2017.05.006.

[5] Pflugfelder SC. Management and therapy of dry eye disease: report of the management and therapy subcommittee of the international dry eye WorkShop. Ocul Surf 2012;5:163–78. https://doi.org/10.1016/s1542-0124(12)70085-x. 2007.

[6] Russo EB. Cannabinoids in the management of difficult to treat pain. Therapeut Clin Risk Manag 2008;4:245–59. https://doi.org/10.2147/TCRM.S1928.

[7] Thapa D, Cairns EA, Szczesniak A-M, Toguri JT, Caldwell MD, Kelly MEM. The cannabinoids Δ8THC, CBD, and HU-308 act via distinct receptors to reduce corneal pain and inflammation. Cannabis Cannabinoid Res 2018;3:11–20. https://doi.org/10.1089/can.2017.0041.

[8] Murataeva N, Miller S, Dhopeshwarkar A, Leishman E, Daily L, Taylor X, et al. Cannabinoid CB2R receptors are upregulated with corneal injury and regulate the course of corneal wound healing. Exp Eye Res 2019;182:74–84. https://doi.org/10.1016/j.exer.2019.03.011.

[9] Hartsel JA, Eades J, Hickory B, Makriyannis A. Cannabis sativa and hemp. Elsevier Inc.; 2016. https://doi.org/10.1016/B978-0-12-802147-7.00053-X.

[10] Gurney SMR, Scott KS, Kacinko SL, Presley BC, Logan BK. Pharmacology, toxicology, and adverse effects of synthetic cannabinoid drugs. Forensic Sci Rev 2014;26:54–76.

[11] Kim D, Thayer SA. Cannabinoids inhibit the formation of new synapses between hippocampal neurons in culture. J Neurosci 2001;21. https://doi.org/10.1523/jneurosci.21-10-j0004.2001. RC146–RC146.

[12] Castillo PE, Younts TJ, Chávez AE, Hashimotodani Y. Endocannabinoid signaling and synaptic function. Neuron 2012;76:70–81. https://doi.org/10.1016/j.neuron.2012.09.020.

[13] Katona I, Sperlágh B, Sík A, Käfalvi A, Vizi ES, Mackie K, et al. Presynaptically located CB1 cannabinoid receptors regulate GABA release from axon terminals of specific hippocampal interneurons. J Neurosci 1999;19:4544–58. https://doi.org/10.1523/JNEUROSCI.19-11-04544.1999.

[14] Ishac EJN, Jiang L, Lake KD, Varga K, Abood ME, Kunos G. Inhibition of exocytotic noradrenaline release by presynaptic cannabinoid CB1 receptors on peripheral sympathetic nerves. Br J Pharmacol 1996;118:2023–8. https://doi.org/10.1111/j.1476-5381.1996.tb15639.x.

[15] Oshita K, Inoue A, Tang H-B, Nakata Y, Kawamoto M, Yuge O. CB(1) cannabinoid receptor stimulation modulates transient receptor potential vanilloid receptor 1 activities in calcium influx and substance P Release in cultured rat dorsal root ganglion cells. J Pharmacol Sci 2005;97:377–85. https://doi.org/10.1254/jphs.FP0040872.

[16] Marsicano G, Goodenough S, Monory K, Hermann H, Eder M, Cannich A, et al. CB1 cannabinoid receptors and on-demand defense against excitotoxicity. Science 2003;302:84–8. https://doi.org/10.1126/science.1088208.

[17] Schwitzer T, Schwan R, Angioi-duprez K, Giersch A, Laprevote V. The endocannabinoid system in the retina: from physiology to practical and therapeutic applications. Neural Plast 2016;2016. https://doi.org/10.1155/2016/2916732.

[18] Toguri JT, Caldwell M, Kelly MEM. Turning down the thermostat: modulating the endocannabinoid system in ocular inflammation and pain. Front Pharmacol 2016;7:1–9. https://doi.org/10.3389/fphar.2016.00304.

[19] Maresz K, Carrier EJ, Ponomarev ED, Hillard CJ, Dittel BN. Modulation of the cannabinoid CB2 receptor in microglial cells in response to inflammatory stimuli. J Neurochem 2005;95:437–45. https://doi.org/10.1111/j.1471-4159.2005.03380.x.

[20] Turcotte C, Blanchet MR, Laviolette M, Flamand N. The CB2receptor and its role as a regulator of inflammation. Cell Mol Life Sci 2016;73:4449–70. https://doi.org/10.1007/s00018-016-2300-4.

[21] Wright K, Rooney N, Feeney M, Tate J, Robertson D, Welham M, et al. Differential expression of cannabinoid receptors in the human colon: cannabinoids promote epithelial wound healing. Gastroenterology 2005;129:437–53. https://doi.org/10.1016/j.gastro.2005.05.026.

[22] Dursun D, Wang M, Monroy D, Li D-QQ, Lokeshwar BL, Stern ME, et al. A mouse model of keratoconjunctivitis sicca. Investig Ophthalmol Vis Sci 2002;43:632–8.
[23] Gehlsen U, Braun T, Notara M, Krösser S, Steven P. A semifluorinated alkane (F4H5) as novel carrier for cyclosporine A: a promising therapeutic and prophylactic option for topical treatment of dry eye. Graefe's Arch Clin Exp Ophthalmol 2017;255:767–75. https://doi.org/10.1007/s00417-016-3572-y.
[24] Niederkorn JY, Stern ME, Pflugfelder SC, De Paiva CS, Corrales RM, Gao J, et al. Desiccating stress induces T cell-mediated Sjögren's syndrome-like lacrimal keratoconjunctivitis. J Immunol 2006;176:3950–7.
[25] Bron AJ, Evans VE, Smith JA. Grading of corneal and conjunctival staining in the context of other dry eye tests. Cornea 2003;22:640–50. https://doi.org/10.1097/00003226-200310000-00008.
[26] Bonin RP, Bories C, De Koninck Y. A simplified up-down method (SUDO) for measuring mechanical nociception in rodents using von Frey filaments. Mol Pain 2014;10:1–10. https://doi.org/10.1186/1744-8069-10-26.
[27] Dixon WJ. The up-and-down method for small samples. J Am Stat Assoc 1965;60:967–78. https://doi.org/10.1080/01621459.1965.10480843.
[28] Stepp MA, Pal-Ghosh S, Tadvalkar G, Williams A, Pflugfelder SC, de Paiva CS. Reduced intraepithelial corneal nerve density and sensitivity accompany desiccating stress and aging in C57BL/6 mice. Exp Eye Res 2018;169:91–8. https://doi.org/10.1016/j.exer.2018.01.024.
[29] Cui W, Guan Z, Zhang Z. An improved region growing algorithm for image segmentation. 2008 int. Conf. Comput. Sci. Softw. Eng., 6. IEEE; 2008. p. 93–6. https://doi.org/10.1109/CSSE.2008.891.
[30] Pal-Ghosh S, Pajoohesh-Ganji A, Tadvalkar G, Kyne BM, Guo X, Zieske JD, et al. Topical Mitomycin-C enhances subbasal nerve regeneration and reduces erosion frequency in the debridement wounded mouse cornea. Exp Eye Res 2016;146:361–9. https://doi.org/10.1016/j.exer.2015.08.023.
[31] Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3—new capabilities and interfaces. Nucleic Acids Res 2012;40. e115–e115.
[32] Koressaar T, Remm M. Enhancements and modifications of primer design program Primer3. Bioinformatics 2007;23:1289–91.
[33] Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C T method. Nat Protoc 2008;3:1101.
[34] De Paiva CS, Chotikavanich S, Pangelinan SB, Pitcher 3rd JD, Fang B, Zheng X, et al. IL-17 disrupts corneal barrier following desiccating stress. Mucosal Immunol 2009;2:243–53.
[35] Straiker AJ, Maguire G, Mackie K, Lindsey J. Localization of cannabinoid CB1 receptors in the human anterior eye and retina. Invest Ophthalmol Vis Sci 1999;40:2442–8.
[36] Iribarne MM, Torbidoni V, Julián K, Prestifilippo JP, Sinha D, Rettori V, et al. Cannabinoid receptors in conjunctival epithelium: identification and functional properties. Investig Ophthalmol Vis Sci 2008;49:4535–44. https://doi.org/10.1167/iovs.07-1319.
[37] Matsuda S, Kanemitsu N, Nakamura A, Mimura Y, Ueda N, Kurahashi Y, et al. Metabolism of anandamide, an endogenous cannabinoid receptor ligand, in porcine ocular tissues. Exp Eye Res 1997;64:707–11. https://doi.org/10.1006/exer.1996.0265.
[38] Niederkorn JY, Stern ME, Pflugfelder SC, De Paiva CS, Corrales RM, Gao J, et al. Desiccating stress induces T cell-mediated Sjogren's syndrome-like lacrimal keratoconjunctivitis. J Immunol 2006;176:3950–7. https://doi.org/10.4049/jimmunol.176.7.3950.
[39] Stern ME, Schaumburg CS, Pflugfelder SC. Dry eye as a mucosal autoimmune disease. Int Rev Immunol 2013;32:19–41. https://doi.org/10.3109/08830185.2012.748052.
[40] Narayanan S, Corrales RM, Farley W, McDermott AM, Pflugfelder SC. Interleukin-1 receptor-1-deficientmiceshowattenuated production of ocular surface inflammatory cytokines in experimental dry eye. Cornea 2008;27:811–7. https://doi.org/10.1097/ICO.0b013e31816bf46c.
[41] Pflugfelder SC, de Paiva CS, Moore QL, Volpe EA, Li DQ, Gumus K, et al. Aqueous tear deficiency increases conjunctival interferon-γ (IFN-γ) expression and goblet cell loss. Investig Ophthalmol Vis Sci 2015;56:7545–50. https://doi.org/10.1167/iovs.15-17627.
[42] Ni X, Geller EB, Eppihimer MJ, Eisenstein TK, Adler MW, Tuma RF. Win 55212-2, a cannabinoid receptor agonist, attenuates leukocyte/endothelial interactions in an experimental autoimmune encephalomyelitis model. Mult Scler J 2004;10:158–64. https://doi.org/10.1191/1352458504ms1009oa.
[43] Masters SL, Mielke LA, Cornish AL, Sutton CE, O'Donnell J, Cengia LH, et al. Regulation of interleukin-1β by interferon-γ is species specific, limited by suppressor of cytokine signalling 1 and influences interleukin-17 production. EMBO Rep 2010;11:640–6. https://doi.org/10.1038/embor.2010.93.
[44] De Paiva CS, Villarreal AL, Corrales RM, Rahman HT, Chang VY, Farley WJ, et al. Dry eye-induced conjunctival epithelial squamous metaplasia is modulated by interferon-γ. Investig Ophthalmol Vis Sci 2007;48:2553–60. https://doi.org/10.1167/iovs.07-0069.
[45] Hu D, Ikizawa K, Lu L, Sanchirico ME, Shinohara ML, Cantor H. Analysis of regulatory CD8 T cells in Qa-1-deficient mice. Nat Immunol 2004;5:516–23. https://doi.org/10.1038/ni1063.
[46] Manuscript A, De Paiva CS, Chotikavanich S, Pangelinan SB, Pitcher 3rd JD, Fang B, et al. IL-17 disrupts corneal barrier following desiccating stress. Mucosal Immunol 2013;2:243–53. https://doi.org/10.1038/mi.2009.5. IL-17.
[47] Chauhan SK, Annan J El, Ecoiffier T, Zhang Q, Saban DR, Dana R, et al. Autoimmunity in dry eye is due to resistance of Th17 to Treg suppression. https://doi.org/10.4049/jimmunol.182.3.1247; 2021.
[48] Roche M, Kelly JP, O'Driscoll M, Finn DP. Augmentation of endogenous cannabinoid tone modulates lipopolysaccharide-induced alterations in circulating cytokine levels in rats. Immunology 2008;125:263–71. https://doi.org/10.1111/j.1365-2567.2008.02838.x.
[49] Mnich SJ, Hiebsch RR, Huff RM, Muthian S. Anti-inflammatory properties of CB1-receptor antagonist involves β2 adrenoceptors. J Pharmacol Exp Therapeut 2010;333:445–53. https://doi.org/10.1124/jpet.109.163998.
[50] Altinsoy A, Dileköz E, Kul O, Ilhan SÖ, Tunccan ÖG, Seven I, et al. A cannabinoid ligand, anandamide, exacerbates endotoxin-induced uveitis in rabbits. J Ocul Pharmacol Therapeut 2011;27:545–52. https://doi.org/10.1089/jop.2011.0049.
[51] Yang Y, Yang H, Wang Z, Varadaraj K, Kumari SS, Mergler S, et al. Cannabinoid receptor 1 suppresses transient receptor potential vanilloid 1-induced inflammatory responses to corneal injury. Cell Signal 2013;25:501–11. https://doi.org/10.1016/j.cellsig.2012.10.015.
[52] Benitez-Del-Castillo JM, Moreno-Montañés J, Jiménez-Alfaro I, Muñoz-Negrete FJ, Turman K, Palumaa K, et al. Safety and efficacy clinical trials for SYL1001, a novel short interfering RNA for the treatment of dry eye disease. Invest Ophthalmol Vis Sci 2016;57:6447–54.
[53] López-Moreno JA, González-Cuevas G, Moreno G, Navarro M. The pharmacology of the endocannabinoid system: functional and structural interactions with other neurotransmitter systems and their repercussions in behavioral addiction. Addiction Biol 2008;13:160–87. https://doi.org/10.1111/j.1369-1600.2008.00105.x.
[54] Lever IJ, Malcangio M. CB1 receptor antagonist SR141716A increases capsaicin-evoked release of Substance P from the adult mouse spinal cord. Br J Pharmacol 2002;135:21–4.
[55] Tzavara ET, Davis RJ, Perry KW, Li X, Salhoff C, Bymaster FP, et al. The CB1 receptor antagonist SR141716A selectively increases monoaminergic neurotransmission in the medial prefrontal cortex: implications for therapeutic actions. Br J Pharmacol 2003;138:544–53.
[56] Belmonte C, Nichols JJ, Cox SM, Brock JA, Begley CG, Bereiter DA, et al. TFOS DEWS II pain and sensation report. Ocul Surf 2017;15:404–37. https://doi.org/10.1016/j.jtos.2017.05.002.
[57] Fakih D, Zhao Z, Nicolle P, Reboussin E, Joubert F, Luzu J, et al. Chronic dry eye induced corneal hypersensitivity, neuroinflammatory responses, and synaptic plasticity in the mouse trigeminal brainstem. J Neuroinflammation 2019;16:1–20. https://doi.org/10.1186/s12974-019-1656-4.
[58] Dieckmann G, Goyal S, Hamrah P. Neuropathic corneal pain: approaches for management. Ophthalmology 2017;124:S34–47. https://doi.org/10.1016/j.ophtha.2017.08.004.
[59] Spierer O, Felix ER, McClellan AL, Parel JM, Gonzalez A, Feuer WJ, et al. Corneal mechanical thresholds negatively associate with dry eye and ocular pain symptoms. Investig Ophthalmol Vis Sci 2016;57:617–25. https://doi.org/10.1167/iovs.15-18133.
[60] Joubert F, Guerrero-Moreno A, Fakih D, Reboussin E, Gaveriaux-Ruff C, Acosta MC, et al. Topical treatment with a mu opioid receptor agonist alleviates corneal allodynia and corneal nerve sensitization in mice. Biomed Pharmacother 2020;132:110794. https://doi.org/10.1016/j.biopha.2020.110794.
[61] Katz J, Costarides AP. Facts vs fiction: the role of cannabinoids in the treatment of glaucoma. Curr Ophthalmol Rep 2019;7:177–81. https://doi.org/10.1007/s40135-019-00214-z.
[62] Braun M, Khan ZT, Khan MB, Kumar M, Ward A, Achyut BR, et al. Selective activation of cannabinoid receptor-2 reduces neuroinflammation after traumatic brain injury via alternative macrophage polarization. Brain Behav Immun 2018;68:224–37.
[63] Racz I, Nadal X, Alferink J, Banos JE, Rehnelt J, Martín M, et al. Interferon-γ is a critical modulator of CB2 cannabinoid receptor signaling during neuropathic pain. J Neurosci 2008;28:12136–45.
[64] Aiello F, Gallo Afflitto G, Li J-PO, Martucci A, Cesareo M, Nucci C. CannabinEYEds: the endocannabinoid system as a regulator of the ocular surface nociception, inflammatory response, neovascularization and wound healing. J Clin Med 2020;9:4036.