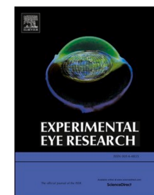
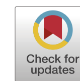
Experimental Eye Research 212 (2021) 108765

Contents lists available at ScienceDirect

Experimental Eye Research

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





Anti-inflammatory and neuroprotective properties of the corticosteroid fludrocortisone in retinal degeneration

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ARTICLE INFO

Keywords:
Corticosteroid
Fludrocortisone
Glucocorticoid receptor
Retinal degeneration
Retinal inflammation
Photooxidative damage
Pro-inflammatory cytokine

ABSTRACT

The pathogenesis of outer retinal degenerations has been linked to the elevation of cytokines that orchestrate pro-inflammatory responses within the retinal milieu, and which are thought to play a role in diseases such as geographic atrophy (GA), an advanced form of AMD. Here we sought investigate the anti-inflammatory and mechanistic properties of fludrocortisone (FA), as well as triamcinolone acetonide (TA), on Müller cell-mediated cytokine expression in response to inflammatory challenge. In addition, we investigated the neuroprotective efficacy of FA and TA in a photo-oxidative damage (PD), a model of outer retinal degeneration. Expression of *CCL2, IL-6,* and *IL-8* with respect to FA and TA were assessed in Müller cells *in vitro*, following simulation with IL-1β or TNF-α. The dependency of this effect on mineralocorticoid and glucocorticoid signaling was also interrogated for both TA and TA via co-incubation with steroid receptor antagonists. For the PD model, C57BL/6 mice were intravitreally injected with FA or TA, and changes in retinal pathology were assessed via electroretinogram (ERG) and optical coherence tomography (OCT). FA and TA were found to dramatically reduce the expression of *CCL2, IL-6,* and *IL-8* in Müller glia *in vitro* after inflammatory challenge with IL-1β or TNF-α ($P < 0.05$). Though FA acts as both a mineralocorticoid and glucocorticoid receptor agonist, co-incubation with selective steroid antagonists revealed that the suppressive effect of FA on *CCL2, IL-6,* and *IL-8* expression is mediated by glucocorticoid signaling ($P < 0.05$). In PD, intravitreal FA was found to ameliorate outer-retinal atrophy as measured by ERG and OCT ($P < 0.05$), while TA had no significant effect ($P > 0.05$). Our data indicate potent anti-inflammatory and mechanistic properties of corticosteroids, specifically FA, in suppressing inflammation and neurodegeneration degeneration associated with outer retinal atrophy. Taken together, our findings indicate that corticosteroids such as FA may have value as a potential therapeutic for outer retinal degenerations where such pro-inflammatory factors are implicated, including AMD.

1. Introduction

Age related macular degeneration (AMD) is a leading cause of blindness worldwide and the most common among developed nations (Reviewed in (Miller, 2013)). There are currently no therapeutic interventions for those suffering from the 'dry' form of the disease, despite the number of individuals afflicted with all forms of AMD being projected to nearly double by 2050 (Rein et al., 2009). In advanced 'dry' AMD, clinically known as geographic atrophy (GA), vision loss in patients is precipitated by a progressive and expanding lesion in the outer retina, causing the death of RPE and photoreceptors, oxidative stress, and breakdown of the blood-retinal barrier (Kauppinen et al., 2016; Tisi et al., 2021).

Inflammation within the outer retinal milieu is associated with the pathogenesis of GA and is regarded as a potential therapeutic and prognostic target (reviewed (Kauppinen et al., 2016; Wang et al.,

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https://doi.org/10.1016/j.exer.2021.108765
Received 13 May 2021; Received in revised form 5 August 2021; Accepted 8 September 2021
Available online 9 September 2021
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2011b)). Mononuclear phagocytes such as macrophages accumulate within GA lesions (Cao et al., 2011; Penfold et al., 1986; Sennlaub et al., 2013), and in concert with the up-regulation of the pro-inflammatory chemoattractant CCL2 in the aqueous humor of GA patients (Sennlaub et al., 2013). Moreover, it has been reported that pro-inflammatory cytokines such as IL-6 and IL-8 are up-regulated in sera of GA patients compared to healthy controls, and at higher levels collectively than neovascular AMD patients (Krogh Nielsen et al., 2019). Elevated serum levels of IL-6 were also shown to predict the progression of GA, offering a potential biomarker of the disease (Krogh Nielsen et al., 2019).

Corticosteroids are steroid hormones with physiological roles in areas such as metabolism, immune regulation, and fluid and electrolyte balance (Reviewed in (Timmermans et al., 2019)). These are broadly placed into 2 classes, glucocorticoids and mineralocorticoids, and exert their biological effects via binding to the glucocorticoid and mineralocorticoid nuclear receptors (Fuller et al., 2000). Glucocorticoids and mineralocorticoids are highly structurally related however, and cross-over binding of these between the 2 corticosteroid receptors occurs to varied degrees (Behar-Cohen and Zhao, 2016; Funder, 2005). Therapeutically, the potent anti-inflammatory properties of synthetic corticosteroids have seen them widely used to treat chronic inflammatory conditions. In the eye, corticosteroids have been pursued for treating diseases such as AMD, diabetic macular edema, pseudophakic cystoid macular edema (reviewed in (Sarao et al., 2014)), including intravitreal administration of triamcinolone acetonide (TA).

It has been established that cytokines such as *CCL2, IL-6*, and *IL-8* are expressed in abundance by retinal cells such as Müller glia (Eastlake et al., 2016; Kauppinen et al., 2016; Rutar et al., 2012; Wang et al., 2011a), and which may be triggered by pro-inflammatory stimuli emanating from activated microglia (Wang et al., 2011a). It has also been demonstrated that activated microglia/macrophages secrete the pro-inflammatory factor IL-1β, and that circumventing IL-1β signaling suppresses cytokine expression in Müller cells and ameliorates pathology in photooxidative damage (PD) (Hu et al., 2015; Natoli et al., 2017b), a model of outer retinal neurodegeneration (Natoli et al., 2016). It is unknown whether corticosteroids can modulate expression of cytokines in retinal cells pertinent to outer retinal degeneration and inflammation, such as *CCL2, IL-6,* and *IL-8*. Moreover, the utility of other corticosteroids such as Fludrocortisone (FA) in modulating ocular neurodegeneration has not been studied. FA, a mineralocorticoid and glucocorticoid receptor agonist (Ajish et al., 2014), has been shown effective in combination with hydrocortisone in reducing mortality in septic shock patients (Annane et al., 2018), and pathology associated with autoimmune hearing loss in mice (Trune and Kempton, 2010).

In the current study, we investigated the efficacy of FA in modulating *CCL2, IL-6,* and *IL-8* expression levels in Müller cells, and its effect on retinal degeneration in mice subjected to PD. In conjunction, we also evaluated the effects of TA in this regard. Our data show that FA and TA and do not induce toxicity in multiple human retinal cell lines, including Müller cell (MIO-M1), RPE (APRE19), and photoreceptor-like (661 W). We also show that addition of either FA or TA drastically reduces the expression of *CCL2, IL-6*, and *IL-8* in Müller cells stimulated with IL-1β or TNF-α, and which is dependent on signaling through the glucocorticoid receptor, and not the mineralocorticoid receptor. Finally, we show that administration of FA improves photoreceptor survival in PD, while TA had no significant effect. Together, our data support a potential repurposing of FA for the treatment of retinal disorders where inflammation is manifested, such as in GA.

2. Materials and methods

2.1. Cell lines

The immortalised retinal cells used were as follows: MIO-M1 cells (Müller cell-derived; Müller 1 Moorefields, Institute of Ophthalmology, Dr A. Limb, Institute of Ophthalmology, University College, UK); ARPE19 (RPE-derived; ATCC CRL-2302, American Tissue Culture Collection, VA, USA); and, 661 W cells (cone photoreceptor-like derived; kindly gifted by Dr Muayyad R. Al-Ubaidi, Department of Cell Biology, University of Oklahoma Health Sciences Centre, Oklahoma City, OK, USA). Cells were authenticated and validated from Cell Bank Australia. All lines were maintained in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Life Technologies) and 3 mM L-glutamine (Life Technologies), in a humidified tissue culture incubator (5% CO2 at 37 °C).

2.2. MTT assay and corticosteroid titration

The MTT assay to was used to assess cell viability in MIO-M1, ARPE19, and 661w cell lines following titration with doses of either FA (10–0.01 μg/μl, provided by Professor Ben Boyd, Monash University, Australia), or TA (10–0.01 μg/μl, Honeywell, USA) for a 12-h. Prior to addition, both FA and TA were dissolved in 20% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, MO, USA) and ultrapure endotoxin-free 0.1 M PBS (Thermo Fisher Scientific, MA, USA), while cultures treated with only the vehicle served as controls. After 12-h, the MTT assay was performed using a kit supplied by Roche (Cell Proliferation Kit I, Roche Applied Science, Penzberg, Germany). After the cells were incubated for 12-h, the MTT assay was applied following the manufacturer's instructions. Quantification of the MTT assay was performed by measuring the absorbance at 570 nm using Tecan Infinite® 200 PRO (Tecan, Männedorf, Switzerland).

2.3. Inflammatory induction and corticosteroid intervention of cell lines

MIO-M1 cells were challenged with either 1 ng/μl of IL-1β or TNF-α (R&D Systems, MN, USA) to stimulate an inflammatory response, and was performed according to our previous methods (Natoli et al., 2017b) with minor adaptation. Briefly, MIO-M1 cells were seeded at 50,000 cells per well in a 24 well plate, after which the cells were stimulated with either IL-1β or TNF-α in serum-deficient DMEM containing 1% FBS for 12-hrs. For groups also receiving corticosteroid treatment, either FA or TA were added to culture media at a concentration of 1 μg/μl, and at the same time as either IL-1β or TNF-α. Further to this, additional treatment groups also received antagonists of various steroid receptors which were added at the same as either TA and FA. The antagonists and their respective concentrations are listed in Table 1. After completion of the 12-h incubation for each treatment group, the samples were immediately prepared for RNA extraction. The culture media was removed and replaced with TRIzol solution (Thermo Fisher Scientific, MA, USA), after which we followed our previously described methodology for extraction, purification, and quantification of the resultant mRNA (Natoli et al., 2017b).

Table 1
Receptor antagonists used in cell culture.

Name	Receptor Antagonist Type	Conc. Series (μM)	Cat. No.	Supplier
RU486	Glucocorticoid receptor	1, 5, 10	S2606	Selleck Chemicals, USA
RU28318	Mineralocorticoid receptor	1, 5, 10	1672	In Vitro Technologies Pty Ltd, VIC, Australia
PF998425	Androgen receptor	1, 5, 10	3923	In Vitro Technologies Pty Ltd, VIC, Australia
ICI182789	Estrogen receptor	0.5, 1, 5	1047	In Vitro Technologies Pty Ltd, VIC, Australia

2.4. Quantitative real time polymerase chain reaction

Following the extraction of mRNA from the cultured cells, cDNA synthesis was performed on each sample using the Tetro cDNA synthesis kit (Bioline, Australia) following to the manufacturer's instructions. Quantitative Real Time Polymerase Chain Reactions (QRT-PCR) was undertaken using TaqMan hydrolysis probes (Applied Biosystems, USA) and an accompanying Gene Expression Master Mix (Applied Biosystems, USA), which were applied according to our previous methods (Rutar et al., 2011). The Taqman Probes using in the study are listed in Table 2. QRT-PCR assays for all samples were then run using the QuantStudio 12 K Flex (Applied Biosystems, USA). The data produced were then analysed following the comparative cycle threshold (Ct) method (ΔΔCt), and which were normalised to the expression of *GAPDH* as validated in our previous study (Natoli et al., 2017b).

2.5. Experimental animals and PD timecourse

All experiments conducted were in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research, and with approval from the ANU Animal Experimentation Ethics Committee (AEEC, protocol #A2014/56). C57BL/6 J mice aged 60 post-natal days were used in the study.

The mouse PD model was conducted according to our established methodology (Natoli et al., 2016). Prior to induction of PD, a group of mice received an intravitreal injection of either TA or FA, which was dissolved in a vehicle solution comprising 0.5% w/v carboxymethyl cellulose and 0.4% v/v Tween 80, while the control group received the vehicle alone. The intravitreal injections were performed as described previously (Natoli et al., 2017a), in which mice were first anaesthetised using an intraperitoneal injection of ketamine (Troy Laboratories, NSW, Australia) and xylazil (Troy Laboratories). Intravitreal injections consisted 3 μL FA or TA in the vehicle solution, equating to a final concentration of 1 μg/uL in the vitreous. After the mice had recovered, they immediately were transferred to individual cages that allow light to enter unimpeded. Animals were then exposed to were exposed to continuous 100 K lux white LED light for 5 days. At the end of the timecourse, Mice were evaluated for changes via Optical Coherence Tomography (OCT) or electroretinography (ERG), then subsequently euthanized with carbon dioxide (CO_2). Each eye was then harvested for histological processing and cryosectioning, according to our standardized methods (Natoli et al., 2016).

2.6. Optical coherence tomography (OCT)

In vivo fundus and cross-sectional images of the mouse retinas following exposure to PD using a Spectralis HRA + OCT device (Heidelberg Engineering, Heidelberg, Germany), as previously detailed (Natoli et al., 2017a) with minor adaptations. Briefly, anaesthetised mice were restrained on a custom-made platform attached to the imaging device, which was adapted for mouse eyes per the manufacturer's specifications. Fundus and cross-sectional images were taken 1-mm intervals at 0–3 mm superior to the optic nerve (ON), where the focal point of the PD lesion is located (Natoli et al., 2016, 2017a). OCT images were analysed using ImageJ software (National Institutes of Health, Bethesda, MD, USA), as previously described. Retinal and ONL thickness were measured for each 1-mm interval, with five equispaced points surveyed across each image. ONL thickness ratios were then calculated as the ONL thickness relative to the distance between the outer and inner limiting membranes, which were then averaged for each animal.

Table 2
TaqMan hydrolysis probes used for QRT-PCR.

Gene	Name	Catalogue Number
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Hs02758991_g1
CCL2	Chemokine (C–C motif) ligand 2	Hs00234140_m1
IL-6	Interleukin 6	Hs00174131_m1
IL-8	Interleukin 8	Hs99999034_m1

2.7. Electroretinography (ERG)

Following corticosteroid treatment and PD, ERG recordings were used to evaluate retinal function in response to full-field flash stimuli under scotopic conditions as detailed previously (Natoli et al., 2016). Animal were subjected to PD for 5 days, after they were removed and darked adapted for 24hrs. Following anaesthetization and placement of mice on the ERG ganzfeld, flash stimuli were then provided by an LED-based system (FS-250 A Enhanced Ganzfeld, Photometric Solutions International, Melbourne) over a stimulus intensity range of 6.3 log cd s m^{-2} (range −4.4 to 1.9 log cd s m^{-2}). The resulting ERG data were processed to extract both mixed a-wave and b-wave responses as previously described (Natoli et al., 2016), which were then expressed as the mean wave amplitude (uV) for each flash intensity.

2.8. Immunohistochemistry and TUNEL labelling

Immunohistochemistry for the macrophage marker IBA1 was performed on mouse retinal cryosections as previously described (Natoli et al., 2016), and utilizing the flowing antibody combination: Rabbit anti-IBA1 (1:500, Wako, Japan), anti-rabbit secondary-Alexa488 (1:1000, Thermo Fisher Scientific, MA, USA). Fluorescence was visualised under an A1 Nikon Confocal Microscope (Nikon, Toyko, Japan), in which a total of 4 equispaced images were acquired across each retina using the accompanying NIS-Elements AR Software (Nikon). For quantification, of immunolabeled IBA1+ cells in the outer retina (ONL-RPE) were counted then averaged for each group, as per our prior methodology (Natoli et al., 2017a).

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) is was used to quantify photoreceptor apoptosis following PD, and was undertaken on retinal cryosections following our previous protocols (Rutar et al., 2012). Fluorescent TUNEL + cells in the ONL were counted across the full length of retinal cryosections cut in the parasagittal plane (supero-inferior), and which were averaged for each experimental group. This images were controlled for location across the retina, with respect to the optic nerve head.

2.9. Statistical analysis

All data was analysed using Prism 6 software (GraphPad Software, CA, USA). The respective statistical analysis utilized for each experimental dataset is detailed in the legend of each Figure. Significant trends in protracted data sets were determined using the 1-way or 2-way analysis of variance (ANOVA), with Tukey's post-hoc test applied for multiple statistical comparisons when desired ($P < 0.05$). Student's t-test was utilized for other single comparisons, where noted.

3. Results

3.1. Cytotoxicity of corticosteroids on retinal cells

The effect of FA or TA on the viability on human retinal cell lines of Müller cells (MIO-M1), RPE (ARPE19), and photoreceptors (661 W) was assessed across a concentration range of 0.01–10 μg/μL, using the MTT assay (Fig. 1). The data show that addition of either TA or FA did not significantly alter viability at most concentrations ($P > 0.05$). A slight reduction in viability was observed at 10 μg/μL for MIO-M1 and 661 W lines ($P < 0.05$), 10-fold higher than the standard concentration. Of particular note, FA or TA did not alter cell viability at 1 μg/μL, which equates to the vitreous concentration for TA that is employed in ophthalmic procedures (Jermak et al., 2007). Consequently, all later experiments interrogating the effect of TA or FA were undertaken at 1

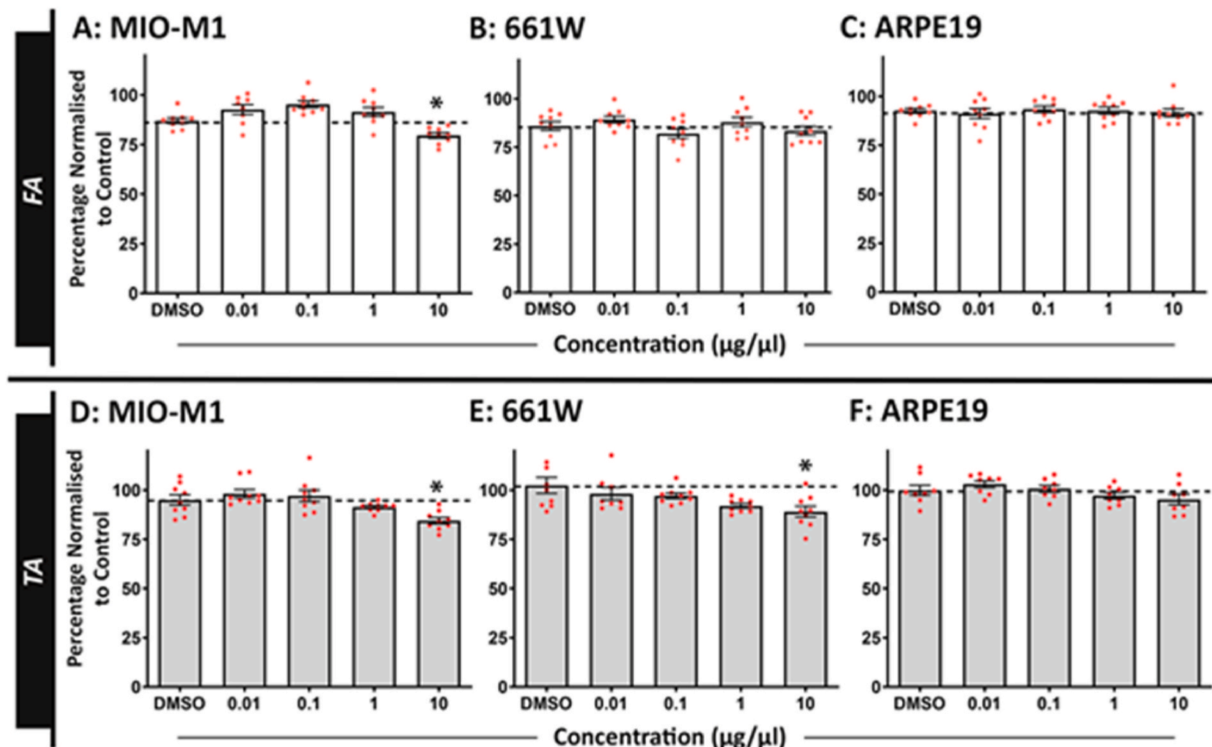

Fig. 1. Effect of FA and TA on the viability of retinal cells *in vitro*. The effect of FA (A–C) and FA (D–F) on cell viability was assessed using the MTT assay on Muller glia (MIO-M1), RPE (ARPE-19), and photoreceptors (661 W) cell lines. **A-C:** The addition of FA had no significant effect on the viability of all cell lines assessed, compared to the DMSO-only group ($P > 0.05$). The exception were MIO-M1 cells (A), which had a slight reduction at the highest concentration (10µg/µL, $P < 0.05$). **D-F:** Introduction of TA similarly did not have significant effect on viability ($P > 0.05$), except for a slight reduction in MIO-M1 (D) and 661 W cells (E) at 10 µg/µL ($P < 0.05$). Statistical significance was determined using a one-way ANOVA and Tukey's post-hoc test, where '*' denotes a significant change. Results presented as the mean ± SEM, N = 8–9.

µg/mL.

3.2. FA and TA modulate cytokine expression in human Müller cells

As has been established that Müller glia are important sources inflammatory cytokines such as *CCL2, IL-6,* and *IL-8* both *in vitro* and *in vivo* (Eastlake et al., 2016; Kauppinen et al., 2016; Rutar et al., 2012; Wang et al., 2011a), we selected MIO-M1 cells to investigate the anti-inflammatory effects TA and FA upon stimulation with either IL-1β or TNF-α (Fig. 2A–D). When challenged with IL-1β, the expression of *CCL2, IL-6,* and *IL-8* were significantly increased in MIO-M1 cells as expected (Fig. 2A, $P < 0.05$). However, in the group where FA was also introduced, the increases *CCL2, IL-6,* and *IL-8* were completely abrogated (Fig. 2A, $P < 0.05$). Incubation with TA similarly reduced the expression of *CCL2, IL-6,* and *IL-8* following stimulation, though to about one-third level compared to IL-1β alone (Fig. 2B, $P < 0.05$). Stimulation of MIO-M1 cells with TNFα also induced increases *CCL2, IL-6,* and *IL-8* expression (Fig. 2C, $P < 0.05$), and which were sharply reduced upon co-incubation with FA to near baseline levels ($P < 0.05$). Similarly, TA abrogated *CCL2, IL-6,* and *IL-8* expression to baseline levels cells following TNF-α stimulation (Fig. 2D, $P < 0.05$).

3.3. FA modulates cytokine expression in human Müller cells via the glucocorticoid receptor

Given that FA can bind both mineralocorticoid and glucocorticoid receptors, we then sought to determine the signalling pathway responsible for the anti-inflammatory effect of FA on *CCL2, IL-6,* and *IL-8* expression. To address this, we co-incubated antagonists of either mineralocorticoid (RU28318) and glucocorticoid (RU486) receptor signalling alongside IL-1β and FA in MIO-M1 cells (Fig. 3A and B). To exclude potential off-target effects of FA, we further tested antagonists of androgen (PF98425) and estrogen (ICI182780) receptor signalling over the same treatment paradigm (Fig. 3C and D). The antagonists were administered at a concentration of 1 µM, which was determined to have no modulatory effect of *CCL2, IL-6,* and *IL-8* expression (Supplementary Figure 1). With the addition of RU486 to the IL-1β +FA treatment group, the suppressive effect of FA on the expression of *CCL2, IL-6,* and *IL-8* was completely abrogated (Fig. 3A, $P < 0.05$). In contrast, co-incubation with RU28318 did not alter gene expression levels compared to IL-1β +FA alone (Fig. 3B, $P > 0.05$). Finally, co-incubation with PF998425 or ICI182780 was not found to alter the suppressive effect of FA on *CCL2, IL-6,* and *IL-8* expression following IL-1β stimulation (Fig. 3C–D, $P > 0.05$). We also examined the effect of these antagonists on TA signalling, which confirmed the immunomodulatory effect on *CCL2, IL-6,* and *IL-8* was partly mediated by the glucocorticoid receptor (Supplementary Figure 2).

3.4. FA is neuroprotective in a PD model of retinal degeneration

To determine the neuroprotective properties of FA, we assessed the effect of intravitreal administration of either FA, TA or vehicle in the PD model outer retinal atrophy, using a combination of OCT, ERG, and TUNEL labeling (Fig. 4). OCT analysis showed that FA treatment preserved ONL thickness in the locations 1–2 mm and 2–3 mm superior to the optic nerve – where the PD lesions are located (Natoli et al., 2017a) – compared to controls (Fig. 4A–D, $P < 0.05$). In TA-treated mice however, there was no significant difference in ONL thickness at all locations assessed following PD (Fig. 4A, $P > 0.05$). Analysis of ERG recording showed that FA-treated mice had significantly higher a- and b-wave responses to following PD compared to either the control or TA-treated groups indicating preservation of photoreceptors by FA (Fig. 4E–F, P >

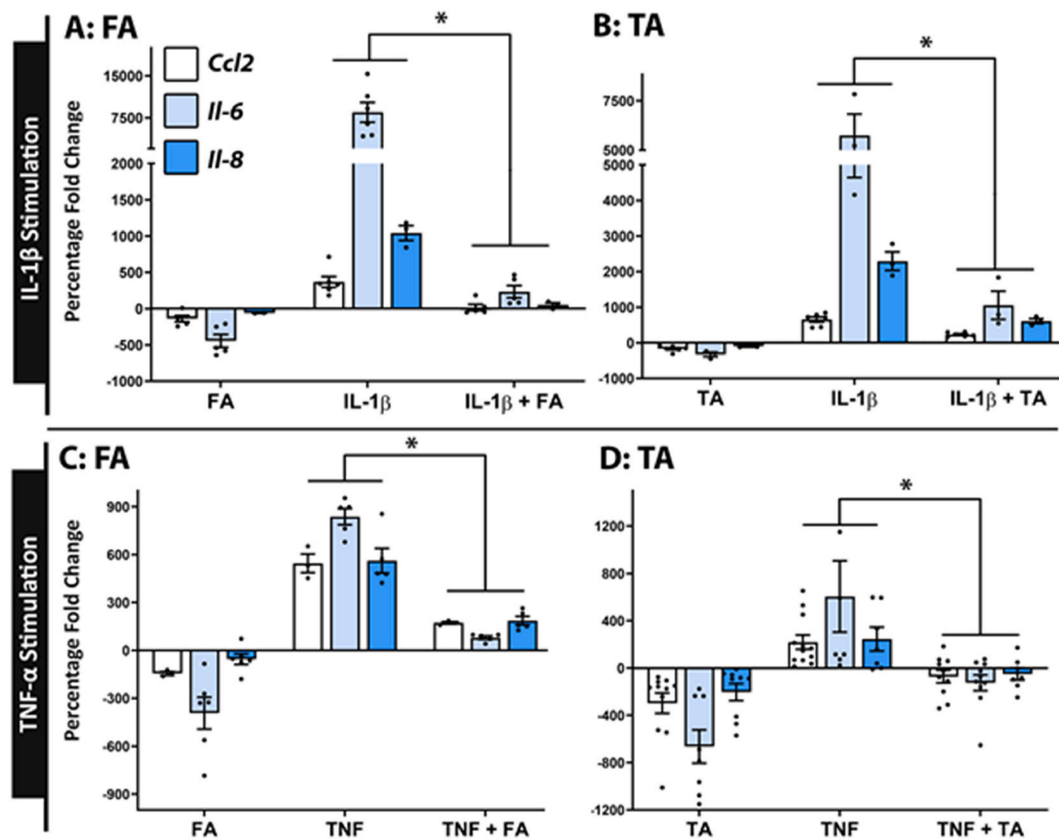

Fig. 2. Modulation of pro-inflammatory cytokine expression by FA and TA *in vitro*. The effect of FA and TA on induction of *CCL2, IL-6* and *IL-8* expression by MIO-M1 cells was assessed following their stimulation with either IL-1β (A–B) or TNF-α (C–D). **A-B:** After stimulation with IL-1β, MIO-M1 cells exhibited profound increases *CCL2, IL-6* and *IL-8* expression ($P < 0.05$). When FA was added to the culture media upon IL-1β stimulation however (A), the up-regulation of *CCL2, IL-6* and *IL-8* was suppressed to near baseline levels ($P < 0.05$). A significant reduction in the expression of *CCL2, IL-6* and *IL-8* was also observed in groups where TA was instead added ($P < 0.05$). **C-D:** Stimulation with TNF-α induced a similar, though less pronounced, up-regulation of *CCL2, IL-6* and *IL-8* in MIO-M1 cells ($P < 0.05$). This effect was also significantly inhibited when FA or TA were added to the culture media ($P < 0.05$). Statistical significance was determined using a one-way ANOVA and Tukey's post-hoc test, where '*' denotes a significant change. Data are presented as the mean ± SEM, N = 5, A-B; N = 5–11, C-D.

0.05). In addition, quantification of apoptosis using TUNEL revealed that FA-treated mice had significantly fewer TUNEL$^+$ photoreceptors following PD, compared to controls ($P < 0.05$, Figure G–J). TA-treated mice also exhibited a slight reduction in TUNEL + photoreceptors, however this was not statistically significant (Fig. 4J, $P < 0.05$). In conjunction, the effect of FA and TA on the accumulation of IBA1$^+$ macrophages in the outer retina was examined (Fig. 5). The following PD, the data show that both formulations suppressed the infiltration of IBA + cells into the ONL compared to controls (Fig. 5A–D, $P < 0.05$).

4. Discussion

The current study identifies beneficial properties of FA in suppressing retinal inflammation and cell death. In addition, we shed light on the mechanism by which FA exerts this effect. First, the data show that FA and TA have no impact on viability across multiple human retinal cell lines over a broad concentration range. Second, we demonstrate that FA and TA dramatically reduce the expression of pro-inflammatory cytokines and chemokines in IL-1β- or TNFα-activated Müller glia, an immunomodulatory cell of the retinal milieu. Third, we reveal that the suppressive effect of FA on *CCL2, IL-6*, and *IL-8*, is dependent on binding through the glucocorticoid receptor, as opposed to the mineralocorticoid receptor. Finally, our data indicate that intravitreal administration FA suppresses photoreceptor death and improves visual function following PD, while TA showed no significant effect.

Though the literature is indeed rich in studies investigating the cytotoxicity and parameters of commonly known corticosteroids, such as TA, on retinal cell culture (Chung et al., 2007; Kuppermann et al., 2014; Shaikh et al., 2006; Yeung et al., 2003), there has been no investigation of the effects of FA in the retinal environment. The cytotoxicity of corticosteroids and their respective vehicle formulations, particularly TA, has been with some controversy (Chang et al., 2007; Kuppermann et al., 2014; Ye et al., 2014; Yeung et al., 2004), highlighting the importance of ascertaining non-cytotoxic concentrations of previously uncharted corticosteroids such as FA in the current study. Our study confirms that FA, as well as TA, are non-cytotoxic over a broad concentration range for all retinal cell cultures examined, including 1 mg/mL. The routine dosage of TA in intravitreal ophthalmic procedures equates to a concentration of 1 mg/ml in the vitreous (Jermak et al., 2007), which underscores the relevance of our findings regarding FA to the application of corticosteroids in the eye previously.

While an anti-inflammatory and protective role of FA has been alluded to in conditions such as septic shock and autoimmune hearing loss (Annane et al., 2018; Trune and Kempton, 2010), this is the first study to (1) dissect the immunomodulatory capacity of FA with respect to retinal cells, and (2) identity the receptor mechanism by which such an effect could be exerted. The data demonstrate an anti-inflammatory effect of FA in Müller cells through inhibition of *CCL2*, *IL-6,* and *IL-8* expression in response IL-1β or TNFα. The presence of glucocorticoid and mineralocorticoid receptors in the eye has been confirmed by previous studies (Wickham et al., 2001), including expression in Müller cells (Bohn et al., 1991). In addition, our data are consistent with previous reports that Müller cells up-regulate expression of *CCL2*, *IL-6* and *IL-8* in response to neurodegenerative and inflammatory stimuli,

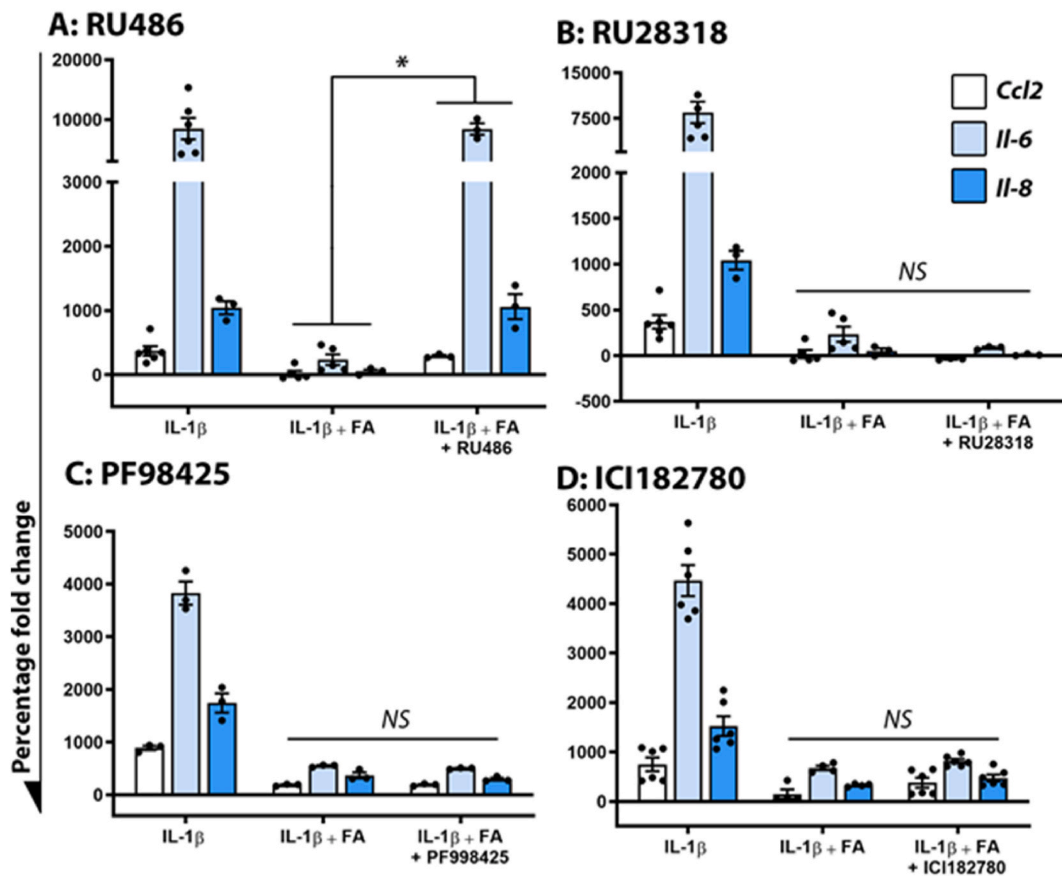

Fig. 3. Contribution of glucocorticoid and mineralocorticoid receptor signaling to the anti-inflammatory effect of FA *in vitro*. The inhibitory effect of FA on *CCL2, IL-6* and *IL-8* expression in IL-1β-stimulated MIO-M1 cells was further investigated through co-incubation with selective antagonists (A–D). **A-B:** The addition of RU486, a glucocorticoid receptor antagonist (A), was found to completely abrogate the FA-mediated suppression of *CCL2, IL-6* and *IL-8* following IL-1β simulation ($P < 0.05$). However, incubation with of RU28318, a mineralocorticoid receptor antagonist (B), did not significantly alter the expression of *CCL2, IL-6* and *IL-8*, compared to MIO-M1 cells treated with IL-1β and FA alone ($P > 0.05$). **C-D:** Incubation of antagonists to either the androgen receptor, PF998425 (C), or the estrogen receptor, ICI182780 (D), did not alter the suppression of *CCL2, IL-6* and *IL*-8 by FA following IL-1β stimulation ($P > 0.05$). Statistical significance was determined using a two-way ANOVA and Tukey's post-hoc test, where '*' denotes a significant change in comparison to FA + IL-1β. Data are presented as the mean ± SEM, N = 5–6, A-B, D; N = 3, C.

including IL-1β, both *in vitro* and in PD (Liu et al., 2014; Rutar et al., 2011, 2012; Wang et al., 2011a). We have also demonstrated that inhibition of IL-1β signaling suppresses the up-regulation of *CCL2* in Müller cells and retinal pathology in PD (Natoli et al., 2017b). In addition, it has been previously demonstrated that IL-1β-mediated up-regulation of IL-8 in Müller cells involves activation of p38 MAPK and ERK1/2 pathways (Liu et al., 2014), which are both known to be modulated by glucocorticoid receptor signaling (Ayroldi et al., 2012).

Though a number of mineralocorticoids have the capacity to bind glucocorticoid receptors, including FA, they commonly bind only to the mineralocorticoid receptor, as seen by examples such as aldosterone (Arriza et al., 1987; Fuller et al., 2000). Examples of crossover in binding of mineralocorticoid to glucocorticoids receptors, and vice-versa, are not unheard in the literature, though the relative contribution of this binding to the physiological functional effect observed in corticosteroids such as FA and TA is poorly characterized (Behar-Cohen and Zhao, 2016; Funder, 2005). Our data reflect an uncommon scenario, indicating that FA, nominally classified as a mineralocorticoid, is exerting an anti-inflammatory effect on *CCL2*, *IL-6* and *IL-8* expression primarily via the glucocorticoid receptor pathway, and is the first study of its kind to demonstrate this finding. There is limited understanding of a potential role for mineralocorticoids in management of retinal inflammation (Jaisser and Farman, 2016), although a study by Bousquet et al. demonstrated a role for aldosterone and the MR pathway in inhibiting inflammation in a rat model of endotoxin-induced inflammation in the retina (Bousquet et al., 2012).

The *in vivo* findings indicate that FA, a mineralocorticoid and glucocorticoid receptor agonist, has a higher neuroprotective profile in PD than TA, predominately a glucocorticoid receptor agonist. We speculate that the divergence in neuroprotective efficacy between FA and TA, in PD, could be due to several factors. Our data suggest that FA has a more pronounced effect on IL-1β-mediated regulation of *CCL2*, *IL-6,* and *IL-8* in Müller glia, which our previous finding show is implicated in promoting deleterious inflammation in the PD model (Natoli et al., 2017b). In addition, previous reports indicate that FA has a higher potency than TA with regard to GR signaling (Parente, 2017), and through which our data indicate that the modulatory effect on *CCL2*, *IL-6,* and *IL*-8 by FA is exerted. On the other hand, a recent report has indicated that Dexamethasone, a glucocorticoid like TA, is protective in PD (Marquioni-Ramella et al., 2020). However, it should be noted that their investigation utilized a damaging light stimulus of a shorter duration and intensity and then the current study, and so we speculate that a more subtle neuroprotective phenotype of TA could be masked by the higher damage profile in our model. Alternatively, or perhaps concurrently, the neuroprotective profile of FA could be partly attributed to mineralocorticoid receptor activity.

While our data indicate that modulation of *CCL2*, *IL-6,* and *IL*-8 by FA is dependent on the glucocorticoid receptor in Müller glia, mineralocorticoid signaling could be exerting an anti-inflammatory effect on other immune pathways pertinent to retinal degeneration, indicated

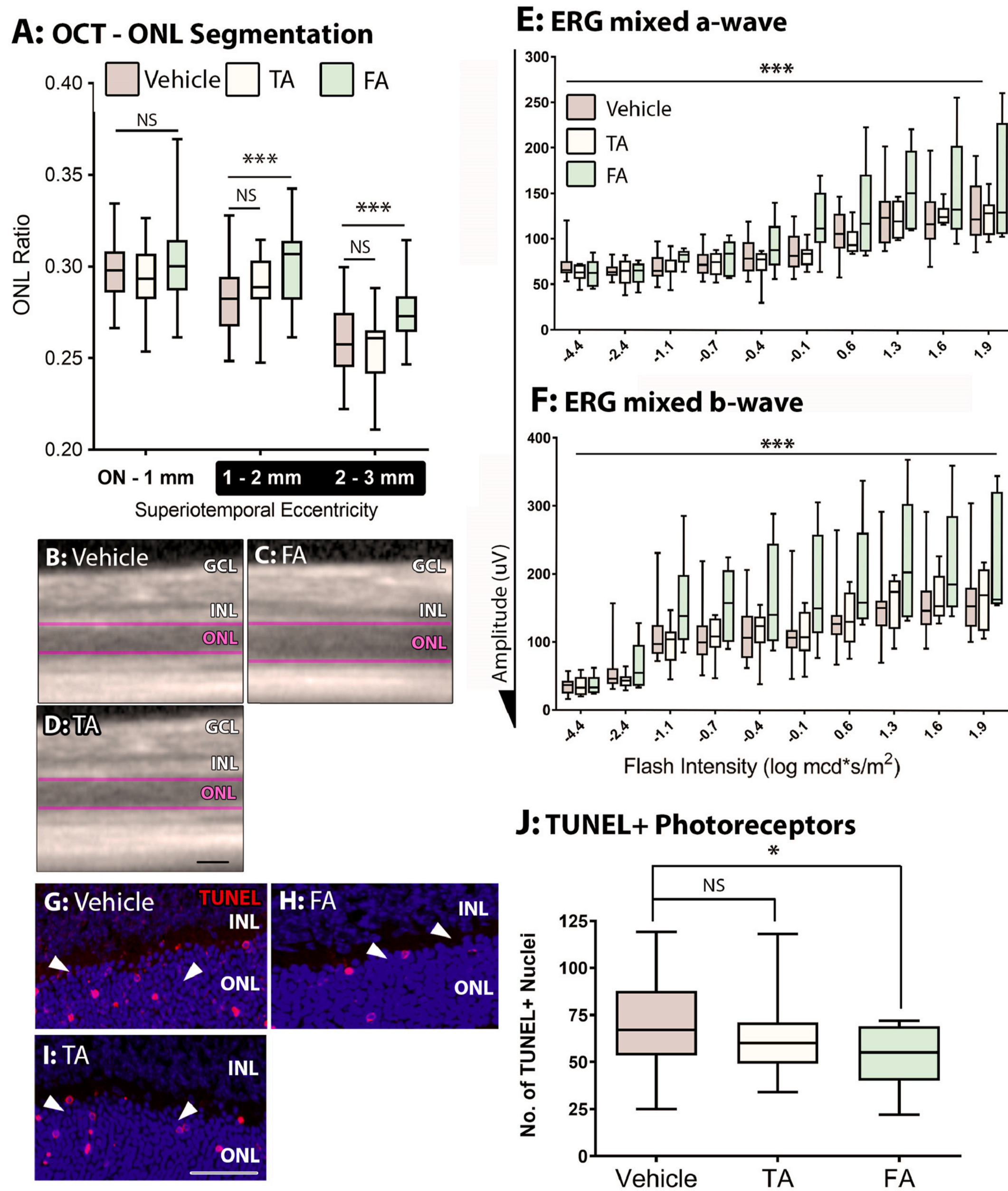

Fig. 4. Neuroprotective properties of FA and TA on retinal cell death in mice following PD. Effect of intravitreal injection of either FA or TA on photoreceptor degeneration following PD was determined with a combination of OCT segmentation (A), ERG recordings (E–F), and abundance of apoptotic TUNEL + photoreceptors (J). **A-D:** ONL measurements quantified from OCT images centered on the lesion area (1–2/2-3 mm eccentricity from the optic nerve) revealed a significant preservation in ONL thickness in the FA treated group (B) compared to controls following PD (C, $P < 0.05$, B). In contrast, there was no significant change in the TA-treated group ($P > 0.05$). **E-F:** FA-treated mice showed significantly larger a-wave (E) and b-wave (F) ERG responses compared to both control and TA-treated groups ($P < 0.05$). **G-J:** Following exposure to PD, mice treated with FA were found to have significantly fewer TUNEL + photoreceptors than vehicle-treated mice ($P < 0.05$, H). Conversely, mice treated with TA exhibited no significant change in the number of TUNEL + photoreceptors ($P > 0.05$, I). Statistical significance was determined using either one-way ANOVA (A, J, Tukey's post-hoc test) or two-way ANOVA (E, F, Tukey's post-hoc test), where '*' denotes a significant change. N = 6–7, A; N = 6–8, E-F, J. Scale bar represents 25 μm. GCL (ganglion cell layer); INL (inner nuclear layer); ONL (outer nuclear layer).

experimental endotoxin-induced Uveitis following aldosterone administration (Bousquet et al., 2012). Though beyond the scope of this investigation, the physiological effect of FA regarding these factors merit further investigation. Though an adverse effect of FA was not observed in our study, it should be noted that over activation of mineralocorticoid receptors via aldosterone may exacerbative pathology in certain diseases, such as retinal edema and central serous chorioretinitis (Behar-Cohen and Zhao, 2016). While aldosterone is more potent than FA in this respect (Heming et al., 2018), and aldosterone administration has indeed been shown beneficial in other conditions such endotoxin-induced Uveitis (Bousquet et al., 2012), it is nevertheless prudent that broad application of mineralocorticoid agonists be approached judiciously, and with regard to safe dosage and potential side effects. Though our data indicate robust neuroprotection with regard to FA in PD, it should also be noted that whether the chronicity this effect extend past 5 days PD is unclear, and a line of investigation worth considering in future studies.

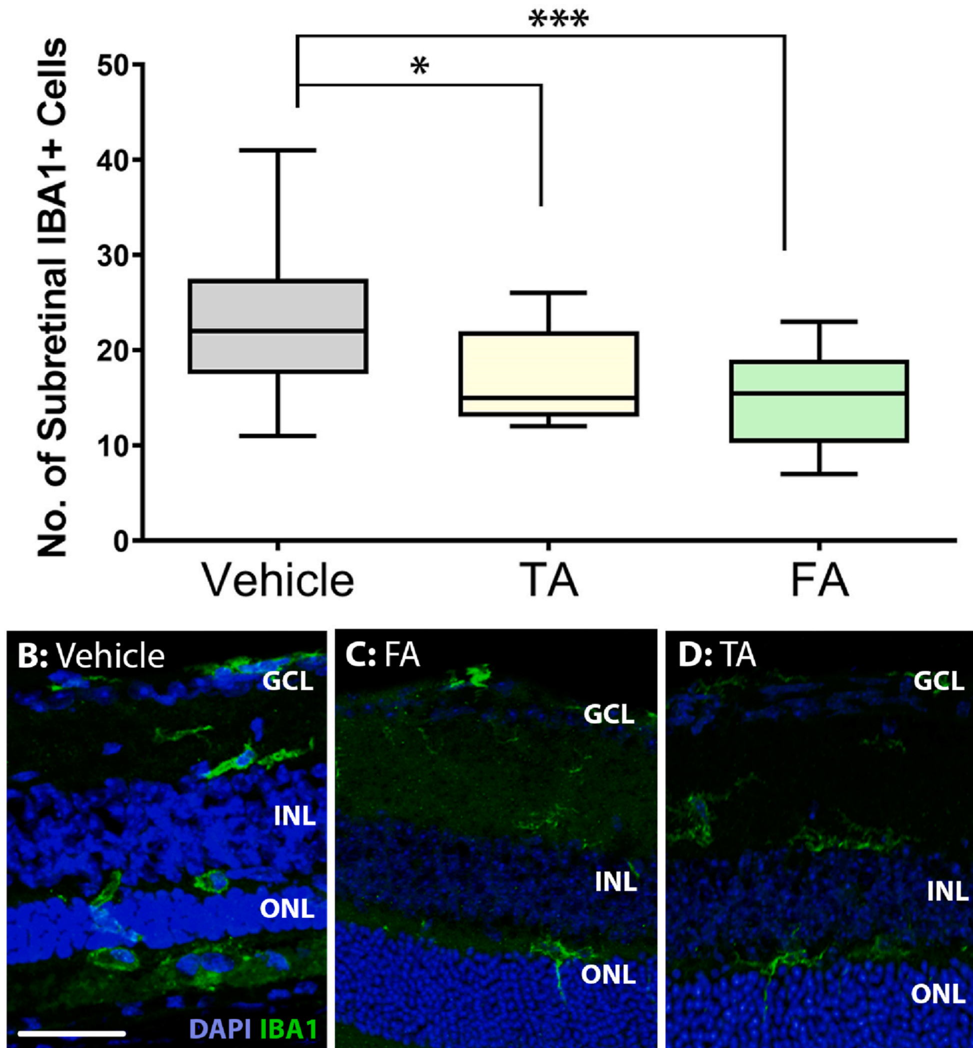

Fig. 5. Effect of FA and TA on macrophage infiltration in mice following PD in mice. The infiltration of activated macrophages to the outer retina following PD was quantified (A) using immunolabeling for IBA1 (B–C, green). **A-D:** There was an abundance of IBA1+ macrophages within the outer retina following PD (A), which coincided spatially with disruption to the ONL and subretinal space (B). In contrast, there was significant reduction of the number IBA1+ cells in both FA- and TA-treated groups (C,D) compared to control (B), with FA showing the most pronounced trend ($P < 0.0001$). Statistical significance was determined using either one-way ANOVA, where '*' denotes a significant change. N = 6–9. Scale bar represents 50 μm. GCL (ganglion cell layer); INL (inner nuclear layer); ONL (outer nuclear layer).

5. Conclusions

The present study offers proof-of-principle support for the application of corticosteroids, specifically FA, in the context of outer retinal atrophy and inflammation. Our data demonstrate the anti-inflammatory and mechanistic properties of FA and TA in Müller cells *in vitro*, via their inhibition of pro-inflammatory factors *CCL2*, *IL-6,* and *IL-8.* Furthermore, our findings demonstrate that FA has higher neuroprotective potency than TA, with FA ameliorating the effects of PD in mice. Our data support further investigation of the mechanistic properties of mineralocorticoid and glucocorticoid agonists such as FA. Though it should be noted that inflammation is one of several pathways implicated in outer retinal dystrophies, such as AMD, our findings nevertheless suggest that FA may be an efficacious strategy for ameliorating neurodegeneration implicated in such disorders.

Financial support

This investigation was supported by grants from the National and Health and Medical Research Council of Australia (NHMRC, GNT1165599), and the Gordon and Gretel Bootes Foundation. Neither of these organizations had any role in the design or undertaking of this research.

Declaration of competing interest

No conflicting relationship exists for any author.

Acknowledgements

The authors would like to thank Professor Ben Boyd for the provision of FA used in the study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exer.2021.108765.

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