The prophylactic value of TNF-α inhibitors against retinal ganglion cell and optic nerve axon loss after corneal surgery or trauma

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Abstract

Background and Purpose: Late secondary glaucoma is an often severe complication after anterior segment surgery, trauma, infection, etc. TNF-α is a major mediator that is rapidly upregulated and causes retinal cell apoptosis and optic nerve axon degeneration (mediating steps to glaucomatous damage). Anti-TNF-α antibodies are in animals very effective in protecting the ganglion cells and the optic nerve—and might therefore be useful prophylactically against secondary glaucoma in patients. Here we evaluate 1) toxicity and 2) efficacy of two TNF-α inhibitors (adalimumab and infliximab), in rabbits by subconjunctival administration.

Methods: For drug *toxicity*, animals with *normal, unburned* corneas were injected with adalimumab (0.4, 4, or 40 mg), or infliximab (1, 10, or 100 mg). For drug *efficacy*, other animals were subjected to alkali burn before such injection, or steroids. The rabbits were evaluated clinically with slit lamp and photography, electroretinography, optical coherence tomography, and intraocular pressure manometry. Some eyes were stained *ex vivo* after 3 days for retinal ganglion cell apoptosis (TUNEL). In other experiments the optic nerves were evaluated with paraphenylenediamine staining after 50 or 90 days. Loss of retinal ganglion cells and optic nerve degeneration were quantified.

Results: Subconjunctival administration of 0.4 mg or 4.0 mg adalimumab were well tolerated, whereas 40.0 mg was toxic to the retina. 1, 10, or 100 mg infliximab were also well tolerated. Analysis of the optic nerve axons after 50 days confirmed the safety of 4.0 mg adalimumab or of 100 mg infliximab.

For *efficacy, 4.0 mg adalimumab subconjunctivally in 0.08 mL* provided practically full protection against ganglion cell apoptosis 3 days following alkali burn, and infliximab 100 mg only slightly less. At 90 days following a burn, the control optic nerve showed about 50% axon loss but only about 8% if protected with adalimumab.

Conclusions: *Subconjunctival injection of 4.0 mg adalimumab* in rabbits shows no eye toxicity and provides excellent neuroprotection, short (3 days) and long-term (90 days). *Our total accumulated data from several studies,z combined with the present paper, suggest that corneal*

injuries, including surgery, might benefit from routine administration of anti-TNF-α biologics to reduce inflammation and future secondary glaucoma.

Introduction

It is well known and well documented by clinical ophthalmologists that acute traumatic events to the cornea (e.g. chemical burns, ruptured globes, infections), as well as standard penetrating corneal surgery (such as transplantations, keratoprosthesis (KPro), lacerations, etc.), often develop a late sight-threatening optic nerve neuropathy that phenotypically appears similar or identical to chronic open-angle or closed-angle glaucomas[1-30]. However, due to having an identifiable triggering cause, this category has often been labeled "secondary glaucoma." Secondary glaucoma is the most consequential complication after corneal surgery.

The magnitude of this secondary glaucomatous complication has most likely been underestimated in the past due to its frequently delayed manifestation, sometimes many years ("time bomb"). Epidemiological studies on secondary glaucoma are therefore limited and numbers citing incidence and outcomes vary substantially with geography and level of economy of the area[31-37]. One source estimated that about 6 million patients in the world have secondary glaucoma compared with 67 million with the primary glaucomas[4]. In totality, the prevalence of secondary glaucoma across the world has been stated to vary from 6 to 22% among various glaucoma studies[31]. WHO in 2002 estimated the prevalence of *blindness* from secondary glaucoma to be 2.7 million people worldwide[37]. Glaucomatous blindness is of course presently irreversible.

The immediate cause of secondary glaucoma, according to many studies, has been primarily attributed to surgery or trauma with corresponding inflammation rather than to chronic diseases[32]—pointing to "an acute single-event episode." Such an acute episode should require only a relatively short period of treatment, including prophylaxis against complications such as secondary glaucoma. The majority of postsurgical glaucoma has been described as unilateral, but the eventual visual outcome has been severe with a very high percentage of angle closure[31].

With regards to the pathophysiology of secondary glaucoma there have been recent shifts of view. The IOP was almost universally blamed in the past, especially in cases of angle closure with markedly elevated pressure. However, difficulties in explaining glaucomatous damage fully in the presence of "normal" pressure has led to a greater interest in neuroinflammation and genetics[38-45]. In fact, an intensive research effort has been directed towards key inflammatory

mediators for glaucoma in general, rather than strictly mechanical (IOP) factors (see a review[39]). The TNF-α pathway has received attention in its involvement in these processes.

Our observations in the 1990ies of the often-dramatic effect of anti-TNF-α antibodies in preventing destructive cornea melt around keratoprostheses in autoimmune patients[43,44,46-49] stimulated a series of experimental studies on the pathophysiology of such treatment[49-53]. With secondary glaucoma in mind, we focused on the model of alkali burns of the cornea. These studies showed that such an alkali burn can upregulate TNF-α anteriorly which will very rapidly diffuse to the retina and result in considerable apoptosis of the ganglion cells (TUNEL), and degeneration of their nerve axons ("the hallmarks of glaucoma"). It had earlier been shown in studies by Kinoshita et al. that other cytokines could reach the retina in early stages after corneal burn[59]. (The alkali itself cannot reach the retina—it is effectively buffered at the iris plane[50,52,60].) These events occur very rapidly while IOP is still normal or low, pointing to the existence of a rapid, inflammatory, IOP-independent pathway to secondary glaucomatous damage after acute events elsewhere in the eye[61]. These results have later been corroborated elswhere[62].

Thus, at present, it is not known clinically how much of any late secondary glaucoma may be due to this newly identified pathway in contrast to the classic IOP-triggered influence, but it would be advisable to probe further, especially since *prophylactic prevention* should be a possibility since drugs are already available and can be promptly applied. Thus, importantly, it has been shown that not only corticosteroids but also monoclonal antibodies (mAbs) to TNF-α, such as etanercept, infliximab, or adalimumab, can be markedly protective of the ganglion cells from damage in animals if administered rapidly enough systemically after the instigating event[50, 52,63]. In recently published animal work, we have further demonstrated that prolonged administration of anti-TNF-α antibody to the retina can be achieved by subconjunctival implantation of a polymer-based drug delivery system (DDS)[64]. Although the DDS was loaded with only 85 µg of infliximab (~30 µg/kg), a biological effect was observed for over a month, manifested by a significant reduction of retinal cell apoptosis after an alkali burn to the cornea[65].

However, of considerable importance are also previous findings in animals that not only the described rapid inflammatory response can be suppressed by the biologics but also, quite likely,

the classic IOP-triggered insult as well, since the optic nerve degeneration found after 3 months could have resulted from either or both pathways. Several of these studies showed a very marked effect of the antibodies to protect the ganglion cells and the optic nerve (50-100% protection), strengthening the possibility of prophylaxis of secondary glaucoma at the time of the inflammatory event (surgery, trauma, etc.), and perhaps also shortly afterwards. These findings alone should have substantial therapeutic promise.

Because of these insights and therapeutic possibilities, it seems that further investigations of the ocular use of anti-inflammatory biologics are warranted. Not only postoperative glaucoma is at stake but also other inflammatory complications such as corneal tissue melt, retroprosthetic membranes, uveitis, vitritis, retinal detachments, etc. This study is meant to focus specifically on the following issues: The risks and benefits of the two presently leading marketed TNF-α inhibitors (adalimumab and infliximab), when administered locally to the eyes, should be clarified. Also, it is still not clear whether a drug delivery system (DDS) is the most practical way for the administration, or whether a single injection of an agent would suffice—and be safer and more practical—or whether intravitreal or systemic route would still be preferable for long-term use. The present study has therefore attempted to investigate the toxicity and efficacy of such anti-inflammatory administration by the *subconjunctival* route.

Materials and Methods

Rabbit model

All animal-based procedures were performed in accordance with the Association For Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and the National Institutes of Health Guidance for the Care and Use of Laboratory Animals. This study was approved by the Animal Care Committee of the Massachusetts Eye and Ear Infirmary and Schepens Eye Research Institute. 32 Dutch-belted pigmented rabbits were used for this study and were obtained from Covance (Dedham, MA, USA). Rabbits were used at the ages of 4-10 months.

Rabbit anesthesia, recovery, and euthanasia

Rabbits were anesthetized with intramuscular injection of ketamine hydrochloride INJ, USP (35 mg/kg; KetaVed, VEDCO, St. Joseph, MO, USA), xylazine (5 mg/ kg; AnaSed, LLOYD, Shenandoah, IA, USA), and acepromazine (0.75 mg/kg; PromAce®, Boehringer Ingelheim Vetmedica, Inc. MO, USA). Reversal of anesthesia was obtained with intravenous (IV) yohimbine (0.1 mg/kg; Yobine, LLOYD) administration in a marginal ear vein. Rabbits were placed on a warm pad until becoming sternal and able to move. Anesthetized rabbits were euthanized at the completion of the experiment with 100 mg/kg Fatal Plus IV injection (sodium pentobarbital).

Retinal injury

Sterile inflammatory retinal injury was accomplished by applying a corneal surface alkali burn, an established retinal injury model[50,52]. Topical anesthetic (0.5% proparacaine hydrochloride, Bausch & Lomb, Tampa, FL, USA) was applied to the study eye while the contralateral eye was protected using GenTeal gel (Alcon, Fort Worth, TX, USA). Alkali burn was performed by using an 8-mm diameter filter paper soaked in 2 N NaOH that was applied to the center of the cornea for 10 seconds followed by immediate eye irrigation with saline solution for 15 minutes. Buprenorphine (0.03 mg/kg; Buprenex Injectable, Reckitt Benckiser Health- care Ltd, United Kingdom) was administered subcutaneously prior to the burn procedure for pain management and a transdermal fentanyl patch (12 mcg/hr; LTS Lohmann Therapy System, Corp., NJ, USA) was placed on the right skin to alleviate pain for 3 days.

Dose titration of anti-TNF-α and monoclonal antibodies

Two FDA approved TNF-α inhibitors were selected: a) infliximab (Remicade® Janssen Biotech Inc., Johnson and Johnson, Titusville, NJ, USA), and b) adalimumab (Humira® Abbvie Inc., North Chicago, IL, U.S.A.). Infliximab was reconstituted in 0.8 mL sterile saline for injection and administered subconjunctivally at 1, 10, or 100 mg dose (n=3) in otherwise intact eyes immediately after the irrigation of alkali injury. Likewise, adalimumab in 0.08 mL saline was

administered subconjunctivally at 0.4 mg, 4 mg, and 40 mg dose (n=3). Two sham injection (control) animals were used (one for each study group) that received 0.8 mL of sterile saline subconjunctivally without drug. Subconjunctival injections of infliximab were performed using a 30-G needle and adalimumab using the pre-fitted syringe needle.

Clinical Evaluation

Clinical evaluation was performed on all rabbits before and after treatment and 0.5% proparacaine hydrochloride was applied to the operated eyes. Eyes were photographed using a digital SLR camera (Nikon, Tokyo, Japan) attached to a surgical microscope (S21; Carl Zeiss, Jena, Germany). Remote photography was performed using an iPhone 7plus (Apple Inc) fitted with a magnifying clip-on lens (12x, Pictek Fisheye Lens, Amazon.com Inc, WA, USA).

IOP measurements

Intraocular pressure measurements were performed in anesthetized rabbits using a custom-made intracameral pressure transducer connected to a 27-gauge needle. The device was designed using a differential microelectromechanical pressure sensor 40 PC (Honeywell, Freeport, IL) connected to a 14-bit, 48 kilo samples per second data acquisition NI USB-6009 (National Instruments, Austin, TX), controlled by a proprietary software algorithm operating in Labview 2017 (National Instruments) environment. A special algorithm was designed to compensate for aqueous humor volume displacement during *in vivo* pressure measurements. The device was assembled using microfluidic components (IDEX Health & Science, Oak Harbor, WA) with minimum dead volume. Before measurements, the remaining dead volume of the syringe was pre-filled with sterile water, thus minimizing air compressibility only within the micro-electromechanical cavity, which was co-evaluated by the software algorithm. To perform measurements, the needle was inserted into the anterior chamber of the eye through a temporal clear corneal puncture, adjacent to the limbus, and the needle was advanced approximately 5 mm toward the center of the chamber[52].

In vivo optical coherence tomography

Posterior segment optical coherent tomography (OCT) was performed in anesthetized animals using the Heidelberg Spectralis (Heidelberg Engineering GmbH, Heidelberg, Germany). A speculum was used to retract the lids. Vertical and horizontal raster scans were performed to acquire images of the 4 retinal quadrants adjacent to the optic nerve (ON). Radial scans were performed to image the ON. Quantification of the retinal thickness was performed with image segmentation using ImageJ software version 1.43 or above (NIH, Bethesda, MD; http://imagej.nih.gov/ij). Thickness was measured from the borders of the ganglion cell layer to the outer nuclear layer. Six images of superior, inferior, temporal, and nasal retina were measured. Baseline images were compared to images obtained 50 days after subconjunctival injection.

Retinal damage and cell death

Cell death was assessed in tissue sections using terminal deoxynucleotidyl transferase-mediated dUTP nick- end labeling (TUNEL, Roche TUNEL kit (12156792910; Roche, Basel, Switzerland), as previously described[50,52]. Mounting medium with DAPI (Ultra- Cruz; sc-24941; Santa Cruz Biotechnology, Dallas, TX) was placed over the tissue, followed by a coverslip. Tile images were taken using an epifluorescent microscope (Zeiss Axio Imager M2; Zeiss, Oberkochen, Germany). DAPI signal (blue) was overlayed with Texas red (TUNEL+ cells) and quantified with ImageJ software version 1.43 or above (NIH, Bethesda, MD; http://imagej.nih.gov/ij) to assess the number of TUNEL+ cells overlapping with DAPI in the areas of interest. At least 3 different tissue sections per eye were analyzed, and data were presented as a percentage of the total DAPI area.

Tissue preparation for H&E

Eyes were enucleated at predetermined time points. The eyes were surgically dissected and fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich, St Louis, MO, USA) solution for 3 days at 4ºC. Following fixation, eyes were sagittally dissected and half of the eye ball was embedded in

optimal cutting temperature (OCT) compound and flash-frozen, and the other half in glycol methacrylate. OCT embedded tissues were sectioned at 10 μm thickness and glycol methacrylate embedded at 3 μm and transferred to positively charged glass slides (Superfrost glass slides, Thermo Fisher, IL, USA). Hematoxylin/eosin (H&E) staining was performed for general histologic observation.

Optic nerve evaluation with paraphenylenediamine staining

Optic nerve axon degeneration was evaluated in explanted rabbit eyes using paraphenylenediamine staining (PPD). Optic nerves were dissected from the explanted eyes, fixed in Karnovsky fixative solution for 24 hours at 4ºC, then processed and embedded in acrylic resin. Tissue cross sections (1 μm thick) were stained with 1% PPD in absolute methanol. Each section was mounted onto a glass slide and imaged using a bright field microscope (Nikon eclipse E800 DIC; Tokyo, Japan) with a 100X objective lens. Tile images of the whole nerve section were obtained, and axon degeneration was quantified using ImageJ software, according to previous protocols[52].

Retinal neuroprotection

The efficacy of subconjunctival anti-TNF-α administration in retinal neuroprotection was assessed using the aforementioned ocular burn injury model[50,52]. Immediately after the burn and lavage, the eye received subconjunctival injection of either infliximab (1, 10, or 100 mg) or adalimumab (0.4, 4, or 40 mg). Sterile saline (sham) subconjunctival injection (0.8 mL, n=3) was used as control. Additional controls included: subconjunctival injection of triamcinolone (20 mg, n=3). Retinal protection was assessed using TUNEL assay. Long-term efficacy of subconjunctival 4 mg adalimumab was assessed in rabbit eyes, 90 days after injury using retinal H&E and ON PPD staining.

Statistical analysis

Results were analyzed with the statistical package of social sciences (SPSS) Version 17.0 (Statistical Package for the Social Sciences Inc., Chicago, IL, USA). The normality of continuous variables was assessed using the Kolmogorov-Smirnov test. Quantitative variables were expressed as mean ± standard error of mean (SEM) and qualitative variables were expressed as frequencies and percentages. The Mann-Whitney test was used to assess differences between groups. All tests were two-tailed, and statistical significance was determined at $p < 0.05$. The independent student t-test was used to compare means between two groups, and pairwise t-test to compare changes within the same group. Analysis of variance (ANOVA) was used for comparisons of multiple groups. Alpha level correction was applied, as appropriate, for multiple comparisons.

Results

To assess toxicity, naive Dutch-Belted pigmented rabbits received subconjunctival injection of adalimumab (0.4, 4, 40 mg) or infliximab (1, 10, 100 mg) in one eye. All injections were uneventful. A conjunctival bleb was observed immediately after the injection of a higher dose of adalimumab (40 mg) or infliximab (100 mg), which resolved within a day **(Fig. 1 A-C)**. No bleb was generated following injection of 4 mg adalimumab in 0.08 mL saline **(Fig. 1 D-F)**. Fifty days after injection of the agents, all eyes looked normal, with intact corneal and conjunctival epithelium and lack of signs of inflammation or vascularization **(Fig. 1 C, F)**.

Nor did subconjunctival injection of adalimumab or infliximab, at various doses, cause immediate or long-term intraocular pressure elevation, as assessed by intracameral manometry 7 days before injection and 1, 3, 7, 28 and 50 days after injection **(Fig. 1 G)**. A slight IOP elevation occurred only 7, 28, and 50 days after injection of sterile saline (sham group) but was not statistically significant **(Fig. 1 G)**.

Additional evaluation was performed to assess potential retinal toxicity following subconjunctival injection of adalimumab (up to 40 mg) or infliximab (up to 100 mg). Dark-adapted and light-adapted electroretinography (ERG) were performed at baseline (7 days prior to injection) and 3, 7, 28, and 45 days post injection. Adalimumab and infliximab, at the highest

doses, did not cause appreciable changes, and ERG responses following flash stimulation with 0.01, 3, or 10 cd.S/m2 were similar between injected and un-injected contralateral eyes**.** Moreover, a and b wave amplitudes and time responses were statistically similar with saline injected eyes **(Supplemental figures 1, 2)**.

In vivo histology was performed by using OCT imaging at baseline and 50 days after subconjunctival injection of adalimumab and infliximab. 4 mg of adalimumab did not cause changes in retinal thickness **(Fig. 2 A)**, while high dose (40 mg) caused increase in superior retinal thickness ($P<0.05$) as compared to baseline measurements **(Fig. 2 B)**. No retinal thickness changes were observed following 100 mg subconjunctival injection of infliximab **(Fig. 2 C)**. Lower dose of adalimumab 0.4 or infliximab 1 and 10 mg likewise did not cause changes in retinal thickness measured by OCT.

Further toxicity assessment was performed by analyzing the optic nerve axons 50 days after subconjunctival injection of either 0.4, 4, 40 mg of adalimumab or 1, 10, 100 mg of infliximab. The results confirmed the safety of 4 mg adalimumab and the adverse effect of 40 mg adalimumab, which resulted in axonal degeneration and drop-out, as compared to the contralateral un-injected eye and control saline (sham) injected eye **(Fig. 2 A, B, D-F, H, I)**. Infliximab 100 mg on the other hand did not cause optic nerve axonal degeneration/drop-out, consistent with the OCT retinal thickness measurements **(Fig. 2 C, G, J)**.

The efficacy of subconjunctival injection of adalimumab and infliximab in acute retinal protection was evaluated 3 days after corneal burn. We have previously shown that corneal alkali burn causes acute uveal inflammation, release of TNF-α, and subsequent retinal cell apoptosis within 3 days in rabbits[52]. Indeed, saline treated (subconjunctival) rabbits exhibited peripheral and central retinal cell apoptosis within 3 days after the injury, extending in all 3 retinal layers **(Fig. 3 A, B, I)**. In contrast, subconjunctival injection of infliximab 100 mg reduced retinal cell apoptosis **(Fig. 3 C, D, I)** but not as efficiently as subconjunctival adalimumab 4 mg, which provided complete protection to the retina **(Fig. 3 G, H, I, J)**. Likewise, subconjunctival triamcinolone 20 mg was very protective **(Fig. 3 E, F, I, J)**. In contrast, high dose (40 mg)

subconjunctival adalimumab was toxic and caused retinal cell apoptosis instead of retinal protection (compared to sham control) **(Fig. 3 I, J)**.

The long-term efficacy of subconjunctival 4 mg adalimumab in retinal and optic nerve protection was evaluated in a separate study, by evaluating the retina and optic nerve 90 days after corneal burn. A single subconjunctival injection of 4 mg adalimumab after the injury was able to prevent retinal ganglion cells loss and change in retinal thickness, as compared to saline treatment that exhibited significant loss in RGCs and increase in retinal thickness **(Fig. 4 A-D)**. Moreover, analysis of the optic nerve axons confirmed the above results, showing that 4 mg adalimumab was able to preserve 92% of the ON axons at 90 days, as compared to saline treatment which exhibited almost 50% axonal loss **(Fig. 4 E-G)**.

Expected retinal bioavailability of adalimumab after subconjunctival administration has been roughly estimated computationally. It is known that about 0.06% of a hydrophilic compound (Gd-DTPA) permeates to the rabbit retina after subconjunctival injection[66]. Similar results were obtained by computational simulation of protein drug permeation from the subconjunctival space to the retina and vitreous (i.e. 0.1–1% of the dose; fraction of 0.001-0.01)[67]. This estimate takes into account permeability in the sclera, choroid, and RPE as well as drug loss to the conjunctival and choroidal blood flows. Average retinal adalimumab concentration after subconjunctival injection can be estimated using equation:

$$C_{ss,av} = (F_{ret} \times D_{sc}) / (\tau \times CL_{ret})$$

where F_{ret} (retinal bioavailability) = 0.001-0.01, D_{sc} (subconjunctival dose) = 4 mg, τ (follow-up period after the dose) = 72 h (Fig. 5), and CL_{ret} (clearance from the retina/vitreous humor compartment) = 0.066 ml/h[68]. Therefore, adalimumab (4 mg; molecular weight 144,190 g/ml) concentration in the retina/vitreous compartment is expected to be 0.8-8 µg/ml (5.8-58 nM); about 10^3-10^4 times higher than its affinity (K_d = 8.6 pM) to soluble TNF-α[69], and 10-100 times higher than adalimumab affinity towards membrane bound TNF-α (K_d = 468 pM). Thus, it is likely that direct permeation of adalimumab from the subconjunctival space to the retina results in therapeutic activity in the retina.

It is known that substantial systemic drug absorption takes place from the subconjunctival injection site. Experimental data[70] and computational follow-up analysis[67] suggest systemic absorption of 72–83% for large molecules. Then, average steady-state concentration of adalimumab ($C_{ss,av}$) in human plasma and adalimumab quantity entering retina during 72 hours can be estimated as

$$Q_{ret} = P_{RPE} \times S_{RPE} \times C_{ss,av} \times t = P_{RPE} \times S_{RPE} (F \times D_{sc}) / (\tau \times CL) \times t$$

where $P_{RPE} = 0.035 \times 10^{-6}$ cm/s (RPE permeability of bevacizumab from)[71], $S_{RPE} = 12$ cm^2 (area of human RPE)[72], $F = 0.72$, $D_{sc} = 4$ mg, $\tau = 72$ h, CL (plasma clearance) = 12 ml/h[73] and $t = 72$ h. Average concentration in plasma is calculated to be ≈ 3.2 mg/L (22 nM) resulting in distribution of ≈ 0.35 µg of adalimumab across the RPE into the retinal compartment within 72 hours. Permeation of proteins across the blood retinal barrier is slow[74] and the result (0.35 µg) is about 0.01% of the subconjunctival dose. *Therefore, we conclude that direct permeation of adalimumab across the ocular tissues to the retina represents about 10-100 higher bioavailability (≈ 0.1–1%) than its retinal entry via systemic blood circulation (≈ 0.01%).*

(In a very recent rabbit study, the development of a thermosensitive biodegradable slow-release DDS was reported. Using this device loaded with 2 mg infliximab reduced the axon loss after alkali burn to the cornea 3 months earlier, from about 50% (n=4) to about 8% (n=3) [Zhou et al[75]]—thus very similar to what is reported in this paper.)

Discussion

The inflammatory cytokine TNF-α seems to be playing a major role in the triggering of the optic neuropathy after acute traumatic events in the eye[50,63]. Most likely other inflammatory cytokines are involved as well, especially IL-6 and IL-1β[52], but the role of TNF-α in glaucoma has been under strong suspicion for over two decades[76,77]. Development of monoclonal antibodies have helped to pinpoint the role of this cytokine in the process. Thus, it has been previously shown in animals that ganglion cell death after short exposure to high intraocular pressure, can be blocked

by etanercept, a TNF-α antibody[63]. In the likely IOP-independent, inflammatory and rapid pathway described by us in animals, infliximab and adalimumab similarly show a high level of ganglion cell protection[50,52]. Whether these seemingly separate pathophysiological pathways to optic nerve damage (IOP-independent and IOP-triggered) are truly separate or merge into one, remains to be determined.

When interpreting the results on past alkali burn or corneal surgery experiments, the timing of events is important. Thus, the TUNEL staining of the retinal ganglion cell apoptosis performed 3 days after the corneal burn, should reflect only the initial, rapid, seemingly IOP-independent events, whereas the optic nerve degeneration after 50 days (in earlier experiments 90 days[52]) might reflect not only this first phase but also on the second, IOP-related phase during the healing process (increased outflow resistance, etc.). Under any circumstances, both phases might be blocked with prompt delivery of TNF-α inhibitors already on the market, and this therapeutic possibility deserves to be subjected to further evaluation. Here we have tried to identify efficacy, toxicity, and optimal tissue for administration.

Potential toxicity of TNF-α antibodies to the retina received attention already two decades ago, triggered by the introduction of the intravitreal route of administration to target wet macular degeneration. Thus, in one such rabbit study, *intravitreal* adalimumab did not appear toxic in concentrations of 0.5 or 5 mg[78]—these results being pertinent here. Infliximab was tested intravitreally in a similar rabbit study and was found to be non-toxic in doses up to 2.0 mg[79]. Likewise, intravitreal infliximab was found safe in another rabbit study at a dose of up to 1.7 mg[80]. However significant toxicity has been encountered in humans after intravitreal injection of infliximab with doses of 0.5–2.0 mg, which underscores the possible importance of species differences[81-83].

Not only biologics but also corticosteroids are neuroprotective against acute ganglion cell apoptosis and should be useful drugs in this respect. In our experiments in animals the short-acting dexamethasone, for unknown reasons, showed less efficacy than the more long-acting Kenalog® preparation of triamcinolone, in clinically most used concentrations[52]. However, the well-known complications of local corticosteroids, particularly their suppressive effect on

corneal wound healing, limit their use post events to modest concentrations or short duration. Toxicity of steroids has also been previously studied in intravitreal rabbit experiments; 0.5 mg or 1.0 mg triamcinolone into the vitreous did not produce morphological changes in the retina but 4.0 mg, 8.0 mg, and 20 mg produced toxic effects in the outer retina[83]. Another study on intravitreal triamcinolone confirmed that 4.0 mg was retinotoxic and suggested that the vehicle was to blame[84].

It is difficult to compare the results from these toxicology studies with the outcomes from our subconjunctival administrations, but they seem grossly compatible with our findings of a single injection of 4.0 mg adalimumab being well-tolerated subconjunctivally, but 40 mg is not. The computerized exercise on bioavailability (see under Results) supports the intuitive assumption that a lower dose of 4.0 mg adalimumab to the eye would have less systemic toxicity than a subcutaneous injection of 40.0 mg of the same drug.

Not only severe trauma but also less invasive standard corneal surgery (PK, KPro, laceration etc.) can lead to some rapid upregulation of TNF-α in the eye and ganglion cell apoptosis, which may, at least in part, be the cause of subsequent secondary glaucoma[85]. Even though such routine surgery rarely triggers as much inflammation as severe trauma, it makes sense to apply similar prophylactic principles as long as the patients are suspected to be at risk.

The clinical implications of the combined clinical experience in the past and more recent animal experiments point to the need for heavier and more sustained anti-inflammatory medication than is presently practiced after acute ocular trauma or routine surgery. Emphasis should be on very prompt delivery after the acute event, with administration of the drug to an easily accessible part of the eye but where it can reliably protect the retinal ganglion cells—pointing to subconjunctival application as practical and relatively safe. A more detailed pharmacokinetic study on the relative efficacy of various routes of administration of TNF-α inhibitors was beyond the scope of this investigation, however, one may cautiously draw some analogies from work on bevacizumab, the full-length monoclonal antibody against vascular endothelial growth factor (VEGF). Intravitreal injection was by far the most effective route of administration for retinal delivery but subconjunctival injection was sufficient and should be less dangerous in a more

primitive clinical setting. Both pathways resulted in similar systemic exposure, with the subconjunctival route better targeting the cornea[86]. Thus, a subconjunctival injection of anti-TNF-α drugs would be safer and more practical than intravitreal injection for a corneal surgeon, and would also be effective against corneal complications.

A limitation of this study should include the fact that the alkali burn rabbit model used in this paper resulted in a lower percentage of affected ganglion cell than in the mice model used in many previous studies. This seems to be attributed to ocular size differences between models. Also, the number of animals used had to be kept low due to the great expense of the biologics used. However, all critical data have been at least in triplicate.

On the positive side, the low level of toxicity of adalimumab corroborates previous findings. Also, the substantial protective effect on the retinal cells by the biologics and the steroids, in various concentrations, is very high, very persuasive (Fig. 4). Thus, particularly 4 mg adalimumab subconjunctivally seems to suppress apoptosis almost completely (and be locally seemingly non-toxic). Subconjunctival injection of 20 mg triamcinolone as Kenalog® also seems very protective in the rapid, IOP-independent pathway. Particularly encouraging are the more final 3 months results on both ganglion cells and nerve axons. Based on the results of these and earlier animal experiments, it seems reasonable to initiate human studies with biologic low-risk anti-inflammatory regimens following corneal surgery or unexpected trauma. In support, we have already shown in patients that infliximab can have a dramatic effect in preventing corneal tissue melt around a keratoprothesis[49]. Also, FDA has approved the successful use of subcutaneous adalimumab in non-infectious uveitis. A pilot clinical study on the protective effects on the eye should be the next logical step.

Significance statement: This study demonstrates that low dose (4 mg) subconjunctival administration of the TNF-α antibody adalimumab can be safe and very effective in protecting against retinal damage in a rabbit model. This suggests the possibility of using this inhibitor as prophylaxis against late secondary glaucoma, with minimal systemic risk for the patient.

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Figures and Legends

Figure 1. Toxicity: Subconjunctival injection of infliximab and adalimumab in normal eyes.

(A) Photograph of subconjunctival injection of **100 mg infliximab (0.8 ml)** showing elevation of the superior and inferior conjunctiva immediately after the injection as the volume was distributed equally in the superior and inferior conjunctiva. **(B)** The same eye the following day showing lack of bleb and **(C)** at 50 days with normal appearance. **(D)** Photograph of the superior subconjunctiva after injection of **4 mg adalimumab** (**0.08 ml)**. No elevation of the conjunctiva is evident immediately after the injection. **(E - F)** Normal appearance of the eye at day one and 50 days post injection.

(G) Longitudinal intraocular pressure measurements using cannulation in eyes injected subconjunctivally with either 1, 10, or 100 mg of infliximab or 0.4, 4, or 40 mg of adalimumab. No IOP elevation is evident up to 50 days post injection. A slight elevation was observed in the saline (sham) injected eyes at day 28 and 50, which did not reach statistical significance.

Figure 2. Toxicity: Retinal and optic nerve histology, 50 days after subconjunctival administration of adalimumab or infliximab in normal eyes.

Quantification of the retinal thickness from the boundaries of the ganglion cell layer (GCL) to the outer nuclear layer (ONL) shows **(A)** 4 mg adalimumab did not cause any change in the retinal thickness. **(B)** 40 mg adalimumab caused significant increase in the thickness of the superior quadrant 50 days post injection. **(C)** 100 mg infliximab did not cause any appreciable change in retinal thickness.

p-Phenylenediamine (PPD) staining of the optic nerves 50 days after subconjunctival injection of **(D)** saline (sham) or **(E, H)** 4 mg adalimumab did not cause optic axon degeneration. **(F, I)** marked axonal degeneration was evident after subconjunctival injection of 40 mg of adalimumab. All comparisons were performed using the contralateral un-injected eye. **(G, J)** 100 mg of infliximab did not cause appreciable optic nerve axon degeneration.

Scale bar: 50 μm. **(A - C)** Two-way ANOVA with Sidak's correction *P<0.05, **(H - J)** Student t-test ****P<0.0001 (sections of one eye).

Figure 3. Efficacy: Pilot experiment. Acute protection (3 days) of the retina with subconjunctival adalimumab after corneal burn.

Evaluation of peripheral and central retinal cell apoptosis using TUNEL assay 3 days after acute corneal surface injury with alkali. **(A, B)** Saline (sham) treatment results in significant cell apoptosis in all 3 retinal layers. **(C - D)** Subconjunctival administration of 100 mg of infliximab 15 minutes after the injury results in appreciable reduction of retinal cell apoptosis. **(E, F)** Subconjunctival injection of 20 mg triamcinolone also shows almost complete protection to the retina, with some apoptosis evident in the ganglion cell layer of the peripheral retina. **(G, H)** Subconjunctival injection of 4 mg adalimumab provides 100% retinal protection in the peripheral and central retina.

(I) Quantification of retinal cell apoptosis following subconjunctival injection of various doses and drugs. Note that 40 mg adalimumab exacerbates cell apoptosis, indicative of its toxic effect at high dose, however, 4 mg adalimumab provides the most optimal retinal protection, as compared in all studied regimens. **(J)** Two-way ANOVA with Tukey's correction. (n=3: 20 mg trim., 4 mg adalim; n=2: 40 mg adalim; n=1: saline, 100 mg inflix).

Figure 4. Efficacy: Pilot experiment. Long-term protection (90 days) of the retina with 4 mg subconjunctival adalimumab after corneal burn. (A-D)

Histologic examination (H&E) of the retinas with hematoxylin and eosin shows significantly increased in retinal thickness (p= 0.03, n= 3) and reduction in retinal ganglion cell count in the saline treated group (n=3), as compared to adalimumab (4 mg) treated group (n=3) which exhibits minimal changes in retinal thickness and RGC count (p= 0.024, n= 3), indicative of long-term neuroprotective effect. **(E - G)** The saline (sham) treated group exhibits significant 55% reduction in optic nerve axons (p=0.014, n=3) as compared to adalimumab treated eyes that exhibit minimal 9% loss of optic nerve axons. **(C, D, G)** Student t-test.

Table 1. Summary of results of testing adalimumab, infliximab and triamcinolone, injected subconjunctivally, for **(A)** toxicity and **(B)** efficacy in reducing inflammation of the retinal ganglion cells and optic nerve degeneration, after alkali burn to the corneas of rabbits. (The results from Zhou, Robert et al. drug delivery systems with infliximab are added for comparison. See p. 14, Refs 65, 75).

Adalim=adalimumab, Inflix=infliximab, Triam=triamcinolone, DDS=drug delivery system (inserted subconjunctivally)

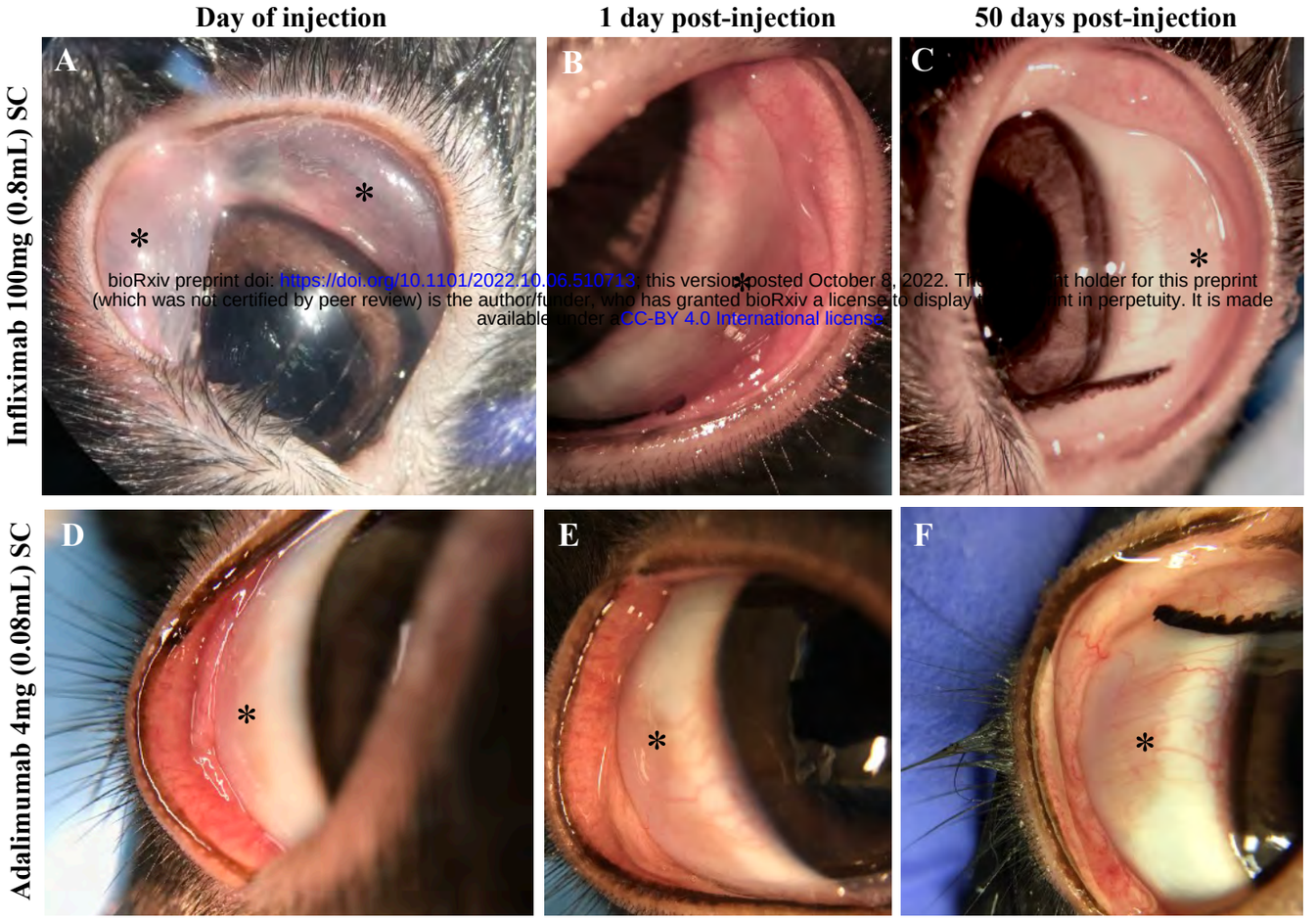
Supplemental Figure 1. **Toxicity: Dark-adapted ERG assessment after subconjunctival injection of adalimumab and infliximab.**

Dark-adapted electroretinography (ERG) using 0.01, 3 and 10 cd.s/m^2 light intensities after subconjunctival injection of **(A - C)** 40 mg adalimumab and **(D - F)** 100 mg infliximab. The contralateral eyes were used as internal controls, while saline (sham) injected eyes served as treatment controls. ERG quantification in **(G)** 40 mg adalimumab injected eyes, **(H)** their corresponding saline control, **(I)** 100 mg infliximab, and **(J)** their corresponding saline control. Measurements were performed at baseline (7 days before injection) and 3, 7, 28, and 45 days after injection. **(G - J)** Quantification was performed for “a" and “b”-wave “A”mplitude and “T”ime responses at“0.01”, “3”, and “10” cd.s/m^2 light intensities in the “OD” injected and “OS” un-injected eyes. High doses of subconjunctival injection of adalimumab and infliximab did not cause appreciable changes in the dark-adapted ERG responses (of optic nerve degeneration after 40 mg of adalimumab).

Supplemental Figure 2. **Toxicity: Light-adapted ERG assessment after subconjunctival injection of adalimumab and infliximab**.

Light-adapted electroretinography (ERG) using 3 cd.s/m^2 flash and flicker light stimulation after subconjunctival injection of **(A, B)** 40 mg adalimumab and **(C, D)** 100 mg infliximab. Their contralateral eyes were used as internal controls, while saline (sham) injected eyes served as treatment controls. ERG quantification in **(E)** 40 mg adalimumab injected eyes, **(F)** their corresponding saline control, **(G)** 100 mg infliximab, and **(H)** their corresponding saline control. Measurements were performed at baseline (7 days before injection) and 3, 7, 28, and 45 days after injection. **(E - H)** Quantification was performed for “a" and “b”-wave “A” amplitude and “T” Time responses at“3” cd.s/m^2 “flash” and “flicker” light intensity in the “OD” injected and “OS” un-injected eyes. High dose subconjunctival injection of adalimumab and infliximab did not cause appreciable changes in the light-adapted ERG responses.

Figure 1



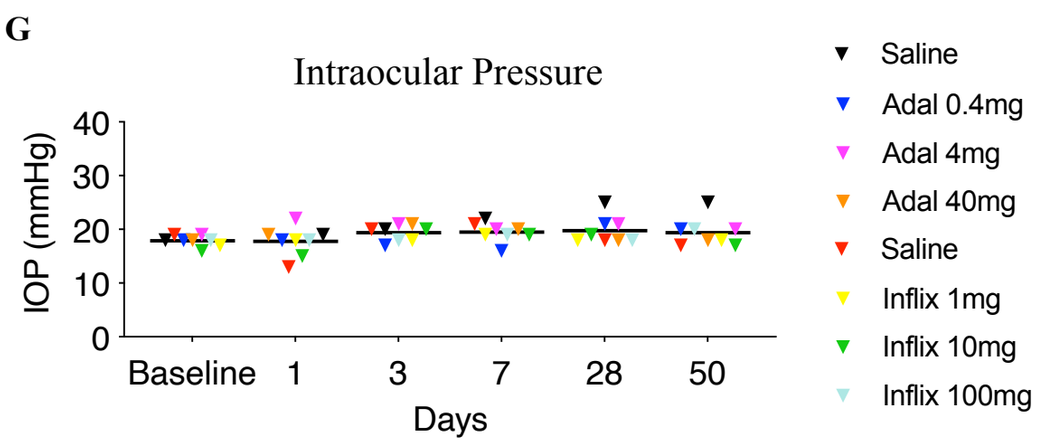


Figure 2

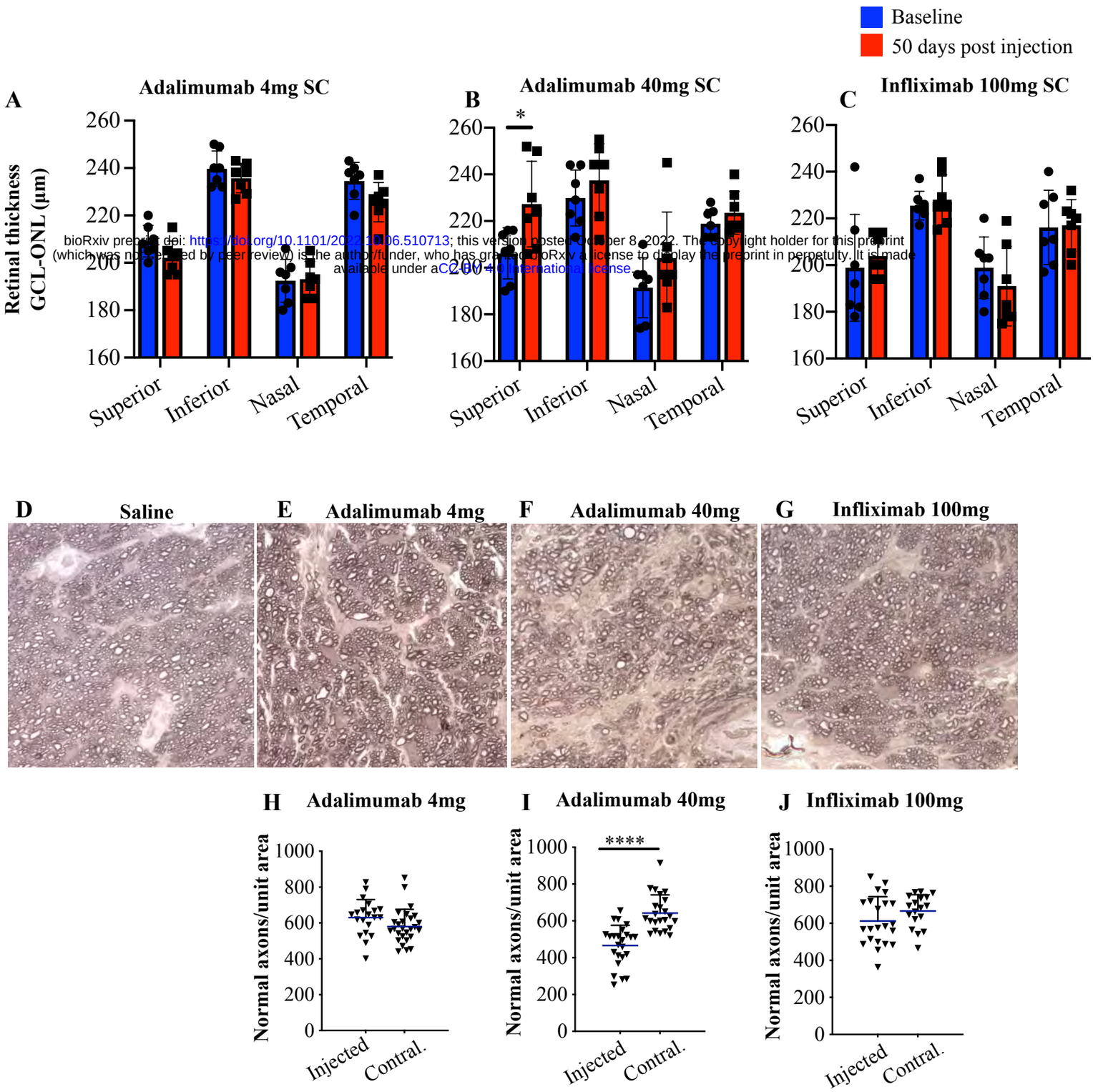
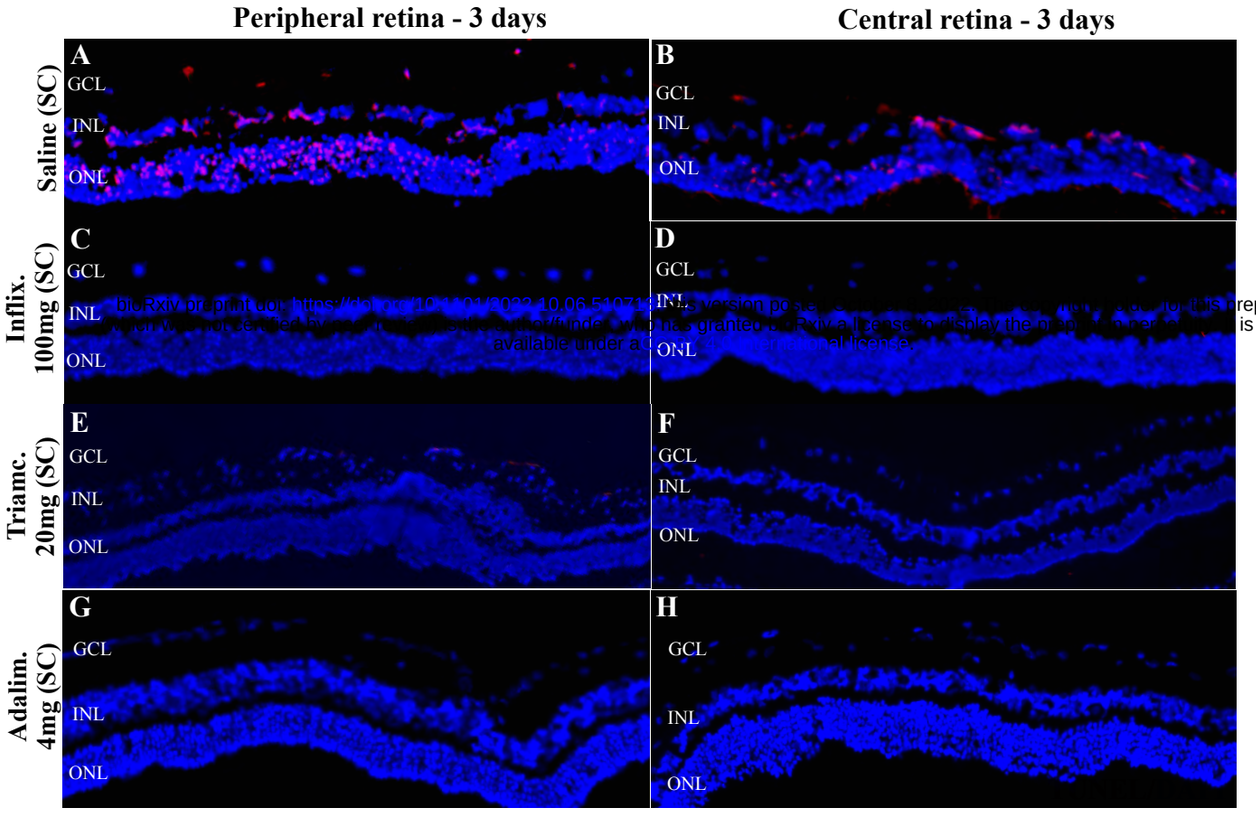
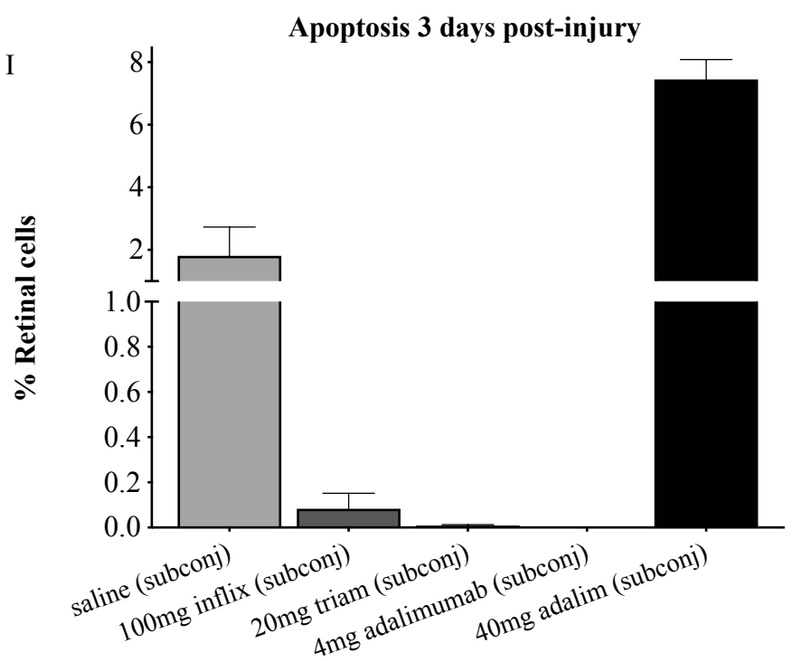


Figure 3





J

Tukey's multiple comparisons test	Adj. P-value
20mg triam (subconj) vs. saline (subconj)	**
20mg triam (subconj) vs. 100mg inflix (subconj)	ns
20mg triam (subconj) vs. 4mg adalimumab (subconj)	ns
20mg triam (subconj) vs. 40mg adalim (subconj)	****
saline (subconj) vs. 100mg inflix (subconj)	*
saline (subconj) vs. 4mg adalimumab (subconj)	**
saline (subconj) vs. 40mg adalim (subconj)	****
100mg inflix (subconj) vs. 4mg adalimumab (subconj)	ns
100mg inflix (subconj) vs. 40mg adalim (subconj)	****
4mg adalimumab (subconj) vs. 40mg adalim (subconj)	****

Figure 4

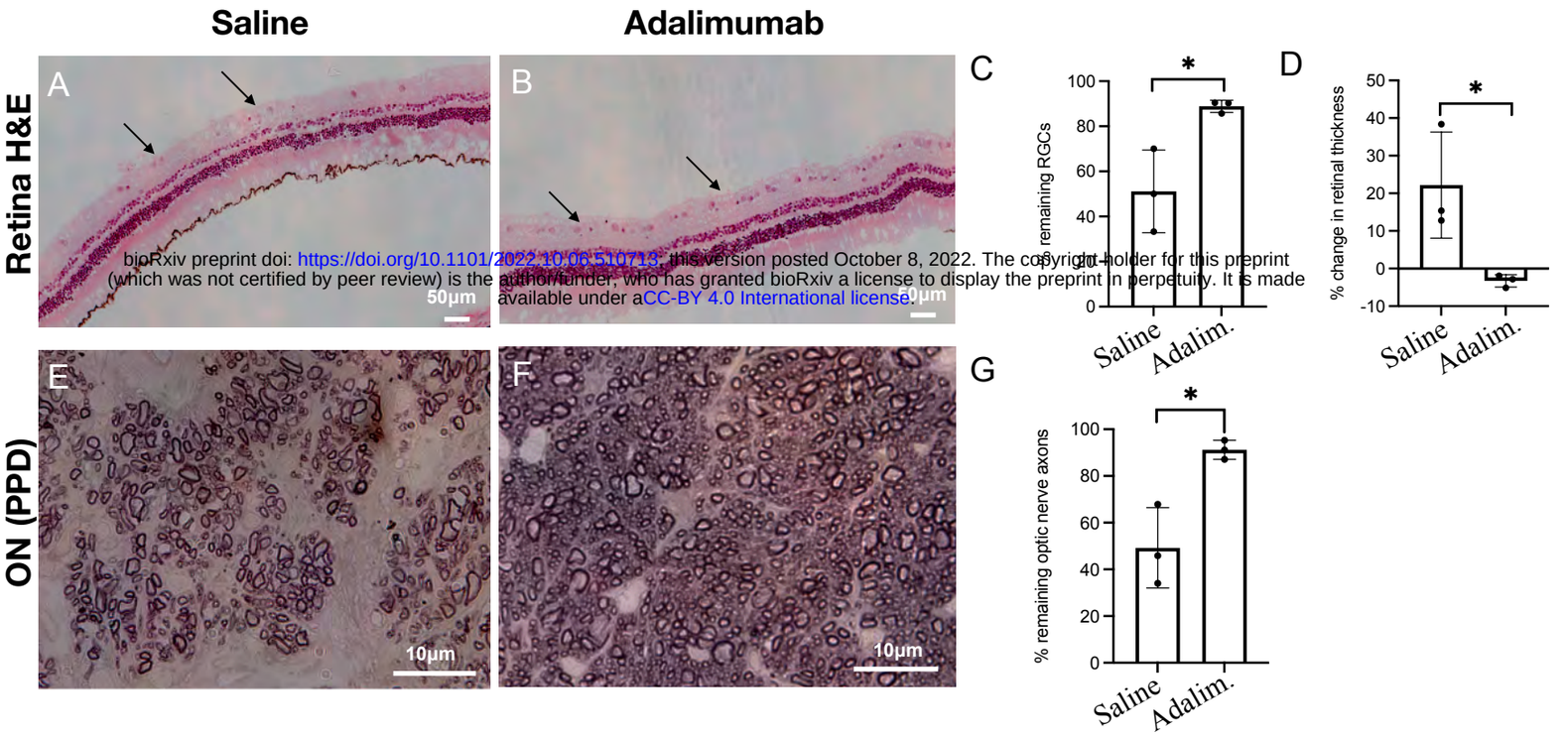


Table 1.

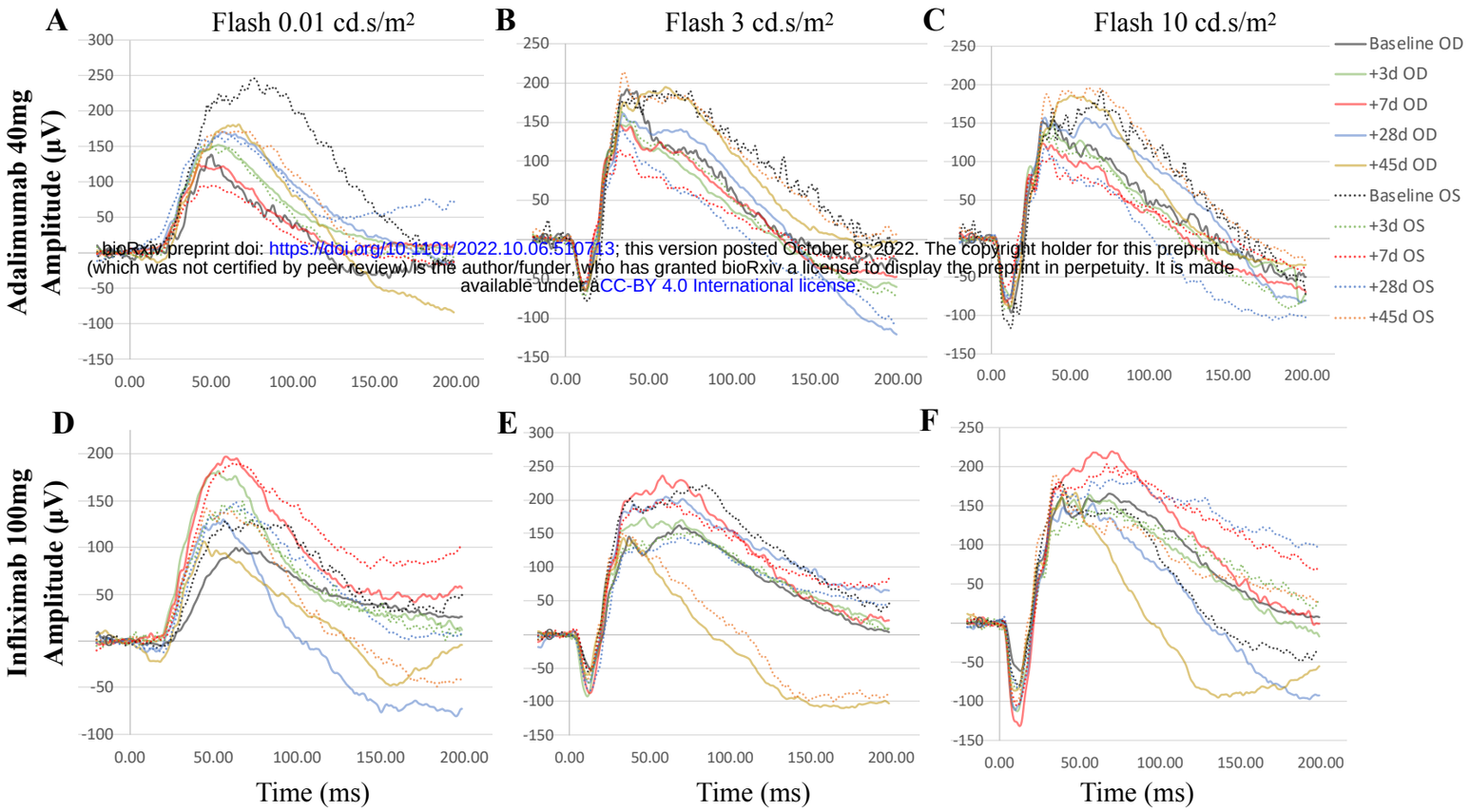
Toxicity to retina or optic nerve from subconjunctival injection of drugs in normal rabbit eyes

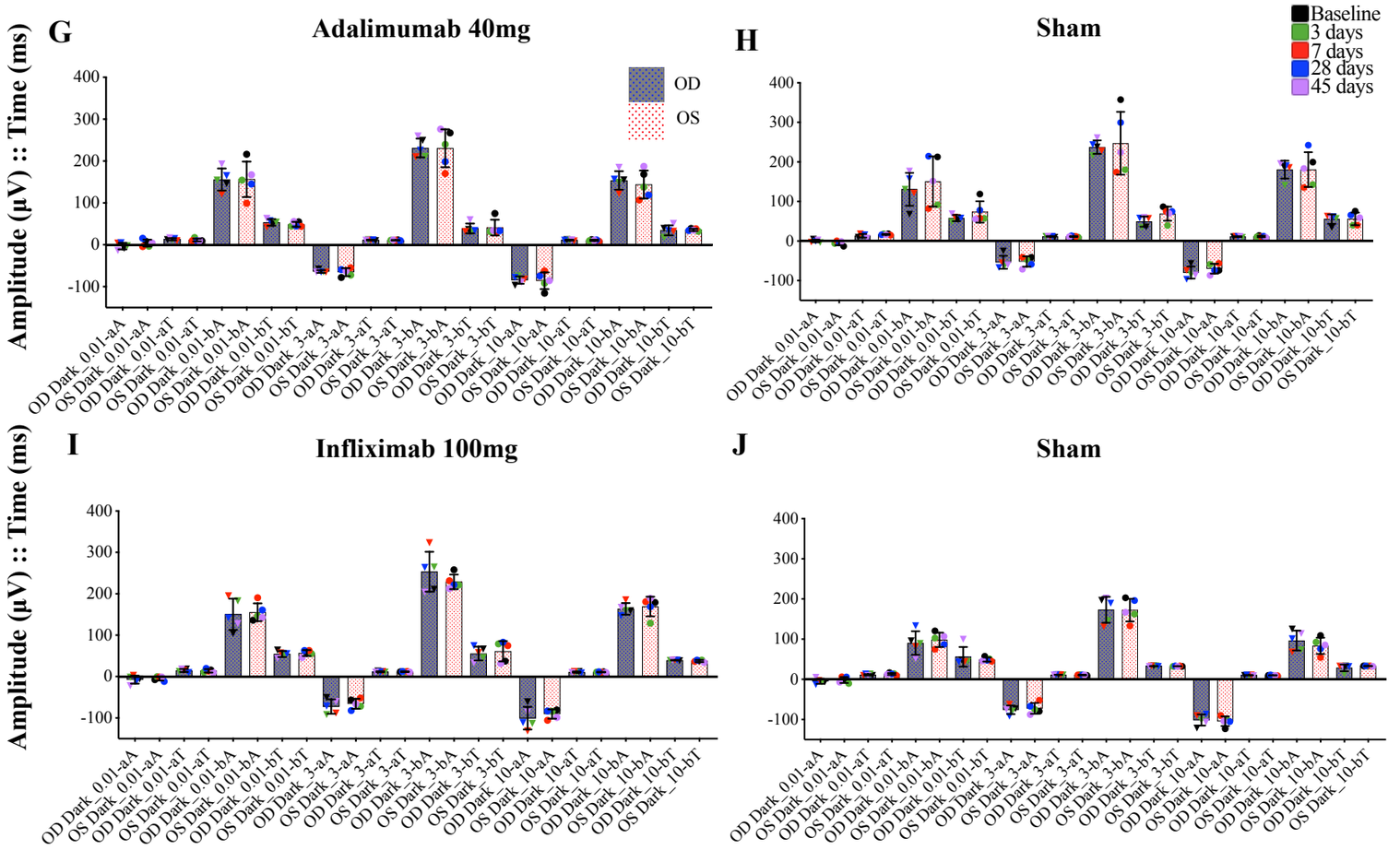
Drug concentration	Observation days/Toxicity		Summarized from
Adalim 0.4 mg	50	-	Fig 1
Adalim 4 mg	50	-	Figs 1, 2
Adalim 40 mg	50	++	Figs 1, 2
Inflix 1 mg	50	-	Fig 1
Inflix 10 mg	50	-	Fig 1
Inflix 100 mg	50	-	Figs 1, 2
Saline (sham)	50	-	Figs 1, 2

Efficacy: Protective effect on retina and optic nerve after burn to cornea

Drug concentration	Observation days/Efficacy				Summarized from
Adalim 0.4 mg	3				
Adalim 4 mg	3	+++	90	+++	Figs 3, 4
Adalim 40 mg	3				
Inflix 1 mg	3				
Inflix 10 mg	3				
Inflix 100 mg	3	++			Fig 3
Triam 20 mg	3	++			Fig 3
Saline (sham)	3	-	90	-	Figs 3, 4
(DDS' Inflix			90	+++	p. 14, refs 65, 75)

Sup. Figure 1. Toxicity evaluation of subconjunctival injection of adalimumab or infliximab at maximum dose with dark-adapted ERG





Sup. Figure 2. Toxicity evaluation of subconjunctival injection of adalimumab or infliximab at maximum dose with light-adapted ERG