The effect of subconjunctival TNF-α inhibitors on retinal ganglion cell apoptosis and optic nerve degeneration after corneal surgery or trauma—of possible prophylactic value against secondary glaucoma.

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Abstract

Background and Purpose: Late secondary glaucoma is an often severe complication after *acute events* like anterior segment surgery, trauma, infection, etc. TNF-α is a major mediator that is rapidly upregulated, diffusing also to the retina and causes apoptosis of the ganglion cells and degeneration of their optic nerve axons (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

121 genetics[38-45]. In fact, an intensive research effort has been directed towards key inflammatory
122 mediators for glaucoma in general, rather than strictly mechanical (IOP) factors (see a review[39]).
123 The TNF-α pathway has received attention in its involvement in these processes.
124
125 Our observations in the 1990ies of the often-dramatic effect of anti-TNF-α antibodies in
126 preventing destructive cornea melt around keratoprostheses in autoimmune patients[43,44,46-49]
127 stimulated a series of experimental studies on the pathophysiology of such treatment[49-53]. With
128 secondary glaucoma in mind, we focused on the model of alkali burns of the cornea. These
129 studies showed that such an alkali burn can upregulate TNF-α anteriorly which will very rapidly
130 diffuse to the retina and result in considerable apoptosis of the ganglion cells (TUNEL), and
131 degeneration of their nerve axons ("the hallmarks of glaucoma"). It had earlier been shown in
132 studies by Kinoshita et al. that other cytokines could reach the retina in early stages after corneal
133 burn[59]. (The alkali itself cannot reach the retina—it is effectively buffered at the iris plane[50,52,60].)
134 These events occur very rapidly while IOP is still normal or low, pointing to the existence of a
135 rapid, inflammatory, IOP-independent pathway to secondary glaucomatous damage after acute
136 events elsewhere in the eye[61]. These results have later been corroborated elswhere[62].
137
138 Thus, at present, it is not known clinically how much of any late secondary glaucoma may be
139 due to this newly identified pathway in contrast to the classic IOP-triggered influence, but it
140 would be advisable to probe further, especially since *prophylactic prevention* should be a
141 possibility since drugs are already available and can be promptly applied. Thus, importantly, it
142 has been shown that not only corticosteroids but also monoclonal antibodies (mAbs) to TNF-α,
143 such as etanercept, infliximab, or adalimumab, can be markedly protective of the ganglion cells
144 from damage in animals if administered rapidly enough systemically after the instigating event[50,
145 52,63]. In recently published animal work, we have further demonstrated that prolonged
146 administration of anti-TNF-α antibody to the retina can be achieved by subconjunctival
147 implantation of a polymer-based drug delivery system (DDS)[64]. Although the DDS was loaded
148 with only 85 μg of infliximab (~30 μg/kg), a biological effect was observed for over a month,
149 manifested by a significant reduction of retinal cell apoptosis after an alkali burn to the cornea[65].
150

151 However, of considerable importance are also previous findings in animals that not only the
152 described rapid inflammatory response can be suppressed by the biologics but also, quite likely,
153 the classic IOP-triggered insult as well, since the optic nerve degeneration found after 3 months
154 could have resulted from either or both pathways. Several of these studies showed a very marked
155 effect of the antibodies to protect the ganglion cells and the optic nerve (50-100% protection),
156 strengthening the possibility of prophylaxis of secondary glaucoma at the time of the
157 inflammatory event (surgery, trauma, etc.), and perhaps also shortly afterwards. These findings
158 alone should have substantial therapeutic promise.
159
160 Because of these insights and therapeutic possibilities, it seems that further investigations of the
161 ocular use of anti-inflammatory biologics are warranted. Not only postoperative glaucoma is at
162 stake but also other inflammatory complications such as corneal tissue melt, retroprosthetic
163 membranes, uveitis, vitritis, retinal detachments, etc. This study is meant to focus specifically on
164 the following issues: The risks and benefits of the two presently leading marketed TNF-α
165 inhibitors (adalimumab and infliximab), when administered locally to the eyes, should be
166 clarified. Also, it is still not clear whether a drug delivery system (DDS) is the most practical
167 way for the administration, or whether a single injection of an agent would suffice—and be safer
168 and more practical—or whether intravitreal or systemic route would still be preferable for long-
169 term use. The present study has therefore attempted to investigate the toxicity and efficacy of
170 such anti-inflammatory administration by the *subconjunctival* route.
171

172 **Materials and Methods**

173 **Rabbit model**

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

181

182 **Rabbit anesthesia, recovery, and euthanasia**

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

191

192 **Retinal injury**

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

203

204 **Dose titration of anti-TNF-α and monoclonal antibodies**

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

209 immediately after the irrigation of alkali injury. Likewise, adalimumab in 0.08 mL saline was
210 administered subconjunctivally at 0.4 mg, 4 mg, and 40 mg dose (n=3). Two sham injection
211 (control) animals were used (one for each study group) that received 0.8 mL of sterile saline
212 subconjunctivally without drug. Subconjunctival injections of infliximab were performed using a
213 30-G needle and adalimumab using the pre-fitted syringe needle.

214

215 **Clinical Evaluation**

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

221

222 **IOP measurements**

223 Intraocular pressure measurements were performed in anesthetized rabbits using a custom-made
224 intracameral pressure transducer connected to a 27-gauge needle. The device was designed using
225 a differential microelectromechanical pressure sensor 40 PC (Honeywell, Freeport, IL)
226 connected to a 14-bit, 48 kilo samples per second data acquisition NI USB-6009 (National
227 Instruments, Austin, TX), controlled by a proprietary software algorithm operating in Labview
228 2017 (National Instruments) environment. A special algorithm was designed to compensate for
229 aqueous humor volume displacement during *in vivo* pressure measurements. The device was
230 assembled using microfluidic components (IDEX Health & Science, Oak Harbor, WA) with
231 minimum dead volume. Before measurements, the remaining dead volume of the syringe was
232 pre-filled with sterile water, thus minimizing air compressibility only within the micro-
233 electromechanical cavity, which was co-evaluated by the software algorithm. To perform
234 measurements, the needle was inserted into the anterior chamber of the eye through a temporal
235 clear corneal puncture, adjacent to the limbus, and the needle was advanced approximately 5 mm
236 toward the center of the chamber[52].

237

238 ***In vivo* optical coherence tomography**

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

249

250 **Retinal damage and cell death**

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

261

262 **Tissue preparation for H&E**

263 Eyes were enucleated at predetermined time points. The eyes were surgically dissected and fixed
264 in 4% paraformaldehyde (PFA) (Sigma-Aldrich, St Louis, MO, USA) solution for 3 days at 4ºC.

265 Following fixation, eyes were sagittally dissected and half of the eye ball was embedded in
266 optimal cutting temperature (OCT) compound and flash-frozen, and the other half in glycol
267 methacrylate. OCT embedded tissues were sectioned at 10 μm thickness and glycol methacrylate
268 embedded at 3 μm and transferred to positively charged glass slides (Superfrost glass slides,
269 Thermo Fisher, IL, USA). Hematoxylin/eosin (H&E) staining was performed for general
270 histologic observation.

271

272 **Optic nerve evaluation with paraphenylenediamine staining**

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

281

282 **Retinal neuroprotection**

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

291

292 **Statistical analysis**

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

303

304 ## Results

305 To assess toxicity, naive Dutch-Belted pigmented rabbits received subconjunctival injection of
306 adalimumab (0.4, 4, 40 mg) or infliximab (1, 10, 100 mg) in one eye. All injections were
307 uneventful. A conjunctival bleb was observed immediately after the injection of a higher dose of
308 adalimumab (40 mg) or infliximab (100 mg), which resolved within a day **(Fig. 1 A-C)**. No bleb
309 was generated following injection of 4 mg adalimumab in 0.08 mL saline **(Fig. 1 D-F)**. Fifty
310 days after injection of the agents, all eyes looked normal, with intact corneal and conjunctival
311 epithelium and lack of signs of inflammation or vascularization **(Fig. 1 C, F)**.
312
313 Nor did subconjunctival injection of adalimumab or infliximab, at various doses, cause
314 immediate or long-term intraocular pressure elevation, as assessed by intracameral manometry 7
315 days before injection and 1, 3, 7, 28 and 50 days after injection **(Fig. 1 G)**. A slight IOP elevation
316 occurred only 7, 28, and 50 days after injection of sterile saline (sham group) but was not
317 statistically significant **(Fig. 1 G)**.
318
319 Additional evaluation was performed to assess potential retinal toxicity following
320 subconjunctival injection of adalimumab (up to 40 mg) or infliximab (up to 100 mg). Dark-
321 adapted and light-adapted electroretinography (ERG) were performed at baseline (7 days prior to
322 injection) and 3, 7, 28, and 45 days post injection. Adalimumab and infliximab, at the highest

323 doses, did not cause appreciable changes, and ERG responses following flash stimulation with
324 0.01, 3, or 10 cd.S/m2 were similar between injected and un-injected contralateral eyes**.**
325 Moreover, a and b wave amplitudes and time responses were statistically similar with saline
326 injected eyes **(Supplemental figures 1, 2)**.
327
328 *In vivo* histology was performed by using OCT imaging at baseline and 50 days after
329 subconjunctival injection of adalimumab and infliximab. 4 mg of adalimumab did not cause
330 changes in retinal thickness **(Fig. 2 A)**, while high dose (40 mg) caused increase in superior
331 retinal thickness ($P<0.05$) as compared to baseline measurements **(Fig. 2 B)**. No retinal thickness
332 changes were observed following 100 mg subconjunctival injection of infliximab **(Fig. 2 C)**.
333 Lower dose of adalimumab 0.4 or infliximab 1 and 10 mg likewise did not cause changes in
334 retinal thickness measured by OCT.
335
336 Further toxicity assessment was performed by analyzing the optic nerve axons 50 days after
337 subconjunctival injection of either 0.4, 4, 40 mg of adalimumab or 1, 10, 100 mg of infliximab.
338 The results confirmed the safety of 4 mg adalimumab and the adverse effect of 40 mg
339 adalimumab, which resulted in axonal degeneration and drop-out, as compared to the
340 contralateral un-injected eye and control saline (sham) injected eye **(Fig. 2 A, B, D-F, H, I)**.
341 Infliximab 100 mg on the other hand did not cause optic nerve axonal degeneration/drop-out,
342 consistent with the OCT retinal thickness measurements **(Fig. 2 C, G, J)**.
343
344 The efficacy of subconjunctival injection of adalimumab and infliximab in acute retinal
345 protection was evaluated 3 days after corneal burn. We have previously shown that corneal alkali
346 burn causes acute uveal inflammation, release of TNF-α, and subsequent retinal cell apoptosis
347 within 3 days in rabbits[52]. Indeed, saline treated (subconjunctival) rabbits exhibited peripheral
348 and central retinal cell apoptosis within 3 days after the injury, extending in all 3 retinal layers
349 **(Fig. 3 A, B, I)**. In contrast, subconjunctival injection of infliximab 100 mg reduced retinal cell
350 apoptosis **(Fig. 3 C, D, I)** but not as efficiently as subconjunctival adalimumab 4 mg, which
351 provided complete protection to the retina **(Fig. 3 G, H, I, J)**. Likewise, subconjunctival
352 triamcinolone 20 mg was very protective **(Fig. 3 E, F, I, J)**. In contrast, high dose (40 mg)

353 subconjunctival adalimumab was toxic and caused retinal cell apoptosis instead of retinal
354 protection (compared to sham control) **(Fig. 3 I, J)**.
355
356 The long-term efficacy of subconjunctival 4 mg adalimumab in retinal and optic nerve protection
357 was evaluated in a separate study, by evaluating the retina and optic nerve 90 days after corneal
358 burn. A single subconjunctival injection of 4 mg adalimumab after the injury was able to prevent
359 retinal ganglion cells loss and change in retinal thickness, as compared to saline treatment that
360 exhibited significant loss in RGCs and increase in retinal thickness **(Fig. 4 A-D)**. Moreover,
361 analysis of the optic nerve axons confirmed the above results, showing that 4 mg adalimumab
362 was able to preserve 92% of the ON axons at 90 days, as compared to saline treatment which
363 exhibited almost 50% axonal loss **(Fig. 4 E-G)**.
364
365 Expected retinal bioavailability of adalimumab after subconjunctival administration has been
366 roughly estimated computationally. It is known that about 0.06% of a hydrophilic compound
367 (Gd-DTPA) permeates to the rabbit retina after subconjunctival injection[66]. Similar results were
368 obtained by computational simulation of protein drug permeation from the subconjunctival space
369 to the retina and vitreous (i.e. 0.1–1% of the dose; fraction of 0.001-0.01)[67]. This estimate takes
370 into account permeability in the sclera, choroid, and RPE as well as drug loss to the conjunctival
371 and choroidal blood flows. Average retinal adalimumab concentration after subconjunctival
372 injection can be estimated using equation:
373
374 $$C_{ss,av} = (F_{ret} \times D_{sc}) / (\tau \times CL_{ret})$$
375
376 where F_{ret} (retinal bioavailability) = 0.001-0.01, D_{sc} (subconjunctival dose) = 4 mg, τ (follow-up
377 period after the dose) = 72 h (Fig. 5), and CL_{ret} (clearance from the retina/vitreous humor
378 compartment) = 0.066 ml/h[68]. Therefore, adalimumab (4 mg; molecular weight 144,190 g/ml)
379 concentration in the retina/vitreous compartment is expected to be 0.8-8 μg/ml (5.8-58 nM);
380 about 10^3-10^4 times higher than its affinity (K_d = 8.6 pM) to soluble TNF-α[69], and 10-100 times
381 higher than adalimumab affinity towards membrane bound TNF-α (K_d = 468 pM). Thus, it is
382 likely that direct permeation of adalimumab from the subconjunctival space to the retina results
383 in therapeutic activity in the retina.

384

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

390

391 $$Q_{ret} = P_{RPE} \times S_{RPE} \times C_{ss,av} \times t = P_{RPE} \times S_{RPE} (F \times D_{sc}) / (\tau \times CL) \times t$$

392

393 where $P_{RPE} = 0.035 \times 10^{-6}$ cm/s (RPE permeability of bevacizumab from)[71], $S_{RPE} = 12$ cm^2 (area
394 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.
395 Average concentration in plasma is calculated to be ≈ 3.2 mg/L (22 nM) resulting in distribution
396 of ≈ 0.35 μg of adalimumab across the RPE into the retinal compartment within 72 hours.
397 Permeation of proteins across the blood retinal barrier is slow[74] and the result (0.35 μg) is about
398 0.01% of the subconjunctival dose. *Therefore, we conclude that direct permeation of*
399 *adalimumab across the ocular tissues to the retina represents about 10-100 higher*
400 *bioavailability (≈ 0.1–1%) than its retinal entry via systemic blood circulation (≈ 0.01%).*

401

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

406

407 **Discussion**

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

414 by etanercept, a TNF-α antibody[63]. In the likely IOP-independent, inflammatory and rapid
415 pathway described by us in animals, infliximab and adalimumab similarly show a high level of
416 ganglion cell protection[50,52]. Whether these seemingly separate pathophysiological pathways to
417 optic nerve damage (IOP-independent and IOP-triggered) are truly separate or merge into one,
418 remains to be determined.
419
420 When interpreting the results on past alkali burn or corneal surgery experiments, the timing of
421 events is important. Thus, the TUNEL staining of the retinal ganglion cell apoptosis performed 3
422 days after the corneal burn, should reflect only the initial, rapid, seemingly IOP-independent
423 events, whereas the optic nerve degeneration after 50 days (in earlier experiments 90 days[52])
424 might reflect not only this first phase but also on the second, IOP-related phase during the
425 healing process (increased outflow resistance, etc.). Under any circumstances, both phases might
426 be blocked with prompt delivery of TNF-α inhibitors already on the market, and this therapeutic
427 possibility deserves to be subjected to further evaluation. Here we have tried to identify efficacy,
428 toxicity, and optimal tissue for administration.
429
430 Potential toxicity of TNF-α antibodies to the retina received attention already two decades ago,
431 triggered by the introduction of the intravitreal route of administration to target wet macular
432 degeneration. Thus, in one such rabbit study, *intravitreal* adalimumab did not appear toxic in
433 concentrations of 0.5 or 5 mg[78]—these results being pertinent here. Infliximab was tested
434 intravitreally in a similar rabbit study and was found to be non-toxic in doses up to 2.0 mg[79].
435 Likewise, intravitreal infliximab was found safe in another rabbit study at a dose of up to 1.7
436 mg[80]. However significant toxicity has been encountered in humans after intravitreal injection of
437 infliximab with doses of 0.5–2.0 mg, which underscores the possible importance of species
438 differences[81-83].
439
440 Not only biologics but also corticosteroids are neuroprotective against acute ganglion cell
441 apoptosis and should be useful drugs in this respect. In our experiments in animals the short-
442 acting dexamethasone, for unknown reasons, showed less efficacy than the more long-acting
443 Kenalog® preparation of triamcinolone, in clinically most used concentrations[52]. However, the
444 well-known complications of local corticosteroids, particularly their suppressive effect on

445 corneal wound healing, limit their use post events to modest concentrations or short duration.
446 Toxicity of steroids has also been previously studied in intravitreal rabbit experiments; 0.5 mg or
447 1.0 mg triamcinolone into the vitreous did not produce morphological changes in the retina but
448 4.0 mg, 8.0 mg, and 20 mg produced toxic effects in the outer retina[83]. Another study on
449 intravitreal triamcinolone confirmed that 4.0 mg was retinotoxic and suggested that the vehicle
450 was to blame[84].
451
452 It is difficult to compare the results from these toxicology studies with the outcomes from our
453 subconjunctival administrations, but they seem grossly compatible with our findings of a single
454 injection of 4.0 mg adalimumab being well-tolerated subconjunctivally, but 40 mg is not. The
455 computerized exercise on bioavailability (see under Results) supports the intuitive assumption
456 that a lower dose of 4.0 mg adalimumab to the eye would have less systemic toxicity than a
457 subcutaneous injection of 40.0 mg of the same drug.
458
459 Not only severe trauma but also less invasive standard corneal surgery (PK, KPro, laceration
460 etc.) can lead to some rapid upregulation of TNF-α in the eye and ganglion cell apoptosis, which
461 may, at least in part, be the cause of subsequent secondary glaucoma[85]. Even though such routine
462 surgery rarely triggers as much inflammation as severe trauma, it makes sense to apply similar
463 prophylactic principles as long as the patients are suspected to be at risk.
464
465 The clinical implications of the combined clinical experience in the past and more recent animal
466 experiments point to the need for heavier and more sustained anti-inflammatory medication than
467 is presently practiced after acute ocular trauma or routine surgery. Emphasis should be on very
468 prompt delivery after the acute event, with administration of the drug to an easily accessible part
469 of the eye but where it can reliably protect the retinal ganglion cells—pointing to subconjunctival
470 application as practical and relatively safe. A more detailed pharmacokinetic study on the
471 relative efficacy of various routes of administration of TNF-α inhibitors was beyond the scope of
472 this investigation, however, one may cautiously draw some analogies from work on
473 bevacizumab, the full-length monoclonal antibody against vascular endothelial growth factor
474 (VEGF). Intravitreal injection was by far the most effective route of administration for retinal
475 delivery but subconjunctival injection was sufficient and should be less dangerous in a more

476 primitive clinical setting. Both pathways resulted in similar systemic exposure, with the
477 subconjunctival route better targeting the cornea[86]. Thus, a subconjunctival injection of anti-
478 TNF-α drugs would be safer and more practical than intravitreal injection for a corneal surgeon,
479 and would also be effective against corneal complications.
480
481 A limitation of this study should include the fact that the alkali burn rabbit model used in this
482 paper resulted in a lower percentage of affected ganglion cell than in the mice model used in
483 many previous studies. This seems to be attributed to ocular size differences between models.
484 Also, the number of animals used had to be kept low due to the great expense of the biologics
485 used. However, all critical data have been at least in triplicate.
486
487 On the positive side, the low level of toxicity of adalimumab corroborates previous findings.
488 Also, the substantial protective effect on the retinal cells by the biologics and the steroids, in
489 various concentrations, is very high, very persuasive (Fig. 4). Thus, particularly 4 mg
490 adalimumab subconjunctivally seems to suppress apoptosis almost completely (and be locally
491 seemingly non-toxic). Subconjunctival injection of 20 mg triamcinolone as Kenalog® also seems
492 very protective in the rapid, IOP-independent pathway. Particularly encouraging are the more
493 final 3 months results on both ganglion cells and nerve axons. Based on the results of these and
494 earlier animal experiments, it seems reasonable to initiate human studies with biologic low-risk
495 anti-inflammatory regimens following corneal surgery or unexpected trauma. In support, we
496 have already shown in patients that infliximab can have a dramatic effect in preventing corneal
497 tissue melt around a keratoprothesis[49]. Also, FDA has approved the successful use of
498 subcutaneous adalimumab in non-infectious uveitis. A pilot clinical study on the protective
499 effects on the eye should be the next logical step.
500
501 **Significance statement:** This study demonstrates that low dose (4 mg) subconjunctival
502 administration of the TNF-α antibody adalimumab can be safe and very effective in protecting
503 against retinal damage in a rabbit model. This suggests the possibility of using this inhibitor as
504 prophylaxis against late secondary glaucoma, with minimal systemic risk for the patient.
505
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Bibliography

1. Becker B, Shaffer RN. Classification of the glaucomas. In: *Diagnosis and Therapy of the Glaucomas*, St. Louis: Mosby. 1961:15-20.

2. Weinreb RN, Aung T, Medeiros FA. The pathophysiology and treatment of glaucoma: a review. *JAMA*. 2014; 311:1901-1911.

3. Quigley HA. Neuronal death in glaucoma. *Prog Retin Eye Res*. 1999; 18:39-57.

4. Quigley HA, Broman AT. The number of people with glaucoma worldwide in 2010 and 2020. *Br J Ophthalmol*. 2006; 90:262-267.

5. Campbell DG. Traumatic glaucoma. In: Bradford J. Shingleton, Peter S. Hersh, Kenneth R. Kenyon eds. *Eye Trauma*, St. Louis: Mosby-Year Book. 1991: 117-125.

6. Kuckelkorn R, Keller GK, Redbrake C. [Glaucoma after extremely severe chemical and thermal eye burns. Surgical possibilities]. *Ophthalmologe*. 2001; 98:1149-1156.

7. Tsai JH, Derby E, Holland EJ, Khatana AK. Incidence and prevalence of glaucoma in severe ocular surface disease. *Cornea*. 2006; 25:530-532.

8. Lin MP, Eksioglu U, Mudumbai RC, Slabaugh MA, Chen PP. Glaucoma in patients with ocular chemical burns. *Am J Ophthalmol*. 2012; 154:481-485.

9. Dohlman CH, Robert MC, Paschalis EI. Treatment of Chemical Burn to the Eye: A Changing Picture. In: Kathryn Colby, Reza Dana eds. *Foundations of Corneal Disease: Past, Present and Future*, Denver, CO: Springer International Publishing. 2020:109-119.

10. Thoft RA, Gordon JM, Dohlman CH. Glaucoma following keratoplasty. *Trans Am Acad Ophthalmol Otolaryngol*. 1974; 78:352-364.

11. Ayyala RS. Penetrating keratoplasty and glaucoma. *Surv Ophthalmol*. 2000; 45:91-105.

12. Aldave AJ, Rudd JC, Cohen EJ, Rapuano CJ, Laibson PR. The role of glaucoma therapy in the need for repeat penetrating keratoplasty. *Cornea*. 2000; 19:772-776.

13. Rumelt S, Bersudsky V, Blum-Hareuveni T, Rehany U. Preexisting and postoperative glaucoma in repeated corneal transplantation. *Cornea*. 2002; 21:759-765.

14. Greenlee EC, Kwon YH. Graft failure: III. Glaucoma escalation after penetrating

547 keratoplasty. *Int Ophthalmol*. 2008; 28:191-207.
548
549 15. Al-Mahmood AM, Al-Swailem SA, Edward DP. Glaucoma and corneal transplant
550 procedures. *J Ophthalmol*. 2012; 2012:576394.
551
552 16. Haddadin RI, Chodosh J. Corneal transplantation and glaucoma. *Semin Ophthalmol*. 2014;
553 29:380-396.
554
555 17. Wu S, Xu J. Incidence and risk factors for post-penetrating keratoplasty glaucoma: A
556 systematic review and meta-analysis. *PLoS One*. 2017; 12:e0176261.
557
558 18. Baltaziak M, Chew HF, Podbielski DW, Ahmed IIK. Glaucoma after corneal replacement.
559 *Surv Ophthalmol*. 2018; 63:135-148.
560
561 19. Liesenborghs I, Schouten J, Berendschot T, Beckers HJM, Nuijts R, Visser N, Webers CAB.
562 Risk factors for the development of ocular hypertension after keratoplasty: A systematic
563 review. *Cornea*. 2020; 39:394-402.
564
565 20. Netland PA, Terada H, Dohlman CH. Glaucoma associated with keratoprosthesis.
566 *Ophthalmology*. 1998; 105:751-757.
567
568 21. Yaghouti F, Nouri M, Abad JC, Power WJ, Doane MG, Dohlman CH. Keratoprosthesis:
569 preoperative prognostic categories. *Cornea*. 2001; 20:19-23.
570
571 22. Harissi-Dagher M, Dohlman CH. The Boston Keratoprosthesis in severe ocular trauma. *Can*
572 *J Ophthalmol*. 2008; 43:165-169.
573
574 23. Cade F, Grosskreutz CL, Tauber A, Dohlman CH. Glaucoma in eyes with severe chemical
575 burn, before and after keratoprosthesis. *Cornea*. 2011; 30:1322-1327.
576
577 24. Crnej A, Paschalis EI, Salvador-Culla B, Tauber A, Drnovsek-Olup B, Shen LQ, Dohlman
578 CH. Glaucoma progression and role of glaucoma surgery in patients with Boston
579 keratoprosthesis. *Cornea*. 2014; 33:349-354.
580
581 25. Kumar RS, Tan DT, Por YM, Oen FT, Hoh ST, Parthasarathy A, Aung T. Glaucoma
582 management in patients with osteo-odonto-keratoprosthesis (OOKP): the Singapore OOKP
583 Study. *J Glaucoma*. 2009; 18:354-360.
584
585 26. Kamyar R, Weizer JS, de Paula FH, Stein JD, Moroi SE, John D, Musch DC, Mian SI.
586 Glaucoma associated with Boston type I keratoprosthesis. *Cornea*. 2012; 31:134-139.

27. Talajic JC, Agoumi Y, Gagne S, Moussally K, Harissi-Dagher M. Prevalence, progression, and impact of glaucoma on vision after Boston type 1 keratoprosthesis surgery. *Am J Ophthalmol*. 2012; 153:267-274.e261.

28. Iyer G, Srinivasan B, Agarwal S, Shetty R, Krishnamoorthy S, Balekudaru S, Vijaya L. Glaucoma in modified osteo-odonto-keratoprosthesis eyes: role of additional stage 1A and Ahmed glaucoma drainage device-technique and timing. *Am J Ophthalmol*. 2015; 159:482-489.

29. Ali MH, Dikopf MS, Finder AG, Aref AA, Vajaranant T, de la Cruz J, Cortina MS. Assessment of Glaucomatous Damage After Boston Keratoprosthesis Implantation Based on Digital Planimetric Quantification of Visual Fields and Optic Nerve Head Imaging. *Cornea*. 2018; 37:602-608.

30. Geoffrion D, Harissi-Dagher M. Glaucoma Risk Factors and Outcomes Following Boston Keratoprosthesis Type 1 Surgery. *Am J Ophthalmol*. 2021; 226:56-67.

31. Dubey S, Jain K, Mukherjee S, Sharma N, Pegu J, Gandhi M, Bhoot M. Current profile of secondary glaucoma in a Northern India tertiary eye care hospital. *Ophthalmic Epidemiol*. 2019; 26:200-207.

32. Gong H, Ren J, Zheng B, Huang X, Liao Y, Zhou Y, Lin M. The Profile of Secondary Glaucoma in China: A Study of Over 10,000 Patients. *J Glaucoma*. 2021; 30:895-901.

33. Suleiman, Y, Amm, M, Duncker, GIW, and Nölle, B. Prognose von Korneatransplantationen nach Bulbusperforation. *Klin Monbl Augenheilkd*. 2004; 221: 658-673.

34. Li, KX, Durrani, AF, Zhou, Y, Zhao, PY, Tannen, BL, Mian, SI, Musch, DC, and Zacks, DN. Outcomes of Penetrating Keratoplasty After Open Globe Injury. *Cornea*. 2021. doi: 10.1097/ico.0000000000002918. Online ahead of print.

35. Doren, GS, Cohen, EJ, Brady, SE, Arentsen, JJ, and Laibson, PR. Penetrating keratoplasty after ocular trauma. *Am J Ophthalmol*. 1990; 110: 408-11.

36. Dana, MR, Moyes, AL, Gomes, JA, Rosheim, KM, Schaumberg, DA, Laibson, PR, Holland, EJ, Sugar, A, and Sugar, J. The indications for and outcome in pediatric keratoplasty. A multicenter study. *Ophthalmology*. 1995; 102(8): 1129-38.

37. World Health Organization, 2004. *International Statistical Classification of Diseases and*

Related Health Problems: Alphabetical index (Vol. 3). World Health Organization.

38. Quigley, HA, Dunkelberger, GR, and Green, WR. Retinal ganglion cell atrophy correlated with automated perimetry in human eyes with glaucoma. *Am J Ophthalmol*. 1989; 107(5): 453-464.

39. Williams, PA, Marsh-Armstrong, N, and Howell, GR. Neuroinflammation in glaucoma: A new opportunity. *Exp Eye Res*. 2017; 157: 20-27.

40. Wiggs, JL. Glaucoma Genes and Mechanisms. *Prog Mol Biol Transl Sci*. 2015; 134: 315-342.

41. Wei X, Cho KS, Thee EF, Jager MJ, Chen DF. Neuroinflammation and microglia in glaucoma: time for a paradigm shift. *J Neurosci Res*. 2019; 97:70-76.

42. Mélik Parsadaniantz S, Réaux-le Goazigo A, Sapienza A, Habas C, Baudouin C. Glaucoma: A Degenerative Optic Neuropathy Related to Neuroinflammation? *Cells*. 2020; 9(3).

43. Dohlman JG, Foster CS, Dohlman CH. Boston Keratoprosthesis in Stevens-Johnson syndrome: a case of using infliximab to prevent tissue necrosis. *Digit J Ophthalmol*. 2009; 15:5-8.

44. Dohlman, CH, Cade, F, Regatieri, CV, Zhou, C, Lei, F, Crnej, A, Harissi-Dagher, M, Robert, MC, Papaliodis, GN, Chen, D, Aquavella, JV, Akpek, EK, Aldave, AJ, Sippel, KC, D'Amico, DJ, Dohlman, JG, Fagerholm, P, Wang, L, Shen, LQ, González-Andrades, M, Chodosh, J, Kenyon, KR, Foster, CS, Pineda, R, Melki, S, Colby, KA, Ciolino, JB, Vavvas, DG, Kinoshita, S, Dana, R, and Paschalis, EI. Chemical Burns of the Eye: The Role of Retinal Injury and New Therapeutic Possibilities. *Cornea*. 2018; 37(2): 248-251.

45. Harwerth RS, Carter-Dawson L, Smith EL, 3rd, Barnes G, Holt WF, Crawford ML. Neural losses correlated with visual losses in clinical perimetry. *Invest Ophthalmol Vis Sci*. 2004; 45:3152-3160.

46. Dohlman CH, Dudenhoefer EJ, Khan BF, Dohlman JG. Corneal blindness from end-stage Sjögren's Syndrome and graft-versus-host disease. In: Sullivan DA, Stern ME, Tsubota K, Dartt DA, Sullivan RM, Bromberg BB eds. *Lacrimal Gland, Tear Film, and Dry Eye Syndromes 3: Basic Science and Clinical Relevance Part B*, Boston: Springer. 2002:1335-1338.

47. Ciralsky J, Papaliodis GN, Foster CS, Dohlman CH, Chodosh J. Keratoprosthesis in autoimmune disease. *Ocul Immunol Inflamm*. 2010; 18:275-280.

48. Robert MC, Črnej A, Shen LQ, Papaliodis GN, Dana R, Foster CS, Chodosh J, Dohlman CH. Infliximab after Boston Keratoprosthesis in Stevens-Johnson Syndrome: An Update. *Ocul Immunol Inflamm*. 2017; 25:413-417.

49. Dohlman CH. The Boston Keratoprosthesis—the first 50 years. Some reminiscences. *Annu Rev Vis Sci.* 2022. in press

50. Cade F, Paschalis EI, Regatieri CV, Vavvas DG, Dana R, Dohlman CH. Alkali burn to the eye: protection using TNF-alpha inhibition. *Cornea*. 2014; 33:382-389.

51. Crnej A, Omoto M, Dohlman TH, Gonzalez-Andrades M, Paschalis EI, Cruzat A, Vu TH, Doorenbos M, Chen DF, Dohlman CH, Dana R. Effect of Penetrating Keratoplasty and Keratoprosthesis Implantation on the Posterior Segment of the Eye. *Invest Ophthalmol Vis Sci*. 2016; 57:1643-1648.

52. Paschalis, EI, Zhou, C, Lei, F, Scott, N, Kapoulea, V, Robert, MC, Vavvas, D, Dana, R, Chodosh, J, and Dohlman, CH. Mechanisms of Retinal Damage after Ocular Alkali Burns. *Am J Pathol*. 2017; 187(6): 1327-1342.

53. Paschalis EI, Taniguchi EV, Chodosh J, Pasquale LR, Colby K, Dohlman CH, Shen LQ. Blood Levels of Tumor Necrosis Factor Alpha and Its Type 2 Receptor Are Elevated in Patients with Boston Type I Keratoprosthesis. *Curr Eye Res*. 2019; 44:599-606.

59. Miyamoto F, Sotozono C, Ikeda T, Kinoshita S. Retinal cytokine response in mouse alkali-burned eye. *Ophthalmic Res*. 1998; 30:168-171.

60. Kompa S, Redbrake C, Hilgers C, Wüstemeyer H, Schrage N, Remky A. Effect of different irrigating solutions on aqueous humour pH changes, intraocular pressure and histological findings after induced alkali burns. *Acta Ophthalmol Scand*. 2005; 83:467-470.

61. Dohlman CH, Zhou C, Lei F, Cade F, Regatieri CV, Crnej A, Dohlman JG, Shen LQ, Paschalis EI. Glaucoma After Corneal Trauma or Surgery-A Rapid, Inflammatory, IOP-Independent Pathway. *Cornea*. 2019; 38:1589-1594.

62. Huang, Y, Yuan, M, Duan, F, Yang, Y, Lou, B, and Lin, X. Inhibition of endoplasmic reticulum stress by 4-phenylbutyrate alleviates retinal inflammation and the apoptosis of retinal ganglion cells after ocular alkali burn in mice. *Inflamm Res*. 2022. doi: 10.1007/s00011-022-01565-3. Online ahead of print.

63. Roh, M, Zhang, Y, Murakami, Y, Thanos, A, Lee, SC, Vavvas, DG, Benowitz, LI, and

Miller, JW. Etanercept, a widely used inhibitor of tumor necrosis factor-alpha (TNF-alpha), prevents retinal ganglion cell loss in a rat model of glaucoma. *PLoS One*. 2012; 7(7): e40065.

64. Robert, MC, Frenette, M, Zhou, C, Yan, Y, Chodosh, J, Jakobiec, FA, Stagner, AM, Vavvas, D, Dohlman, CH, and Paschalis, EI. A Drug Delivery System for Administration of Anti-TNF-alpha Antibody. *Transl Vis Sci Technol*. 2016; 5:11.

65. Zhou C, Robert MC, Kapoulea V, Lei F, Stagner AM, Jakobiec FA, Dohlman CH, Paschalis EI. Sustained Subconjunctival Delivery of Infliximab Protects the Cornea and Retina Following Alkali Burn to the Eye. *Invest Ophthalmol Vis Sci*. 2017; 58:96-105.

66. Kim, H, Robinson, MR, Lizak, MJ, Tansey, G, Lutz, RJ, Yuan, P, Wang, NS, and Csaky, KG. Controlled drug release from an ocular implant: an evaluation using dynamic three-dimensional magnetic resonance imaging. *Invest Ophthalmol Vis Sci*. 2004; 45: 2722-31.

67. Ranta, VP, Mannermaa, E, Lummepuro, K, Subrizi, A, Laukkanen, A, Antopolsky, M, Murtomäki, L, Hornof, M, and Urtti, A. Barrier analysis of periocular drug delivery to the posterior segment. *J Control Release*. 2010; 148(1): 42-48.

68. Subrizi, A, Del Amo, EM, Korzhikov-Vlakh, V, Tennikova, T, Ruponen, M, and Urtti, A. Design principles of ocular drug delivery systems: importance of drug payload, release rate, and material properties. *Drug Discov Today*. 2019; 24(8): 1446-1457.

69. Kaymakcalan, Z, Sakorafas, P, Bose, S, Scesney, S, Xiong, L, Hanzatian, DK, Salfeld, J, and Sasso, EH. Comparisons of affinities, avidities, and complement activation of adalimumab, infliximab, and etanercept in binding to soluble and membrane tumor necrosis factor. *Clin Immunol*. 2009; 131(2): 308-16.

70. Kim, SH, Csaky, KG, Wang, NS, and Lutz, RJ. Drug elimination kinetics following subconjunctival injection using dynamic contrast-enhanced magnetic resonance imaging. *Pharm Res*. 2008; 25(3): 512-20.

71. Ramsay, E, Hagström, M, Vellonen, KS, Boman, S, Toropainen, E, Del Amo, EM, Kidron, H, Urtti, A, and Ruponen, M. Role of retinal pigment epithelium permeability in drug transfer between posterior eye segment and systemic blood circulation. *Eur J Pharm Biopharm*. 2019; 143: 18-23.

72. Panda-Jonas, S, Jonas, JB, Jakobczyk, M, and Schneider, U. Retinal photoreceptor count, retinal surface area, and optic disc size in normal human eyes. *Ophthalmology*. 1994; 101(3): 519-23.

73. Mascelli, MA, Zhou, H, Sweet, R, Getsy, J, Davis, HM, Graham, M, and Abernethy, D. Molecular, biologic, and pharmacokinetic properties of monoclonal antibodies: impact of these parameters on early clinical development. *J Clin Pharmacol*. 2007; 47(5): 553-65.

74. Vellonen, KS, Soini, EM, Del Amo, EM, and Urtti, A. Prediction of Ocular Drug Distribution from Systemic Blood Circulation. *Mol Pharm*. 2016; 13(9): 2906-11.

75. Zhou C, Hui PC, Wolkow N, Dohlman CH, Vavvas DG, Paschalis EI. Corneal and retinal protection using a novel slow-release injectable anti-TNF-α/anti-VEGF drug delivery system. *Invest Ophthalmol Vis Sci*. 2021; ARVO Abstract 3546816.

76. Madigan MC, Sadun AA, Rao NS, Dugel PU, Tenhula WN, Gill PS. Tumor necrosis factor-alpha (TNF-alpha)-induced optic neuropathy in rabbits. Neurol Res. 1996; 18(2):176-184.

77. Yuan, L and Neufeld, AH. Tumor necrosis factor-alpha: a potentially neurodestructive cytokine produced by glia in the human glaucomatous optic nerve head. *Glia*. 2000; 32(1): 42-50.

78. Tsilimbaris M, Diakonis VF, Naoumidi I, Charisis S, Kritikos I, Chatzithanasis G, Papadaki T, Plainis S. Evaluation of potential retinal toxicity of adalimumab (Humira). Graefes Arch Clin Exp Ophthalmol. 2009; 247(8):1119-1125.

79. Theodossiadis PG, Liarakos VS, Sfikakis PP, Charonis A, Agrogiannis G, Kavantzas N, Vergados IA. Intravitreal administration of the anti-TNF monoclonal antibody infliximab in the rabbit. Graefes Arch Clin Exp Ophthalmol. 2009; 247(2):273-281.

80. Giansanti F, Ramazzotti M, Vannozzi L, Rapizzi E, Fiore T, Iaccheri B, Degl' Innocenti D, Moncini D, Menchini U. A pilot study on ocular safety of intravitreal infliximab in a rabbit model. Invest Ophthalmol Vis Sci. 2008; 49(3):1151-1156.

81. Rosenfeld PJ, Goodman KW. When is off-label drug use in the patient's best interest? Am J Ophthalmol. 2009; 147(5):761-763.

82. Giganti M, Beer PM, Lemanski N, Hartman C, Schartman J, Falk N. Adverse events after intravitreal infliximab (Remicade). Retina. 2010; 30(1):71-80.

83. Yu SY, Damico FM, Viola F, D'Amico DJ, Young LH. Retinal toxicity of intravitreal triamcinolone acetonide: a morphological study. Retina. 2006; 26(5):531-536.

84. Lang Y, Zemel E, Miller B, Perlman I. Retinal toxicity of intravitreal kenalog in albino rabbits. Retina. 2007; 27(6):778-788.

85. Chen X, Lei F, Zhou C, Chodosh J, Wang L, Huang Y, Dohlman CH, Paschalis EI. Glaucoma after Ocular Surgery or Trauma: The Role of Infiltrating Monocytes and Their Response to Cytokine Inhibitors. Am J Pathol. 2020; 190(10):2056-2066.

86. Nomoto H, Shiraga F, Kuno N, Kimura E, Fujii S, Shinomiya K, Nugent AK, Hirooka K, Baba T. Pharmacokinetics of bevacizumab after topical, subconjunctival, and intravitreal administration in rabbits. Invest Ophthalmol Vis Sci. 2009; 50(10):4807-4813.

830 ## Figures and Legends

831 **Figure 1. Toxicity: Subconjunctival injection of infliximab and adalimumab in normal**
832 **eyes.**
833 **(A)** Photograph of subconjunctival injection of **100 mg infliximab (0.8 ml)** showing elevation of
834 the superior and inferior conjunctiva immediately after the injection as the volume was
835 distributed equally in the superior and inferior conjunctiva. **(B)** The same eye the following day
836 showing lack of bleb and **(C)** at 50 days with normal appearance. **(D)** Photograph of the superior
837 subconjunctiva after injection of **4 mg adalimumab** (**0.08 ml)**. No elevation of the conjunctiva is
838 evident immediately after the injection. **(E - F)** Normal appearance of the eye at day one and 50
839 days post injection.
840 **(G)** Longitudinal intraocular pressure measurements using cannulation in eyes injected
841 subconjunctivally with either 1, 10, or 100 mg of infliximab or 0.4, 4, or 40 mg of adalimumab.
842 No IOP elevation is evident up to 50 days post injection. A slight elevation was observed in the
843 saline (sham) injected eyes at day 28 and 50, which did not reach statistical significance.
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860 **Figure 2. Toxicity: Retinal and optic nerve histology, 50 days after subconjunctival**
861 **administration of adalimumab or infliximab in normal eyes.**
862 Quantification of the retinal thickness from the boundaries of the ganglion cell layer (GCL) to
863 the outer nuclear layer (ONL) shows **(A)** 4 mg adalimumab did not cause any change in the
864 retinal thickness. **(B)** 40 mg adalimumab caused significant increase in the thickness of the
865 superior quadrant 50 days post injection. **(C)** 100 mg infliximab did not cause any appreciable
866 change in retinal thickness.
867 p-Phenylenediamine (PPD) staining of the optic nerves 50 days after subconjunctival injection
868 of **(D)** saline (sham) or **(E, H)** 4 mg adalimumab did not cause optic axon degeneration. **(F, I)**
869 marked axonal degeneration was evident after subconjunctival injection of 40 mg of
870 adalimumab. All comparisons were performed using the contralateral un-injected eye. **(G, J)** 100
871 mg of infliximab did not cause appreciable optic nerve axon degeneration.
872 Scale bar: 50 µm. **(A - C)** Two-way ANOVA with Sidak's correction *P<0.05, **(H - J)** Student t-
873 test ****P<0.0001 (sections of one eye).
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891 **Figure 3. Efficacy: Pilot experiment. Acute protection (3 days) of the retina with**
892 **subconjunctival adalimumab after corneal burn.**
893 Evaluation of peripheral and central retinal cell apoptosis using TUNEL assay 3 days after acute
894 corneal surface injury with alkali. **(A, B)** Saline (sham) treatment results in significant cell
895 apoptosis in all 3 retinal layers. **(C - D)** Subconjunctival administration of 100 mg of infliximab
896 15 minutes after the injury results in appreciable reduction of retinal cell apoptosis. **(E, F)**
897 Subconjunctival injection of 20 mg triamcinolone also shows almost complete protection to the
898 retina, with some apoptosis evident in the ganglion cell layer of the peripheral retina. **(G, H)**
899 Subconjunctival injection of 4 mg adalimumab provides 100% retinal protection in the peripheral
900 and central retina.
901 **(I)** Quantification of retinal cell apoptosis following subconjunctival injection of various doses
902 and drugs. Note that 40 mg adalimumab exacerbates cell apoptosis, indicative of its toxic effect
903 at high dose, however, 4 mg adalimumab provides the most optimal retinal protection, as
904 compared in all studied regimens. **(J)** Two-way ANOVA with Tukey's correction. (n=3: 20 mg
905 trim., 4 mg adalim; n=2: 40 mg adalim; n=1: saline, 100 mg inflix).
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922 **Figure 4. Efficacy: Pilot experiment. Long-term protection (90 days) of the retina with 4**
923 **mg subconjunctival adalimumab after corneal burn. (A-D)**
924 Histologic examination (H&E) of the retinas with hematoxylin and eosin shows significantly
925 increased in retinal thickness (p= 0.03, n= 3) and reduction in retinal ganglion cell count in the
926 saline treated group (n=3), as compared to adalimumab (4 mg) treated group (n=3) which
927 exhibits minimal changes in retinal thickness and RGC count (p= 0.024, n= 3), indicative of
928 long-term neuroprotective effect. **(E - G)** The saline (sham) treated group exhibits significant
929 55% reduction in optic nerve axons (p=0.014, n=3) as compared to adalimumab treated eyes that
930 exhibit minimal 9% loss of optic nerve axons. **(C, D, G)** Student t-test.
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952 **Table 1**. Summary of results of testing adalimumab, infliximab and triamcinolone, injected
953 subconjunctivally, for **(A)** toxicity and **(B)** efficacy in reducing inflammation of the retinal
954 ganglion cells and optic nerve degeneration, after alkali burn to the corneas of rabbits. (The
955 results from Zhou, Robert et al. drug delivery systems with infliximab are added for comparison.
956 See p. 14, Refs 65, 75).

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

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975 **Supplemental Figure 1**. **Toxicity: Dark-adapted ERG assessment after subconjunctival**
976 **injection of adalimumab and infliximab.**
977 Dark-adapted electroretinography (ERG) using 0.01, 3 and 10 cd.s/m^2 light intensities after
978 subconjunctival injection of **(A - C)** 40 mg adalimumab and **(D - F)** 100 mg infliximab.
979 The contralateral eyes were used as internal controls, while saline (sham) injected eyes served as
980 treatment controls. ERG quantification in **(G)** 40 mg adalimumab injected eyes, **(H)** their
981 corresponding saline control, **(I)** 100 mg infliximab, and **(J)** their corresponding saline control.
982 Measurements were performed at baseline (7 days before injection) and 3, 7, 28, and 45 days
983 after injection. **(G - J)** Quantification was performed for “a" and “b”-wave “A”mplitude and
984 “T”ime responses at“0.01”, “3”, and “10” cd.s/m^2 light intensities in the “OD” injected and “OS”
985 un-injected eyes. High doses of subconjunctival injection of adalimumab and infliximab did not
986 cause appreciable changes in the dark-adapted ERG responses (of optic nerve degeneration after
987 40 mg of adalimumab).
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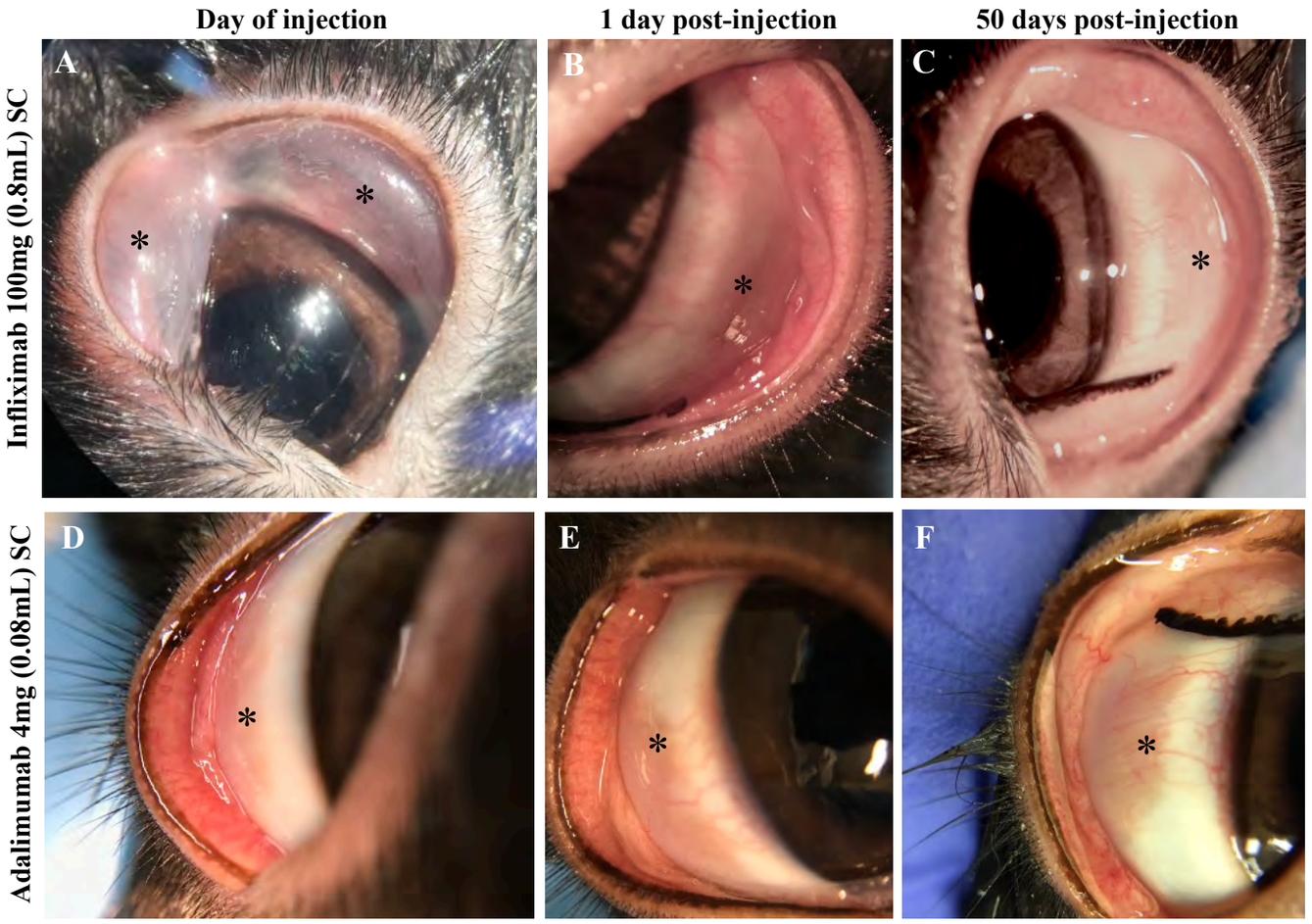
999 **Supplemental Figure 2**. **Toxicity: Light-adapted ERG assessment after subconjunctival**
1000 **injection of adalimumab and infliximab**.
1001 Light-adapted electroretinography (ERG) using 3 cd.s/m^2 flash and flicker light stimulation after
1002 subconjunctival injection of **(A, B)** 40 mg adalimumab and **(C, D)** 100 mg infliximab.
1003 Their contralateral eyes were used as internal controls, while saline (sham) injected eyes served
1004 as treatment controls. ERG quantification in **(E)** 40 mg adalimumab injected eyes, **(F)** their
1005 corresponding saline control, **(G)** 100 mg infliximab, and **(H)** their corresponding saline control.
1006 Measurements were performed at baseline (7 days before injection) and 3, 7, 28, and 45 days
1007 after injection. **(E - H)** Quantification was performed for “a" and “b”-wave “A” amplitude and
1008 “T” Time responses at“3” cd.s/m^2 “flash” and “flicker” light intensity in the “OD” injected and
1009 “OS” un-injected eyes. High dose subconjunctival injection of adalimumab and infliximab did
1010 not cause appreciable changes in the light-adapted ERG responses.
1011

Figure 1


Day of injection
1 day post-injection
50 days post-injection
Infliximab 100mg (0.8mL) SC
Adalimumab 4mg (0.08mL) SC
A
B
C
D
E
F

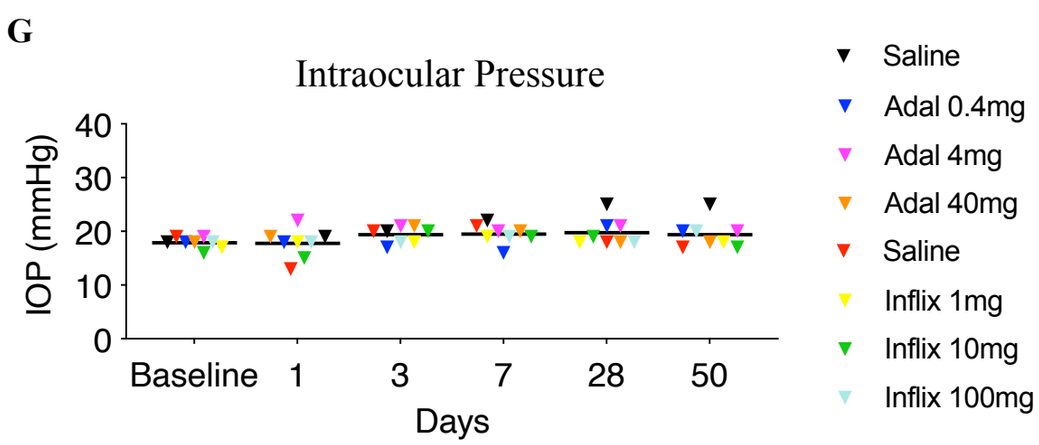

G
Intraocular Pressure
IOP (mmHg)
40
30
20
10
0
Baseline
1
3
7
28
50
Days
Saline
Adal 0.4mg
Adal 4mg
Adal 40mg
Saline
Inflix 1mg
Inflix 10mg
Inflix 100mg

Figure 2

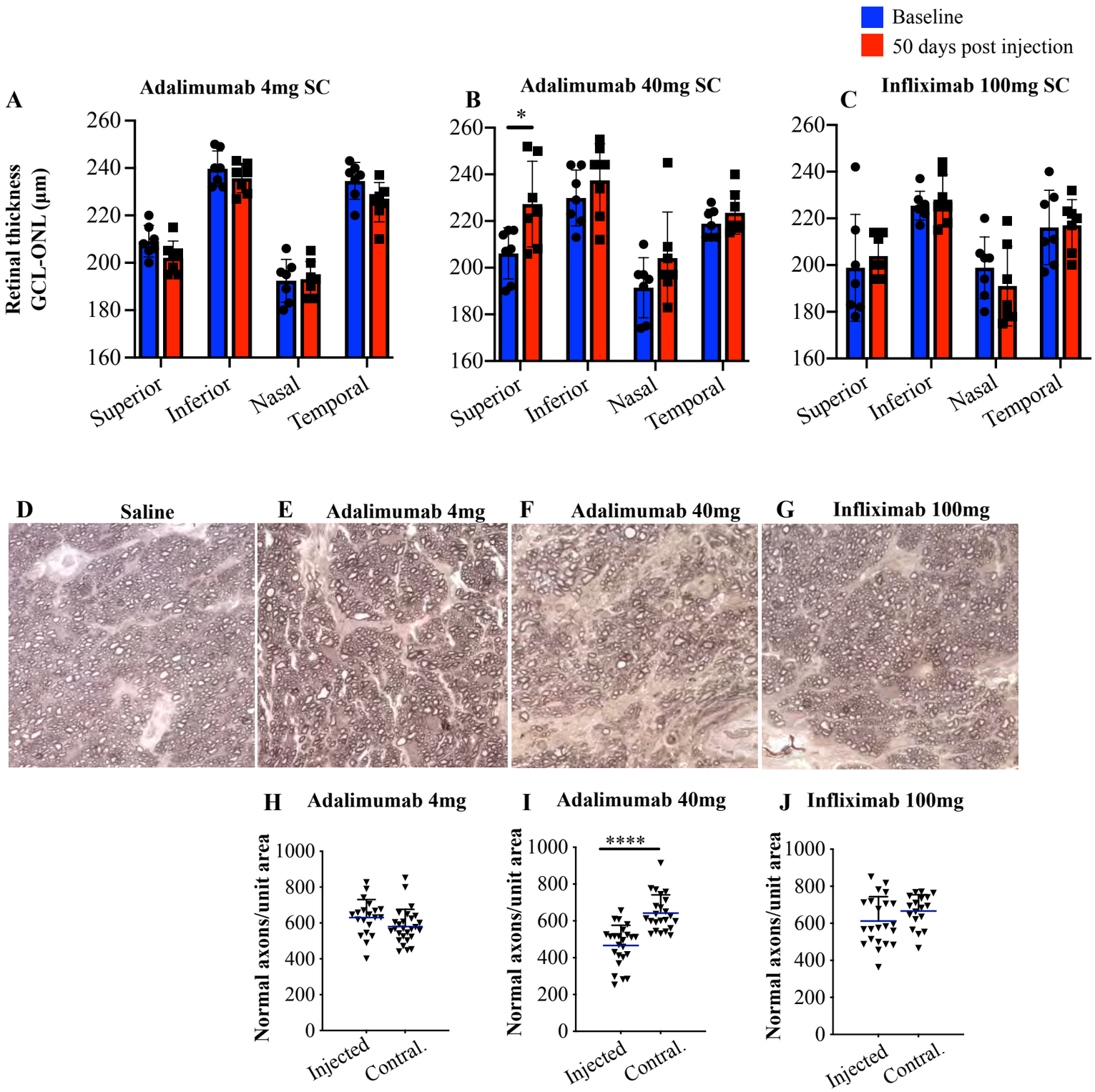
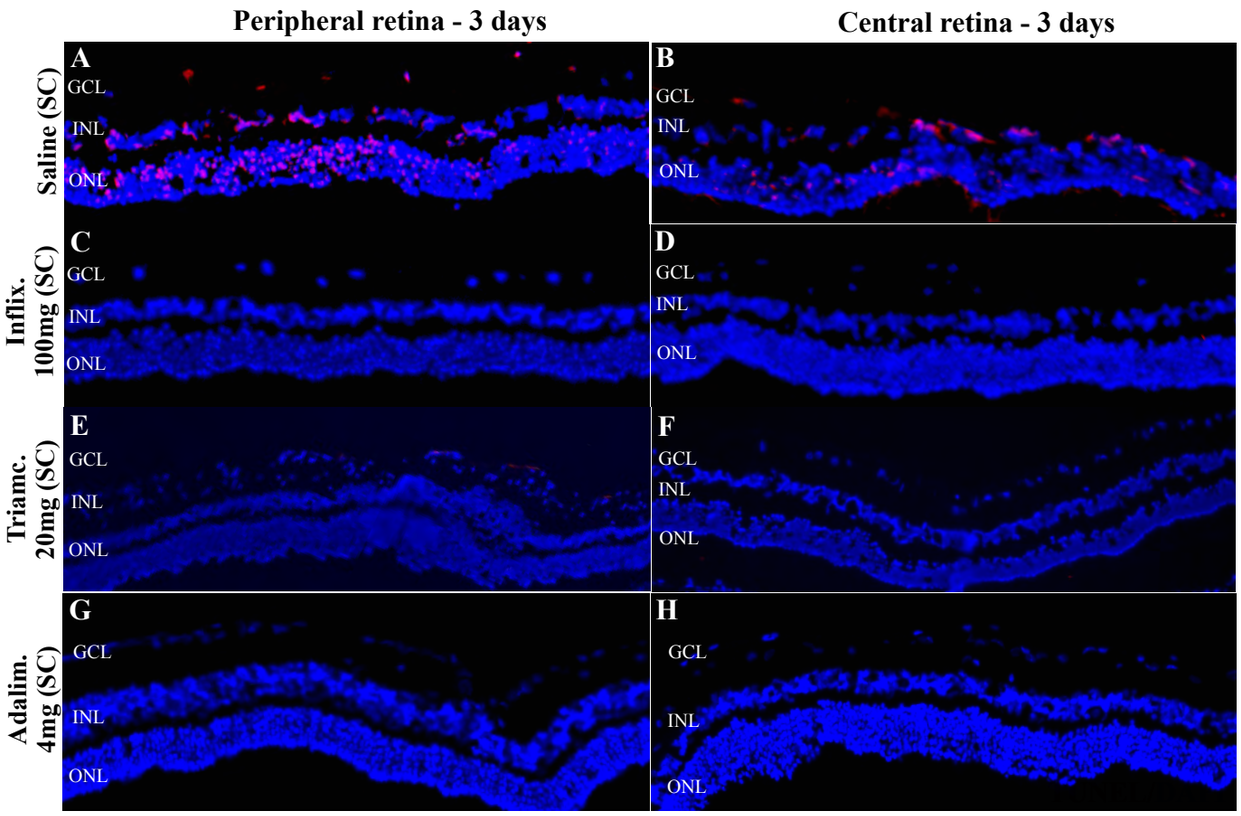
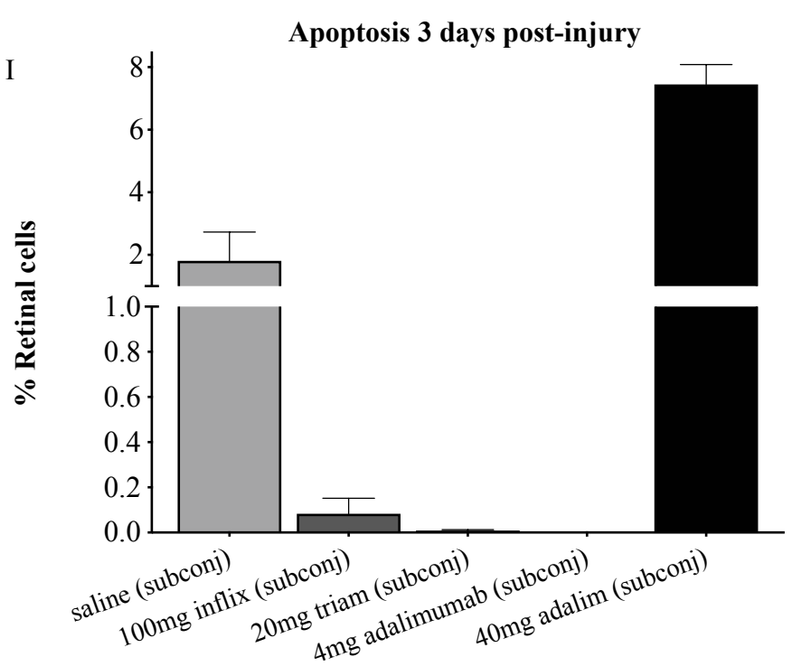

Baseline
50 days post injection
A
Adalimumab 4mg SC
B
Adalimumab 40mg SC
C
Infliximab 100mg SC
Retinal thickness GCL-ONL (µm)
260
240
220
200
180
160
*
Superior
Inferior
Nasal
Temporal
D
Saline
E
Adalimumab 4mg
F
Adalimumab 40mg
G
Infliximab 100mg
H
Adalimumab 4mg
I
Adalimumab 40mg
J
Infliximab 100mg
Normal axons/unit area
1000
800
600
400
200
0
****
Injected
Contral.

Figure 3



I



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

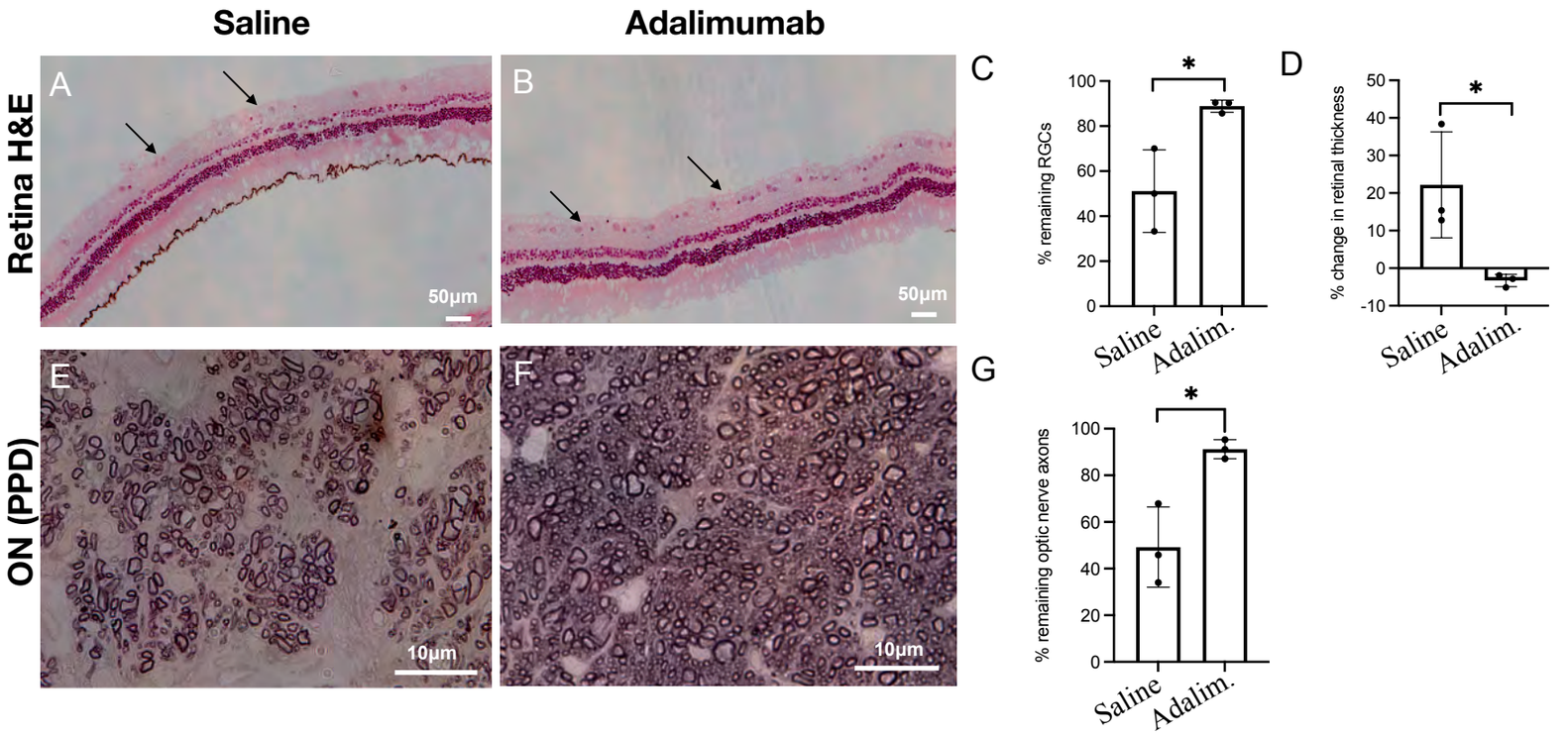

Saline
Adalimumab
Retina H&E
ON (PPD)
A
B
C
D
E
F
G
50µm
50µm
10µm
10µm
% remaining RGCs
% change in retinal thickness
% remaining optic nerve axons
Saline
Adalim.
*

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)

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

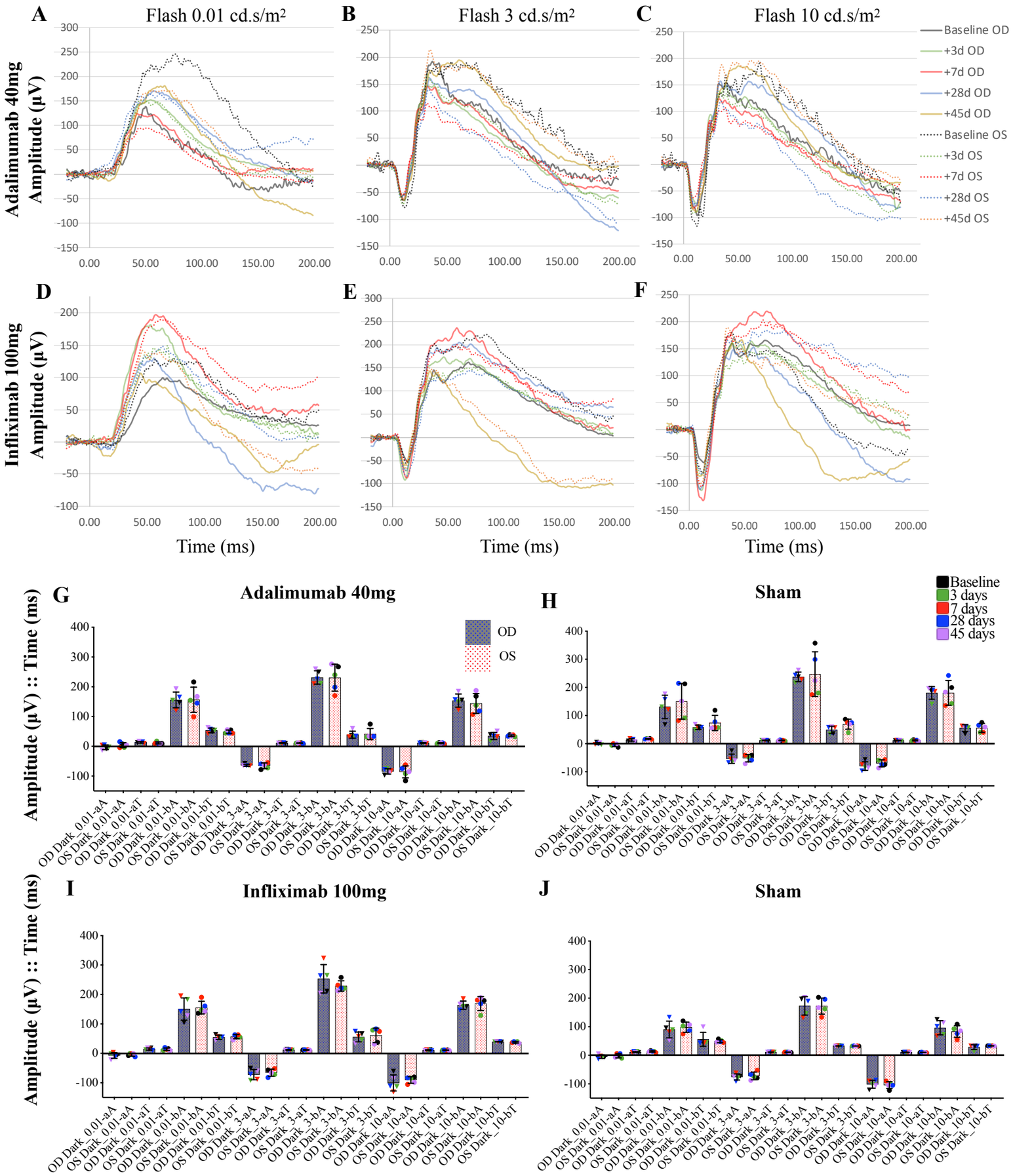
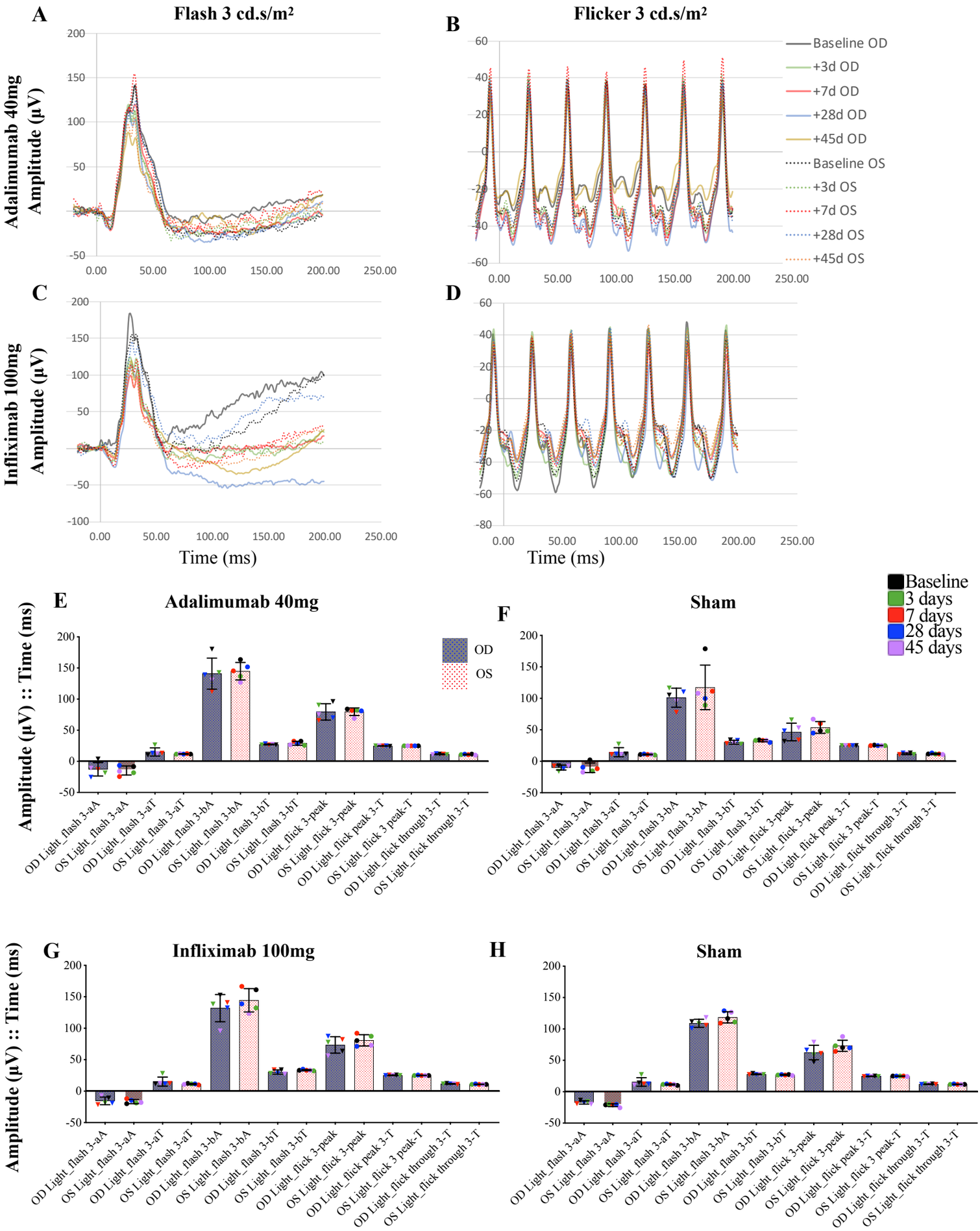



Figure 2. Toxicity evaluation of subconjunctival injection of adalimumab or infliximab at maximum dose with light-adapted ERG