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

88 It is well known and well documented by clinical ophthalmologists that acute traumatic events to
89 the cornea (e.g. chemical burns, ruptured globes, infections), as well as standard penetrating
90 corneal surgery (such as transplantations, keratoprosthesis (KPro), lacerations, etc.), often
91 develop a late sight-threatening optic nerve neuropathy that phenotypically appears similar or
92 identical to chronic open-angle or closed-angle glaucomas[1-30]. However, due to having an
93 identifiable triggering cause, this category has often been labeled "secondary glaucoma."
94 Secondary glaucoma is the most consequential complication after corneal surgery.
95
96 The magnitude of this secondary glaucomatous complication has most likely been
97 underestimated in the past due to its frequently delayed manifestation, sometimes many years
98 ("time bomb"). Epidemiological studies on secondary glaucoma are therefore limited and
99 numbers citing incidence and outcomes vary substantially with geography and level of economy
100 of the area[31-37]. One source estimated that about 6 million patients in the world have secondary
101 glaucoma compared with 67 million with the primary glaucomas[4]. In totality, the prevalence of
102 secondary glaucoma across the world has been stated to vary from 6 to 22% among various
103 glaucoma studies[31]. WHO in 2002 estimated the prevalence of *blindness* from secondary
104 glaucoma to be 2.7 million people worldwide[37]. Glaucomatous blindness is of course presently
105 irreversible.
106
107 The immediate cause of secondary glaucoma, according to many studies, has been primarily
108 attributed to surgery or trauma with corresponding inflammation rather than to chronic
109 diseases[32]—pointing to "an acute single-event episode." Such an acute episode should require
110 only a relatively short period of treatment, including prophylaxis against complications such as
111 secondary glaucoma. The majority of postsurgical glaucoma has been described as unilateral, but
112 the eventual visual outcome has been severe with a very high percentage of angle closure[31].
113
114 With regards to the pathophysiology of secondary glaucoma there have been recent shifts of
115 view. The IOP was almost universally blamed in the past, especially in cases of angle closure
116 with markedly elevated pressure. However, difficulties in explaining glaucomatous damage fully
117 in the presence of "normal" pressure has led to a greater interest in neuroinflammation and
118 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,

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

169 **Materials and Methods**

170 **Rabbit model**

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

178

179 **Rabbit anesthesia, recovery, and euthanasia**

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

188

189 **Retinal injury**

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

200

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

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

207 administered subconjunctivally at 0.4 mg, 4 mg, and 40 mg dose (n=3). Two sham injection
208 (control) animals were used (one for each study group) that received 0.8 mL of sterile saline
209 subconjunctivally without drug. Subconjunctival injections of infliximab were performed using a
210 30-G needle and adalimumab using the pre-fitted syringe needle.

211

212 **Clinical Evaluation**

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

218

219 **IOP measurements**

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

234

235 ***In vivo* optical coherence tomography**

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

246

247 **Retinal damage and cell death**

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

258

259 **Tissue preparation for H&E**

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

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

268

269 **Optic nerve evaluation with paraphenylenediamine staining**

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

278

279 **Retinal neuroprotection**

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

288

289 **Statistical analysis**

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

300

301 **Results**

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

320 doses, did not cause appreciable changes, and ERG responses following flash stimulation with
321 0.01, 3, or 10 cd.S/m2 were similar between injected and un-injected contralateral eyes**.**
322 Moreover, a and b wave amplitudes and time responses were statistically similar with saline
323 injected eyes **(Supplemental figures 1, 2)**.
324
325 *In vivo* histology was performed by using OCT imaging at baseline and 50 days after
326 subconjunctival injection of adalimumab and infliximab. 4 mg of adalimumab did not cause
327 changes in retinal thickness **(Fig. 2 A)**, while high dose (40 mg) caused increase in superior
328 retinal thickness ($P<0.05$) as compared to baseline measurements **(Fig. 2 B)**. No retinal thickness
329 changes were observed following 100 mg subconjunctival injection of infliximab **(Fig. 2 C)**.
330 Lower dose of adalimumab 0.4 or infliximab 1 and 10 mg likewise did not cause changes in
331 retinal thickness measured by OCT.
332
333 Further toxicity assessment was performed by analyzing the optic nerve axons 50 days after
334 subconjunctival injection of either 0.4, 4, 40 mg of adalimumab or 1, 10, 100 mg of infliximab.
335 The results confirmed the safety of 4 mg adalimumab and the adverse effect of 40 mg
336 adalimumab, which resulted in axonal degeneration and drop-out, as compared to the
337 contralateral un-injected eye and control saline (sham) injected eye **(Fig. 2 A, B, D-F, H, I)**.
338 Infliximab 100 mg on the other hand did not cause optic nerve axonal degeneration/drop-out,
339 consistent with the OCT retinal thickness measurements **(Fig. 2 C, G, J)**.
340
341 The efficacy of subconjunctival injection of adalimumab and infliximab in acute retinal
342 protection was evaluated 3 days after corneal burn. We have previously shown that corneal alkali
343 burn causes acute uveal inflammation, release of TNF-α, and subsequent retinal cell apoptosis
344 within 3 days in rabbits[52]. Indeed, saline treated (subconjunctival) rabbits exhibited peripheral
345 and central retinal cell apoptosis within 3 days after the injury, extending in all 3 retinal layers
346 **(Fig. 3 A, B, I)**. In contrast, subconjunctival injection of infliximab 100 mg reduced retinal cell
347 apoptosis **(Fig. 3 C, D, I)** but not as efficiently as subconjunctival adalimumab 4 mg, which
348 provided complete protection to the retina **(Fig. 3 G, H, I, J)**. Likewise, subconjunctival
349 triamcinolone 20 mg was very protective **(Fig. 3 E, F, I, J)**. In contrast, high dose (40 mg)

350 subconjunctival adalimumab was toxic and caused retinal cell apoptosis instead of retinal
351 protection (compared to sham control) **(Fig. 3 I, J)**.
352
353 The long-term efficacy of subconjunctival 4 mg adalimumab in retinal and optic nerve protection
354 was evaluated in a separate study, by evaluating the retina and optic nerve 90 days after corneal
355 burn. A single subconjunctival injection of 4 mg adalimumab after the injury was able to prevent
356 retinal ganglion cells loss and change in retinal thickness, as compared to saline treatment that
357 exhibited significant loss in RGCs and increase in retinal thickness **(Fig. 4 A-D)**. Moreover,
358 analysis of the optic nerve axons confirmed the above results, showing that 4 mg adalimumab
359 was able to preserve 92% of the ON axons at 90 days, as compared to saline treatment which
360 exhibited almost 50% axonal loss **(Fig. 4 E-G)**.
361
362 Expected retinal bioavailability of adalimumab after subconjunctival administration has been
363 roughly estimated computationally. It is known that about 0.06% of a hydrophilic compound
364 (Gd-DTPA) permeates to the rabbit retina after subconjunctival injection[66]. Similar results were
365 obtained by computational simulation of protein drug permeation from the subconjunctival space
366 to the retina and vitreous (i.e. 0.1–1% of the dose; fraction of 0.001-0.01)[67]. This estimate takes
367 into account permeability in the sclera, choroid, and RPE as well as drug loss to the conjunctival
368 and choroidal blood flows. Average retinal adalimumab concentration after subconjunctival
369 injection can be estimated using equation:
370
371 $$C_{ss,av} = (F_{ret} \times D_{sc}) / (\tau \times CL_{ret})$$
372
373 where F_{ret} (retinal bioavailability) = 0.001-0.01, D_{sc} (subconjunctival dose) = 4 mg, τ (follow-up
374 period after the dose) = 72 h (Fig. 5), and CL_{ret} (clearance from the retina/vitreous humor
375 compartment) = 0.066 ml/h[68]. Therefore, adalimumab (4 mg; molecular weight 144,190 g/ml)
376 concentration in the retina/vitreous compartment is expected to be 0.8-8 μg/ml (5.8-58 nM);
377 about 10^3-10^4 times higher than its affinity (K_d = 8.6 pM) to soluble TNF-α[69], and 10-100 times
378 higher than adalimumab affinity towards membrane bound TNF-α (K_d = 468 pM). Thus, it is
379 likely that direct permeation of adalimumab from the subconjunctival space to the retina results
380 in therapeutic activity in the retina.

381

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

387

388 $$Q_{ret} = P_{RPE} \times S_{RPE} \times C_{ss,av} \times t = P_{RPE} \times S_{RPE} (F \times D_{sc}) / (\tau \times CL) \times t$$

389

390 where $P_{RPE} = 0.035 \times 10^{-6}$ cm/s (RPE permeability of bevacizumab from)[71], $S_{RPE} = 12$ cm^2 (area
391 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.
392 Average concentration in plasma is calculated to be ≈ 3.2 mg/L (22 nM) resulting in distribution
393 of ≈ 0.35 µg of adalimumab across the RPE into the retinal compartment within 72 hours.
394 Permeation of proteins across the blood retinal barrier is slow[74] and the result (0.35 µg) is about
395 0.01% of the subconjunctival dose. *Therefore, we conclude that direct permeation of*
396 *adalimumab across the ocular tissues to the retina represents about 10-100 higher*
397 *bioavailability (≈ 0.1–1%) than its retinal entry via systemic blood circulation (≈ 0.01%).*

398

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

403

404 Discussion

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

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

442 corneal wound healing, limit their use post events to modest concentrations or short duration.
443 Toxicity of steroids has also been previously studied in intravitreal rabbit experiments; 0.5 mg or
444 1.0 mg triamcinolone into the vitreous did not produce morphological changes in the retina but
445 4.0 mg, 8.0 mg, and 20 mg produced toxic effects in the outer retina[83]. Another study on
446 intravitreal triamcinolone confirmed that 4.0 mg was retinotoxic and suggested that the vehicle
447 was to blame[84].
448
449 It is difficult to compare the results from these toxicology studies with the outcomes from our
450 subconjunctival administrations, but they seem grossly compatible with our findings of a single
451 injection of 4.0 mg adalimumab being well-tolerated subconjunctivally, but 40 mg is not. The
452 computerized exercise on bioavailability (see under Results) supports the intuitive assumption
453 that a lower dose of 4.0 mg adalimumab to the eye would have less systemic toxicity than a
454 subcutaneous injection of 40.0 mg of the same drug.
455
456 Not only severe trauma but also less invasive standard corneal surgery (PK, KPro, laceration
457 etc.) can lead to some rapid upregulation of TNF-α in the eye and ganglion cell apoptosis, which
458 may, at least in part, be the cause of subsequent secondary glaucoma[85]. Even though such routine
459 surgery rarely triggers as much inflammation as severe trauma, it makes sense to apply similar
460 prophylactic principles as long as the patients are suspected to be at risk.
461
462 The clinical implications of the combined clinical experience in the past and more recent animal
463 experiments point to the need for heavier and more sustained anti-inflammatory medication than
464 is presently practiced after acute ocular trauma or routine surgery. Emphasis should be on very
465 prompt delivery after the acute event, with administration of the drug to an easily accessible part
466 of the eye but where it can reliably protect the retinal ganglion cells—pointing to subconjunctival
467 application as practical and relatively safe. A more detailed pharmacokinetic study on the
468 relative efficacy of various routes of administration of TNF-α inhibitors was beyond the scope of
469 this investigation, however, one may cautiously draw some analogies from work on
470 bevacizumab, the full-length monoclonal antibody against vascular endothelial growth factor
471 (VEGF). Intravitreal injection was by far the most effective route of administration for retinal
472 delivery but subconjunctival injection was sufficient and should be less dangerous in a more

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

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

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

839 **(G)** Longitudinal intraocular pressure measurements using cannulation in eyes injected
840 subconjunctivally with either 1, 10, or 100 mg of infliximab or 0.4, 4, or 40 mg of adalimumab.
841 No IOP elevation is evident up to 50 days post injection. A slight elevation was observed in the
842 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 mg**
923 **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 exhibits
927 minimal changes in retinal thickness and RGC count (p= 0.024, n= 3), indicative of long-term
928 neuroprotective effect. **(E - G)** The saline (sham) treated group exhibits significant 55% reduction
929 in optic nerve axons (p=0.014, n=3) as compared to adalimumab treated eyes that exhibit minimal
930 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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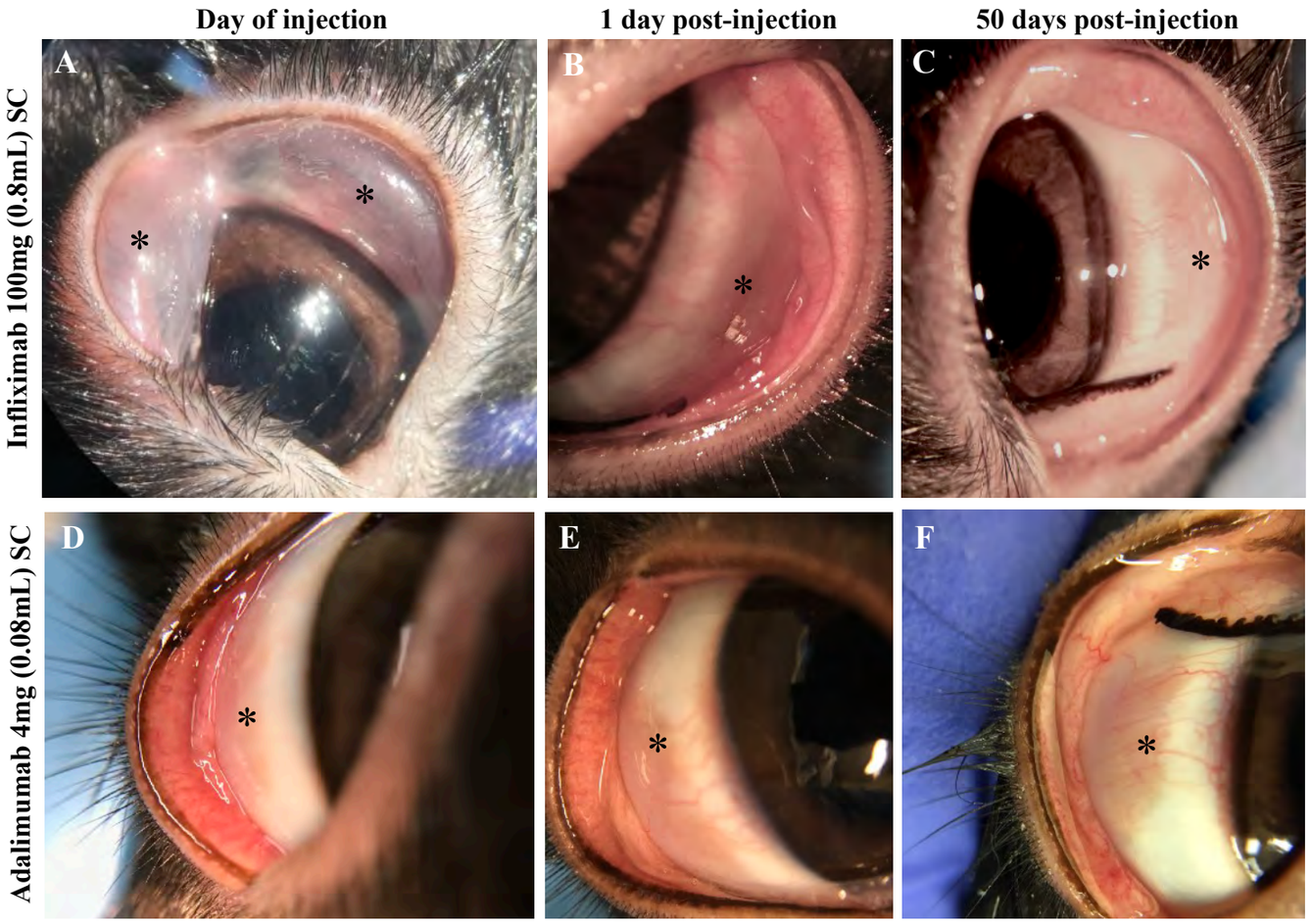
999 **Supplemental Figure 2**. **Toxicity: Light-adapted ERG assessment after subconjunctival**
1000 **injection of adalimumab and infliximab**.
1001 Light-adapted electroretinography (ERG) using 3 cd.s/m^2 flash and flicker light stimulation after
1002 subconjunctival injection of **(A, B)** 40 mg adalimumab 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

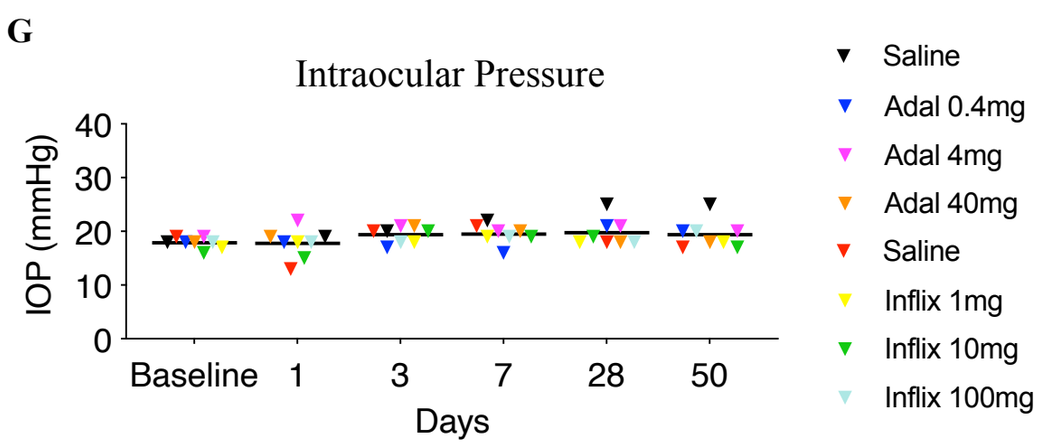

G
Intraocular Pressure
IOP (mmHg)
40
30
20
10
0
Baseline
1
3
7
28
50
Days
Saline
Adal 0.4mg
Adal 4mg
Adal 40mg
Saline
Inflix 1mg
Inflix 10mg
Inflix 100mg

Figure 2

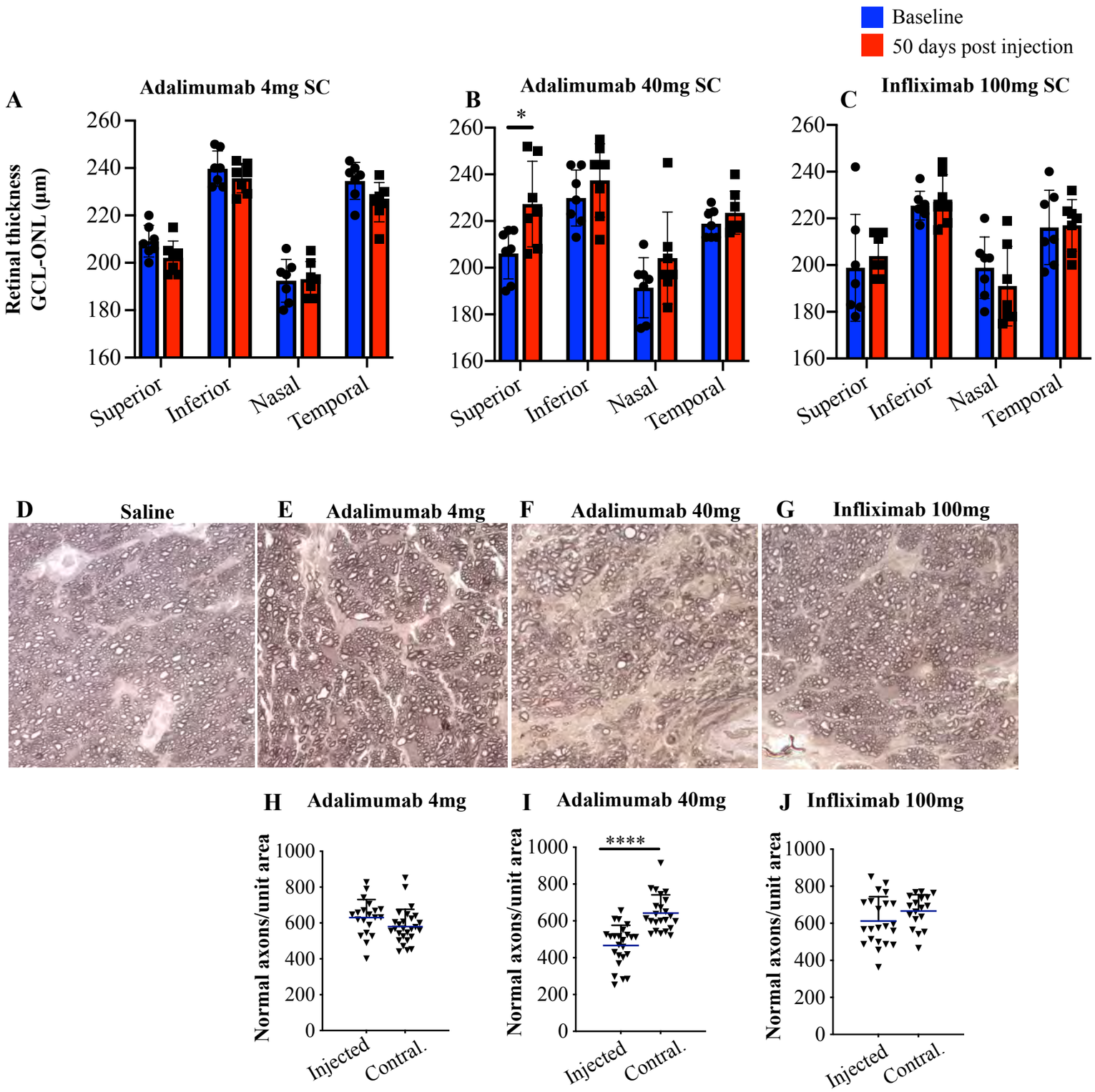
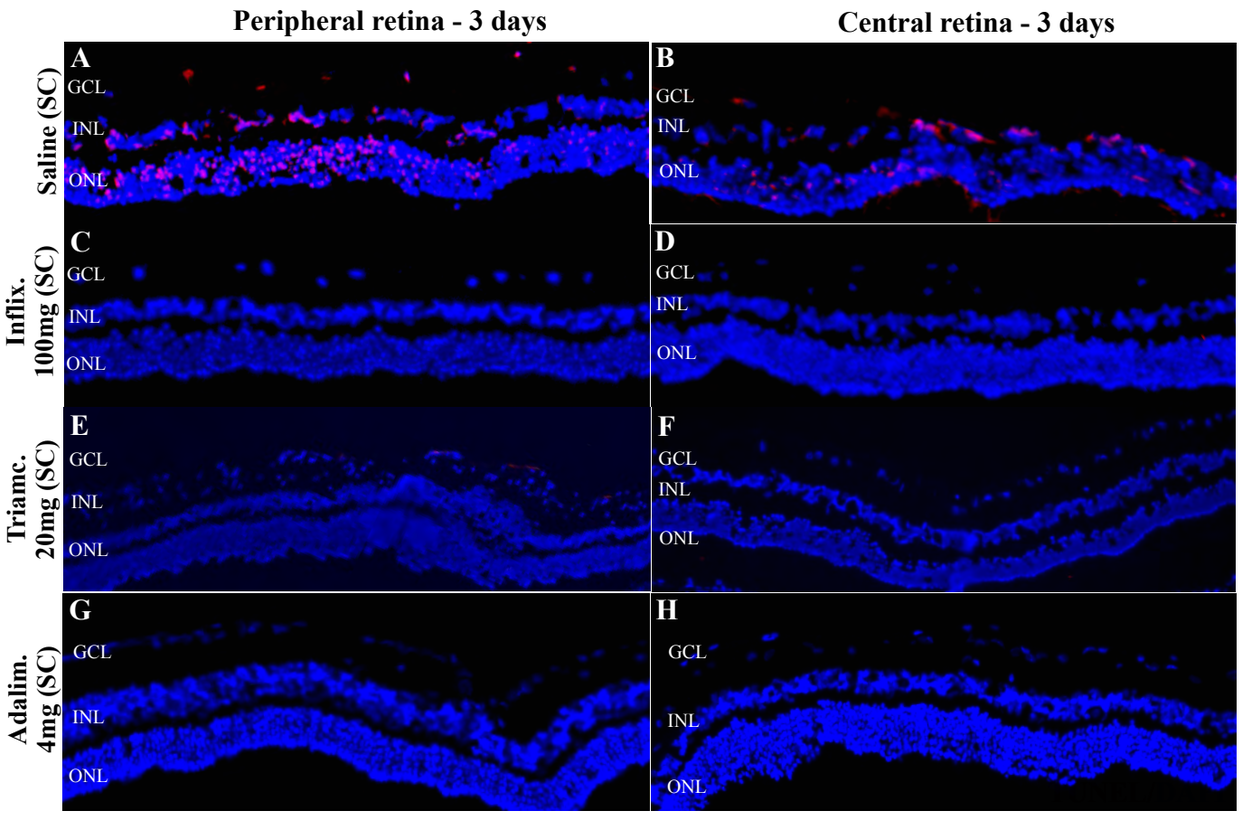
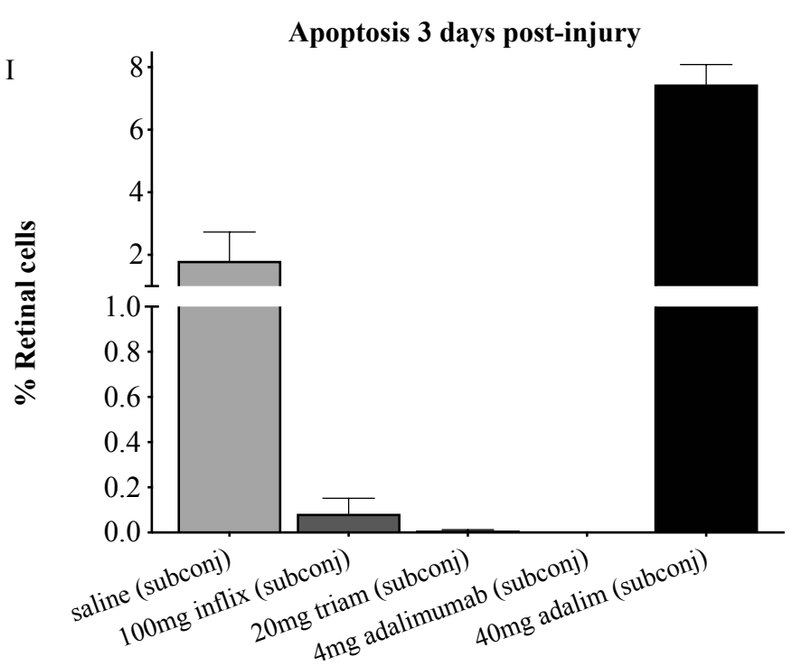

Baseline
50 days post injection
A
Adalimumab 4mg SC
B
Adalimumab 40mg SC
C
Infliximab 100mg SC
Retinal thickness GCL-ONL (µm)
260
240
220
200
180
160
*
Superior
Inferior
Nasal
Temporal
D
Saline
E
Adalimumab 4mg
F
Adalimumab 40mg
G
Infliximab 100mg
H
Adalimumab 4mg
I
Adalimumab 40mg
J
Infliximab 100mg
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

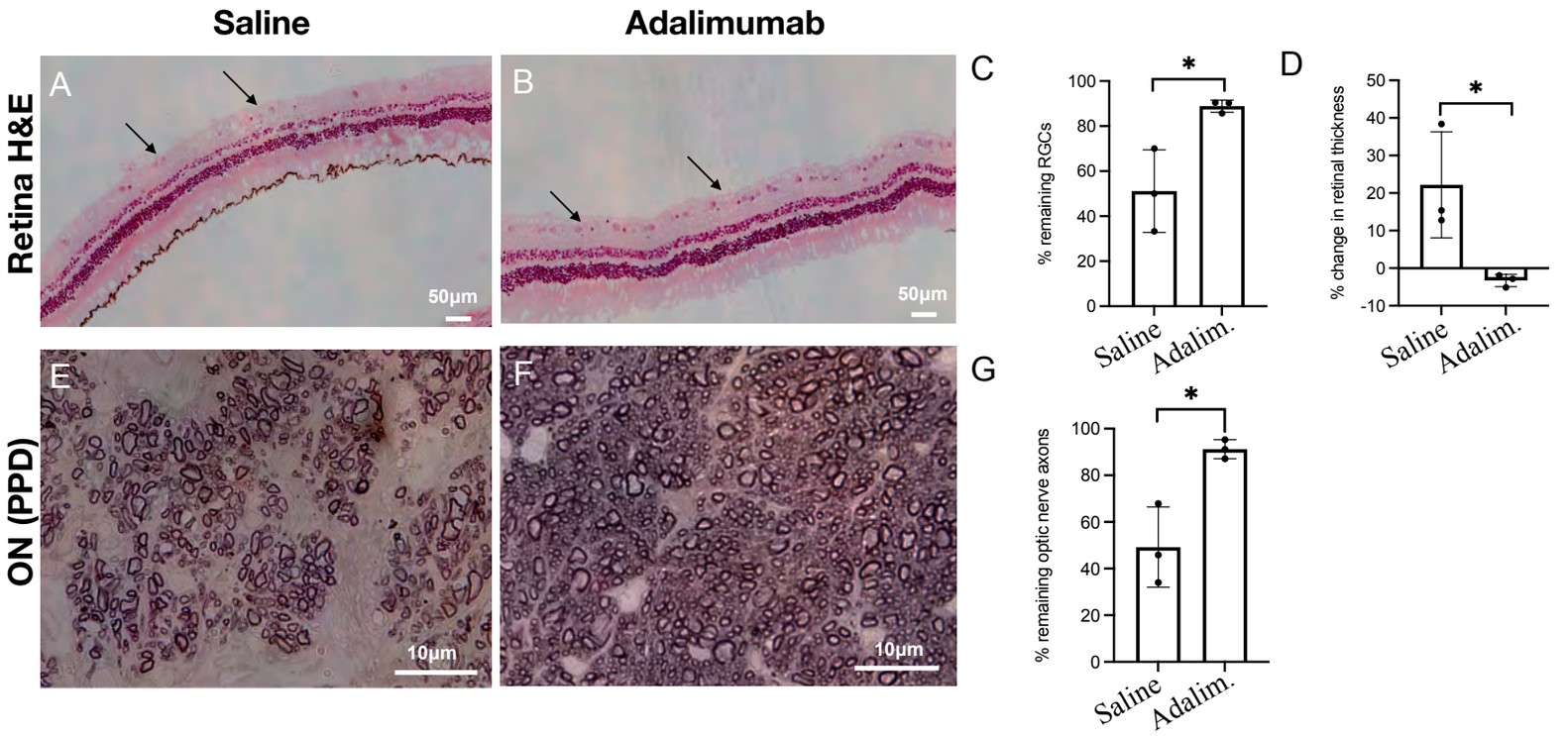

Saline
Adalimumab
Retina H&E
ON (PPD)
A
B
C
D
E
F
G
50µm
50µm
10µm
10µm
% remaining RGCs
% change in retinal thickness
% remaining optic nerve axons
Saline
Adalim.
*

Table 1.

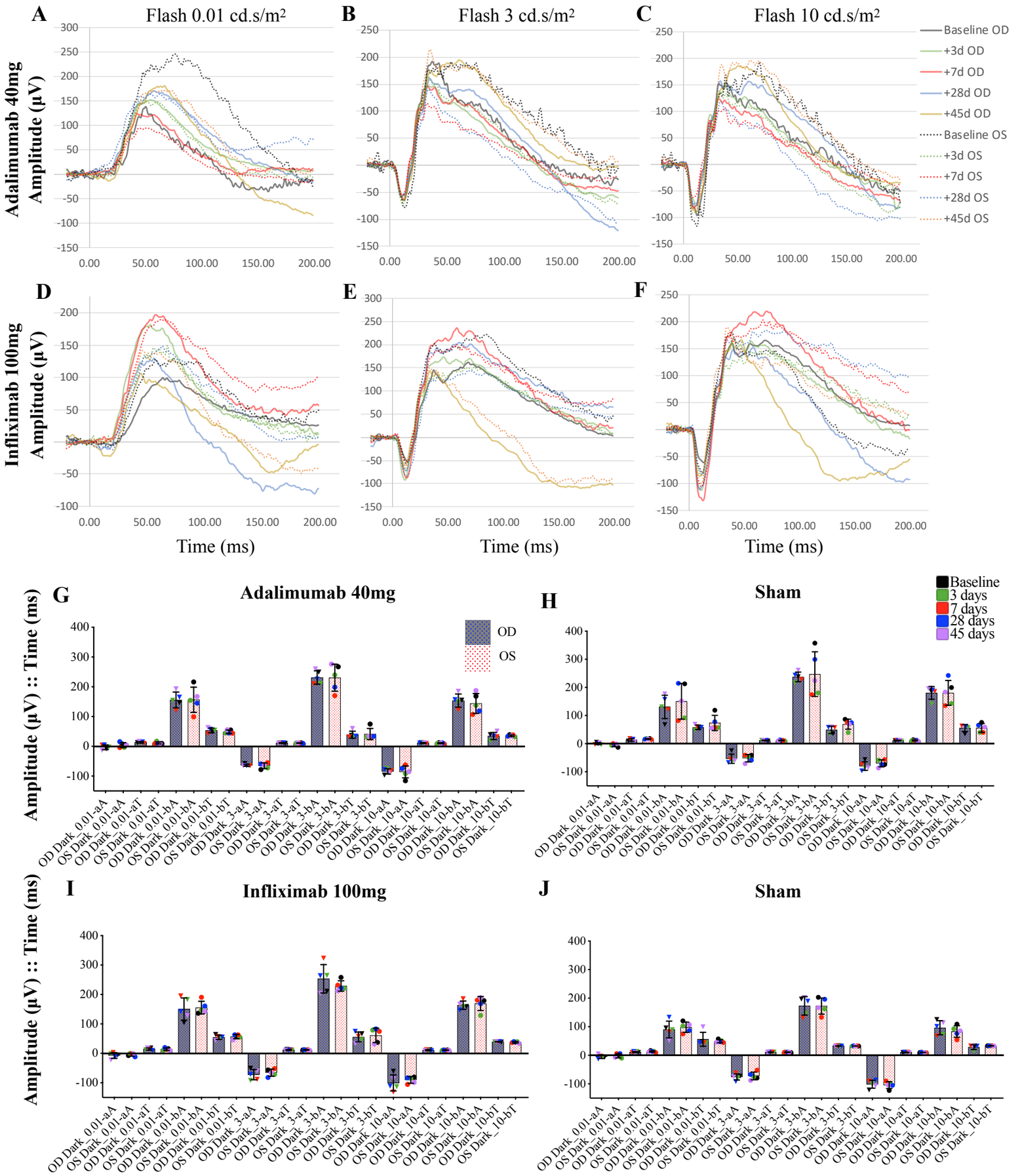
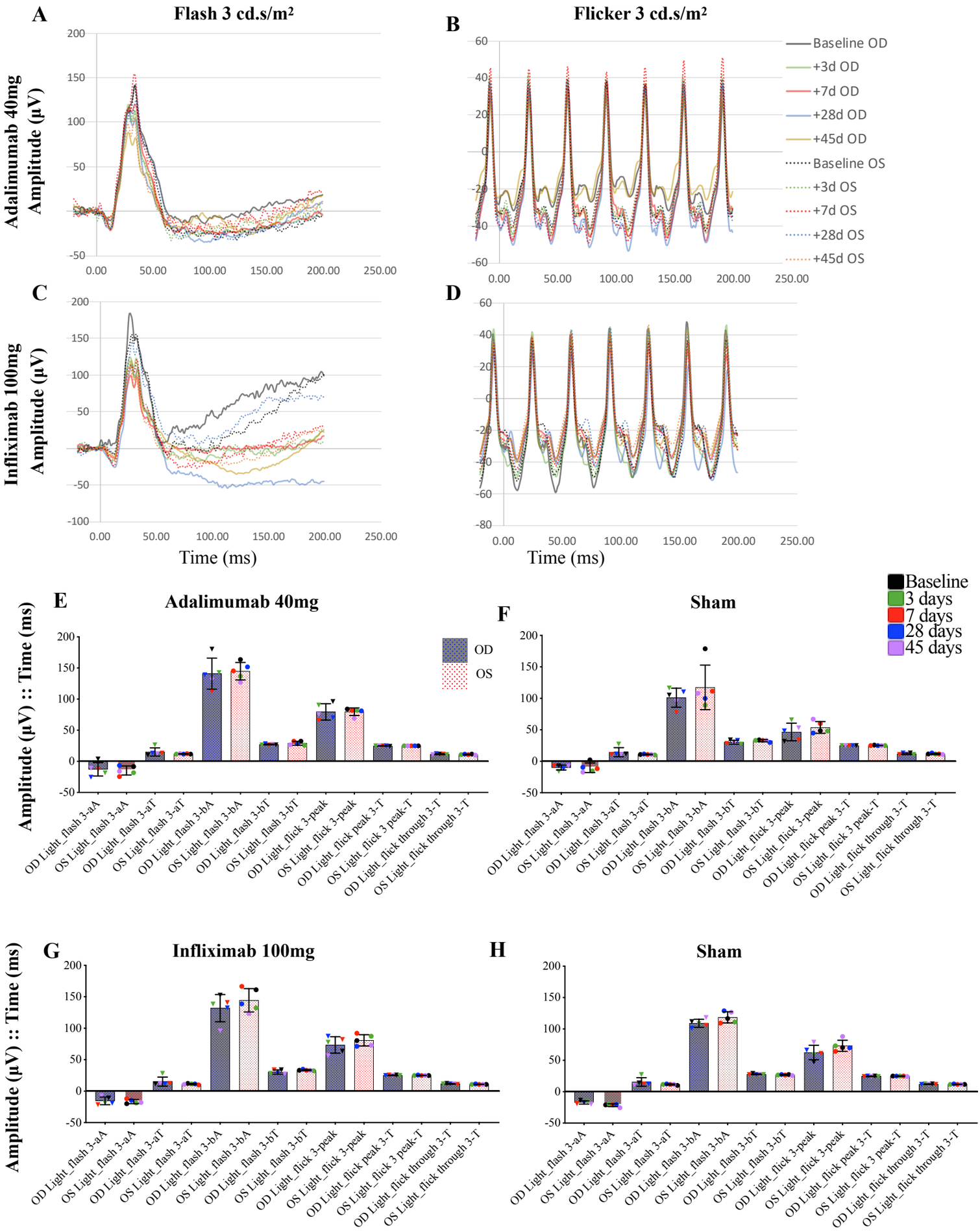
Toxicity to retina or optic nerve from subconjunctival injection of drugs in normal rabbit eyes

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)

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