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# Infliximab reduces Zaprinst-induced retinal degeneration in cultures of porcine retina

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## Abstract

**Background:** cGMP-degrading phosphodiesterase 6 (PDE6) mutations cause around 4 to 5% of retinitis pigmentosa (RP), a rare form of retinal dystrophy. Growing evidence suggests that inflammation is involved in the progression of RP. The aims of this study were to corroborate the presence of high TNF $\alpha$  concentration in the eyes of RP patients and to evaluate whether the blockade of TNF $\alpha$  with Infliximab, a monoclonal anti-TNF $\alpha$  antibody, prevented retinal degeneration induced by PDE6 inhibition in cultures of porcine retina.

**Methods:** Aqueous humor from 30 patients with RP and 13 healthy controls were used to quantify the inflammatory mediators IL-6, TNF $\alpha$ , IL-1 $\beta$ , IL-10 by a multiplex enzyme-linked immunosorbent assay (ELISA) system. Retinal explants from pig were exposed to Zaprinst, a PDE6 inhibitor, for 24 hours in the absence or the presence of Infliximab. Cell death was evaluated by TUNEL assay. The number and distribution of *caspase-3* positive cells, indirect poly(ADP)ribose polymerase (PARP) activation and glial fibrillary acidic protein (GFAP) content were visualized by immunolabeling. Antioxidant total capacity, nitrites and thiobarbituric acid reactive substances (TBARS) formation were determined to evaluate antioxidant-oxidant status.

**Results:** IL-6 and TNF $\alpha$  concentrations were higher in the aqueous humor of RP patients than in controls. Infliximab prevented retinal degeneration, as judging by the reduced presence of TUNEL-positive cells, the reduction of *caspase-3* activation and also reduction of glial activation, in an *ex vivo* model of porcine retina. Additionally, Infliximab partially reduced oxidative stress in retinal explants exposed to Zaprinst.

**Conclusions:** Inflammatory mediators IL-6 and TNF $\alpha$  were elevated in the aqueous humor of RP patients corroborating previous studies suggesting sustained chronic inflammation. Our study suggests that TNF $\alpha$  is playing an important role in cell death in an *ex vivo* model of retinal degeneration by activating different cell pathways at different cell layers of the retina that should be further studied.

**Keywords:** Retinal degeneration, Inflammation, Infliximab, Oxidative stress, TNF $\alpha$ , Poly(ADP-ribose), *caspase-3*, Retinitis pigmentosa, Photoreceptor death

## Background

Retinitis pigmentosa (RP) is a common form of rod-cone dystrophy, constituting the largest Mendelian genetic cause of blindness in the developed world. Patients with RP typically lose night vision in adolescence, peripheral vision in young adulthood, and central vision later in life due to progressive loss of rod and cone photoreceptor cells. Photoreceptor cell death starts with rod photoreceptor

degeneration and eventually cone cell death that is the major problem affecting RP patients, because it leads to loss of central vision [1]. More than 60 genes, including phosphodiesterase 6 (*PDE6*) subunit genes, have been identified to date that, when mutated, cause different forms of non-syndromic RP [2-7].

Although RP is a genetic disease, increasing evidence in patients and animal models suggests that oxidative stress and inflammation, especially TNF $\alpha$ , contribute to its pathogenesis, independently of the genes mutated [8-10]. Some reports show the presence of sustained chronic inflammatory reaction including elevated TNF $\alpha$

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levels in the eyes of RP patients [11] and *rd10* mice [12]. TNF $\alpha$  is a pleiotropic cytokine essential for the induction and maintenance of the inflammatory immune responses [13] that is also upregulated in inflammatory ocular diseases, including Adamantiades-Behcet disease [14], retinal vascular tumors [15], neovascular age-related macular degeneration [16], uveitis [17], glaucoma [18] and ischemic retinopathy [19].

TNF $\alpha$  mediates a broad range of cellular activities, including proliferation, survival, differentiation, inflammation and cell death. In the retina, TNF $\alpha$  is likely to be secreted from activated macrophages, astrocytes, microglial cells and retinal Müller glial cells. TNF $\alpha$  can trigger several well-characterized death-promoting (caspase-dependent and caspase-independent cell death) and survival-promoting pathways, depending upon the predominating signaling pathway in the particular cell type [20]. TNF $\alpha$  binding to cell surface receptors such as TNFR1 mediates activation of initiator caspases (*caspase-8*, *caspase-10*) and finally triggers cleavage of effector caspases (extrinsic pathway of cell death) [21]. TNF $\alpha$  is also involved in the intrinsic pathway of cell death that is initiated by cellular and DNA damage which particularly involves mitochondria. Finally, TNF $\alpha$  can also activate a subset of programmed necrosis called necroptosis. The mechanism that leads cells to undergo apoptosis or necroptosis and the mechanism that mediates the execution of necroptosis still remains unclear. The poly(ADP-ribose) polymerase (PARP) pathway can also activate this mode of programmed necrosis. PARP-1 activation in response to excessive DNA damage results in the massive synthesis of poly(ADP-ribose) polymers (PAR), NAD<sup>+</sup> depletion and subsequent release of apoptosis inducing factor (AIF) from mitochondria, which translocates to the nucleus where it forms an active DNA-degrading complex (caspase-independent pathway). The PARP pathway has been considered as an integral part of TNF-induced necroptosis; however, it has been recently described that both pathways represent distinct and independent routes to programmed necrosis [22].

The mechanisms responsible for photoreceptor cell death in RP are still unclear. However, increasing evidence suggests that inflammation [11,12,23,24] and especially TNF $\alpha$  could contribute to the pathogenesis of RP. Therefore, inhibition of TNF $\alpha$  and downstream cellular signaling mechanisms, following interaction of TNF $\alpha$  with its receptors, could be a possible target in the treatment of retinal neurodegenerative disorders such as RP.

In the current study we found that IL-6 and TNF $\alpha$  were increased in the aqueous humor of RP patients. We also observed that pharmacological inhibition of TNF $\alpha$  with Infliximab, a specific monoclonal antibody against TNF $\alpha$ , prevented retinal degeneration in cultures of porcine retina exposed to Zaprinas. This model reproduces some events of the degeneration found in murine models

of RP caused by non-functional PDE6 [25]. We also observed in our model a reduction of *caspase-3* activation, GFAP reactivity and partially oxidative stress, caused by Infliximab treatment. These results suggest that inflammation, especially TNF $\alpha$  upregulation, is playing an important role in retinal degeneration and, importantly, that strategies that promote its blockade could be promising therapies.

## Methods

### Participants in the study

Human samples were obtained, informed consent from all subjects previously having been given. The procedure was in accordance with the tenets of the Declaration of Helsinki and was approved by the IRB of La Fe University Hospital (Valencia, Spain). Thirty adult patients with typical forms of RP characterized by an elevated final dark-adaptation threshold, retinal arteriolar narrowing, and a reduced and delayed electroretinogram were enrolled in the study. Thirteen Caucasian patients suffering from cataracts without any other ocular disease served as controls. Further details of the patients enrolled in the study are shown in Table 1.

Patients diagnosed of RP were recruited from *Retina Comunidad Valenciana - Asociación Afectados por Retinosis Pigmentaria* and also from the department of Ophthalmology of La Fe University Hospital (Valencia, Spain). Healthy controls were recruited from La Fe University Hospital (Valencia, Spain).

### Ophthalmic examination

The best-corrected visual acuity (BCVA) and automated visual field (VF) were measured in RP patients as previously described [8]. Individual data for each patient is shown in Additional file 1: Table S1. Macular edema secondary to RP was only present in one patient.

### Aqueous humor extraction

Aqueous humor samples from 30 RP patients and from 13 patients with cataracts without any other ocular disease (controls) were collected as previously described [8]. Undiluted aqueous humor samples were collected from each patient, placed in sterile tubes, and stored immediately at  $-80^{\circ}\text{C}$  until use. All specimens were assayed to evaluate cytokine concentration in a double-blind arrangement with respect to their group. For each patient,

**Table 1 Description of the participants included in the study**

	Control	RP
Number of subjects	13	30
Males	7	21
Females	6	9
Age (years)	60 $\pm$ 3	48 $\pm$ 2

aqueous humors were collected from the eye with the more severe retinopathy.

#### Cytokine levels in aqueous humor

The concentrations of cytokines in aqueous humor were measured using a multiplex enzyme-linked immunosorbent assay (ELISA) system. To measure the concentrations of IL-1 $\beta$ , IL-6, IL-10 and TNF $\alpha$ , the SearchLight Custom Human Cytokine-Inflammation Q-Plex Array (Aushon Biosystems, MA, USA) was used. Array was used according to the manufacturer's instructions. The signal of the cytokine array was determined by a cooled CCD camera (Fujifilm, Tokyo, Japan) using chemiluminescence. SearchLight CCD Imaging and Analysis System were used to quantify cytokine concentrations. The cytokine levels were expressed as pg/mL.

#### Porcine retinal explant cultures

Seventy eyes (both left and right eyes from each animal) from small miniature pigs aged 3 to 7 months were obtained from the local slaughterhouse. Neuroretinal explants were carried out as recently described [25]. Treatments were added the day of the culture and maintained for 24 hours. To inhibit PDE6 and induce retinal degeneration, we used a final concentration of 100 nmol/L Zaprinast [25,26]. Zaprinast (Sigma-Aldrich, Madrid, Spain) was diluted in dimethyl sulfoxide (DMSO) (AppliChem, Darmstadt, Germany). The equivalent amount of DMSO was added to the culture medium of controls. To evaluate the possible neuroprotective effect of TNF $\alpha$  blockade we used Infliximab (2  $\mu$ g/mL, alone or combined with Zaprinast) as TNF $\alpha$  blocker (Remicade<sup>®</sup>, Schering-Plough, Madrid, Spain). Infliximab is a chimeric human immunoglobulin G1 with a mouse variable fragment having high TNF $\alpha$  affinity and neutralizing capacity.

#### Tissue processing and histology

Retinal explants were fixed in 4% filtered paraformaldehyde (Sigma-Aldrich, Madrid, Spain) in 0.1 M PBS (pH 7.4) and cryoprotected in a saccharose gradient (15-20-30%) (Panreac Química, Barcelona, Spain). Samples were frozen embedded in Tissue-Tek<sup>®</sup> OCT<sup>™</sup> Compound (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands). After this, 10- $\mu$ m sections were cut with a cryostat (Leica CM1900, Nussloch, Germany) and placed on Super Frost Ultra Plus treated slides (Thermo Scientific, Barcelona, Spain).

#### TUNEL assay

To evaluate apoptosis the terminal deoxynucleotidyl transferase dUTP nick and labeling (TUNEL) assay was used as previously described [25]. The apoptotic (TUNEL-positive) nuclei per field were counted in at least three fields per retinal explant using NIS-Elements imaging software (NIKON

Instruments, Badhoevedorp, The Netherlands). The number of apoptotic nuclei was normalized to the SYTOX Green-labeled cell nuclei. Results are given as percentage of apoptotic nuclei/total nuclei. Data are expressed as mean  $\pm$  SEM.

#### Immunofluorescence of caspase-3, GFAP and PAR

Immunofluorescence was carried out on 10- $\mu$ m cryosections. Sections were post-fixed for 15 minutes at room temperature in 4% filtered paraformaldehyde (Sigma-Aldrich, Madrid, Spain) in 0.1 M PBS (pH 7.4). Sections were incubated for 1 hour in blocking solution containing 5% normal goat serum, 1% BSA and 0.25% Triton X-100. They were then incubated with primary antibody against cleaved *caspase-3* (1:200, Cell Signaling Technology, Barcelona, Spain), glial fibrillary acidic protein (GFAP, 1:400, Sigma-Aldrich, Madrid, Spain) or PAR (1:200, Enzo Life Science, Madrid, Spain) overnight at 4°C in blocking solution. After this samples were incubated for one hour at room temperature with the fluorescence-conjugated secondary antibody Alexa Fluor 647 (Invitrogen, Life Technologies, Madrid, Spain) and observed under a confocal microscope (Leica TCS SP5 Confocal microscope, Leica Microsystemas SLU, Barcelona, Spain) belonging to the Microscopy Unit of the IIS-La Fe (Valencia, Spain). Cells were counted at 40 $\times$  magnification, and the number of *caspase-3* positive cells was counted manually in 4 fields per retinal explant. The number of cells positive for the cleaved *caspase-3* immunolabeling was normalized to the SYTOX Green-labeled cell nuclei (Molecular Probes, Paisley, UK). Results are given as percentage of *caspase-3* positive cell/total nuclei. Data are expressed as mean  $\pm$  SEM.

GFAP and PAR positive cells were difficult to count in different retinal layers. For the quantification we used the following formula to calculate the corrected fluorescence (CF) for each cell layer [27]:

$$\text{CF} = \text{Integrated density of the selected area} \\ - (\text{area of selected area} \times \text{mean fluorescence of background})$$

Data are expressed as mean  $\pm$  SEM.

For co-localization of cleaved *caspase-3* (combined with Alexa Fluor 647) and PAR (combined with Alexa Fluor 488 (Invitrogen, Life Technologies, Madrid, Spain)) staining was followed by TUNEL staining.

#### *caspase-3* activity assay

*caspase-3* activity was measured with a colorimetric tetrapeptide (DEVD-*p*NA) cleavage assay kit following the manufacturer's instructions (Bio-Vision, Mountain View, CA, USA). Total retinal protein was extracted from retinal explants and measured by the bicinchoninic acid

(BCA) protein assay. *caspase-3* activity was expressed as arbitrary units (au)/mg of protein.

#### Nitrites and nitrates (NOX) determination

Intracellular nitrites (stable end-product of nitric oxide (NO)) and nitrates (NOX) were measured in retinal explants by spectrophotometric GRIESS reaction using nitrate reductase [28]. The tissue NOX levels were expressed as nmol/mg protein.

#### Oxidative stress evaluation

Retinal explants were assayed for total antioxidant capacity (TAC) and thiobarbituric acid reactive substances (TBARS) formation as indicator of malonyldialdehyde (MDA) formation.

Retinal explants were homogenized in 5 mM phosphate buffer pH 7, 0.9% NaCl, 0.1% glucose, centrifuged at  $10,000 \times g$  for 15 minutes at 4°C, and then the supernatants were used to determine TAC and TBARS. Protein concentrations were measured by the BCA protein assay.

TAC was measured using a commercial kit (Cayman Chemical, Ann Arbor, MI, USA) [29]. The tissue TAC levels were expressed as nmol/mg protein.

MDA levels were detected by a colorimetric method involving thiobarbituric acid (TBA) adduct formation (Cayman Chemical, Ann Arbor, MI, USA). Tissue TBARS levels were expressed as nmol/mg protein.

Values for *caspase-3* activity, NOX and oxidative markers are given as the mean  $\pm$  SEM of at least eight different cultures. For each experiment samples were measured in duplicate.

#### Statistical analyses

All statistical analyses were done using R software (version 2.15.3) (Foundation for Statistical Computing, Vienna, Austria). Multivariate analysis of covariance (MANCOVA) and multiple linear regression models were used to analyze human data. For parametric data, ANOVA followed by Newman-Keuls *post hoc* test was used. For non-parametric data, Kruskal-Wallis test followed by Dunn's Multiple Comparison test was used. Significance levels were set at  $\alpha = 0.05$ .

## Results

### Increased levels of TNF $\alpha$ and IL-6 in aqueous humor of RP patients

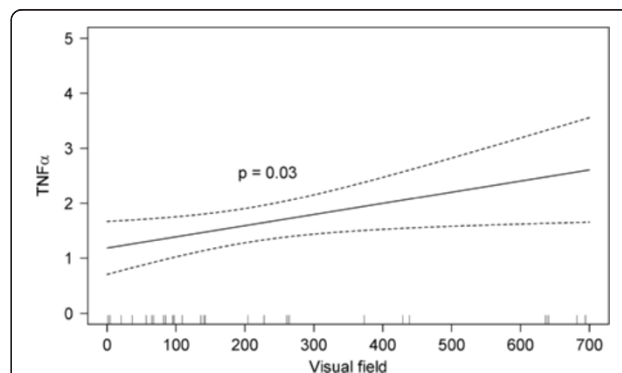
We performed a multiplex ELISA to determine the concentration of TNF $\alpha$ , IL-6, IL-1 $\beta$  and IL-10 in aqueous humor of RP patients. IL-1 $\beta$  and IL-10 were below detectable levels. Descriptive statistics of the results of the measurements of IL-6 and TNF $\alpha$  are shown in Table 2. We performed a MANCOVA with the results of TNF $\alpha$  and IL-6 as dependent variables while disease, age and gender were taken as predictive variables.

**Table 2 Protein levels of cytokines in aqueous humor from retinitis pigmentosa (RP) patients and healthy controls**

	Control	RP
TNF- $\alpha$ (pg/mL)	1.1 $\pm$ 0.2	1.7 $\pm$ 0.2
95% CI	(0.8 to 1.4)	(1.4 to 2.0)
Detectable samples	13/13	28/30
IL-6 (pg/mL)	10.8 $\pm$ 3.4	23.5 $\pm$ 3.8
95% CI	(3.2- to 18.5)	(15.8 to 31.3)
Detectable samples	13/13	30/30

Note: values are expressed as mean  $\pm$  SEM; CI: confidence interval.

This analysis revealed that RP significantly increased inflammatory mediators IL-6 and TNF $\alpha$  in aqueous humor ( $P = 0.03$ ) (See Additional file 2: Table S2). We found no statistical evidence for gender or age effects. Further analysis of each of the response variables indicated that IL-6 is increased in RP patients ( $P = 0.018$ ). TNF $\alpha$  showed a tendency to increase in RP patients ( $P = 0.09$ ). We assessed the possible association between inflammatory status (measured as TNF $\alpha$  and IL-6 levels) and stage of the disease (measured as VF and BCVA values) using a MANOVA with VF, BCVA, sex and age as predictors and TNF $\alpha$  and IL-6 levels as response variables. Our results showed no evidence of association between VF and BCVA and inflammatory status ( $P = 0.09$  for VF and  $P = 0.94$  for visual acuity). Additionally, we also analyzed separately the associations among these predictor variables and each of the two cytokine using linear models. In these analyses we found a statistically significant association between higher VF values and higher levels of TNF $\alpha$  ( $P = 0.03$ ) (Figure 1).



**Figure 1 Relation between visual field and TNF $\alpha$  concentration in aqueous humor of retinitis pigmentosa (RP) patients.** Statistical analysis revealed a positive relation between visual field and TNF $\alpha$  values controlling the other predictive variables (sex, age and acuity). Ninety-five percent confidence intervals are defined by dotted lines.

### Infliximab prevents Zaprinst-induced cell death in cultured porcine retina

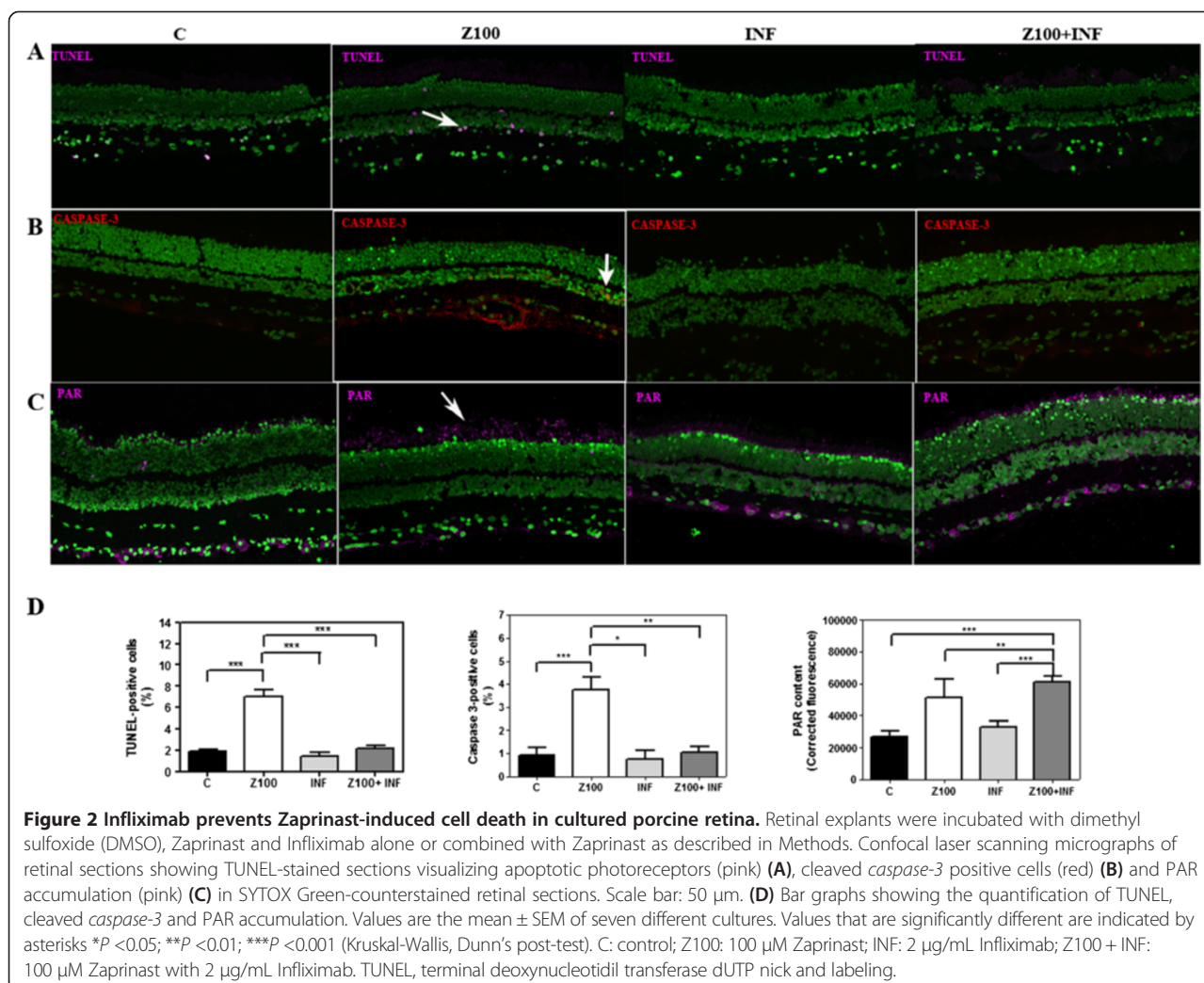
We previously described that PDE6 inhibition by Zaprinst triggered retinal degeneration and induced oxidative stress and inflammatory mediators such as TNF $\alpha$  and IL-6 in cultured porcine retina after 24 hours. In particular, TNF $\alpha$  and IL-6 content increased to twice control content [25].

We tested whether incubation with 2  $\mu$ g/mL Infliximab, a TNF $\alpha$  blocker, for 24 hours prevented Zaprinst-induced retinal degeneration. Firstly, we checked the effect of Infliximab on the TNF $\alpha$  signaling cascade. As we could not measure TNF $\alpha$ , because Infliximab interferes with the ELISA assay as previously described [30], we evaluated its receptor TNF-R1 whose activation is involved in multiple apoptotic pathways. TNF-R1 relative expression increased up to  $1.36 \pm 0.08$  arbitrary units (ANOVA Newman-Keuls post-test,  $P < 0.0001$ ) in Zaprinst-treated explants compared to control explants ( $1.00 \pm 0.08$  arbitrary units). However, Infliximab normalized Zaprinst-induced overexpression of TNF-R1 ( $0.90 \pm 0.08$  arbitrary units,

ANOVA Newman-Keuls post-test,  $P < 0.0001$ ). No significant changes were found in explants treated only with Infliximab ( $0.91 \pm 0.06$  arbitrary units).

As shown in Figure 2, Infliximab significantly reduced the number of TUNEL-positive cells in Zaprinst-treated explants from  $7.0 \pm 0.7\%$  to  $2.2 \pm 0.3\%$  (Kruskal-Wallis, Dunn's post-test,  $P < 0.001$ ) (Figure 2A). As shown in Table 3, this reduction occurred mainly in the outer nuclear layer (ONL).

As mentioned above, TNF $\alpha$  can trigger programmed cell death by activating the extrinsic and intrinsic apoptotic pathways that converges on the execution pathway, which is initiated by the cleavage of *caspase-3* [31]. The activity of *caspase-3* in Zaprinst-treated explants was  $2.3 \pm 0.2$  au/mg protein (ANOVA Newman-Keuls post-test,  $P < 0.01$ ) and  $1.3 \pm 0.2$  au/mg protein in control explants. Infliximab almost normalized *caspase-3* activity ( $1.7 \pm 0.2$  au/mg protein) compared to Zaprinst-treated explants (ANOVA Newman-Keuls post-test,  $P < 0.05$ ) and the percentage of cleaved *caspase-3* positive cells ( $1.1 \pm 0.3\%$ ) compared



**Table 3 Effect of Infliximab treatment on cell death markers in Zaprinast-treated retinal explants**

Layer	TUNEL-positive cells (%)				caspase-3 positive cells (%)				PAR content (CF)			
	C	Z100	INF	Z100 + INF	C	Z100	INF	Z100 + INF	C	Z100	INF	Z100 + INF
ONL	0.3 ± 0.1	3.0 ± 1.1 <sup>a</sup>	0.4 ± 0.2 <sup>b</sup>	0.2 ± 0.1 <sup>c</sup>	0.07 ± 0.04	0.2 ± 0.1	0.01 ± 0.01 <sup>b</sup>	0.04 ± 0.03 <sup>c</sup>	7,647 ± 676	22,925 ± 5111 <sup>a</sup>	15,982 ± 2,019	24,970 ± 1,807 <sup>d,e</sup>
INL	1.1 ± 0.2	2.5 ± 0.4 <sup>a</sup>	0.8 ± 0.3 <sup>b</sup>	1.1 ± 0.3	0.2 ± 0.1	2.1 ± 0.3 <sup>a</sup>	0.4 ± 0.3 <sup>b</sup>	0.4 ± 0.1 <sup>c</sup>	7,019 ± 1,163	9,348 ± 2,288	11,156 ± 1,879	21,511 ± 2,251 <sup>c,d,e</sup>
GCL	0.7 ± 0.1	2.0 ± 0.4 <sup>a</sup>	0.6 ± 0.3 <sup>b</sup>	1.0 ± 0.3	0.6 ± 0.3	1.3 ± 0.2 <sup>a</sup>	0.3 ± 0.1	0.6 ± 0.2 <sup>c</sup>	9,891 ± 2,011	10,019 ± 2,212	10,204 ± 1,496	17,134 ± 2,274 <sup>c,e</sup>

Note: Kruskal-Wallis test and Dunn's Multiple Comparisons were used. Values different from control are shown by <sup>a</sup>( $P < 0.05$ ). Superscripts represent statistical differences ( $P < 0.05$ ) between <sup>b</sup>Z100 and INF; <sup>c</sup>Z100 and Z100 + INF; <sup>d</sup>INF and Z100 + INF; <sup>e</sup>C and Z100 + INF respectively. ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion nuclear layer; PAR, poly(ADP-ribose) polymers; C: control; Z100: 100 μM Zaprinast; INF: 2 μg/mL Infliximab; Z100 + INF: 100 μM Zaprinast with 2 μg/mL Infliximab; CF: corrected fluorescence.

to Zaprinast-treated explants ( $3.8 \pm 0.6\%$ , Kruskal-Wallis, Dunn's post-test,  $P < 0.01$ ). Moreover, immunostaining of cleaved *caspase-3* revealed that Infliximab treatment reduced the percentage of *caspase-3* positive cells at all cell layers (outer, inner and ganglion layer (ONL, INL and GCL)) (Kruskal-Wallis, Dunn's post-test,  $P < 0.05$ ) (Table 3 and Figure 2B).

We have previously observed an over activation of poly(ADP-ribose) polymerase (PARP) in our model of porcine retinal degeneration [25]. Moreover, other authors have described similar results in other animal models of retinal degeneration [32,33]. Therefore, we investigated whether TNF $\alpha$  mediated cell death via the PARP pathway. Accumulation of poly(ADP-ribose) polymers (PAR) was used to analyze indirectly PARP activity indirectly. Immunostaining of PAR revealed a significant accumulation of these polymers in ONL and outer segments (OS) in Zaprinast-treated explants (Kruskal-Wallis, Dunn's post-test,  $P < 0.05$ ) that were not prevented by Infliximab treatment (Table 3 and Figure 2C). Infliximab treatment increased PAR accumulation at all cell layers of Zaprinast-treated explants. Thus, the inhibition of TNF $\alpha$  by Infliximab is not causally linked to PARP activation, and therefore does not prevent the secondary PAR accumulation.

To determine whether cleaved *caspase-3* or PAR accumulation co-localize with TUNEL-positive cells, we performed triple labeling (Figure 3). In Zaprinast-treated explants PAR immunostaining co-localized with TUNEL-positive cells in some cells of ONL, in a few cells of the INL and in several cells of GCL. This co-localization disappeared after Infliximab treatment in ONL and GCL but remained in a subset of cells of the INL. PAR accumulation remained high, and even increased, at all cell layers, although the number of TUNEL-positive cells decreased.

However, *caspase-3* positive cells did not co-localize with TUNEL-positive cells except for a subset of cells in INL in Zaprinast-treated explants. Co-localization of *caspase-3* with TUNEL-positive cells disappeared after Infliximab treatment but increased co-localization of *caspase-3* with PAR in INL.

#### **Infliximab ameliorates Zaprinast-induced glial activation in cultured porcine retina**

Gliosis commonly involves upregulation of the intermediate filament protein, GFAP, in Müller glial cells. We studied whether Zaprinast-induced retinal degeneration was accompanied by altered glial reactivity, and if it was the case, whether the blockade of TNF $\alpha$  could prevent it.

In control explants, GFAP were located in the inner half of the retinal Müller cells and their endfeet (GCL layer). However, Zaprinast-treated explants exhibited strong GFAP-positive staining of Müller cells. After PDE6 inhibition, GFAP was massively upregulated throughout

the retinal explant. After Infliximab treatment the GFAP-positive labeling was significantly decreased (Figure 4).

#### **Infliximab partially prevents Zaprinast-induced oxidative stress in cultured porcine retina**

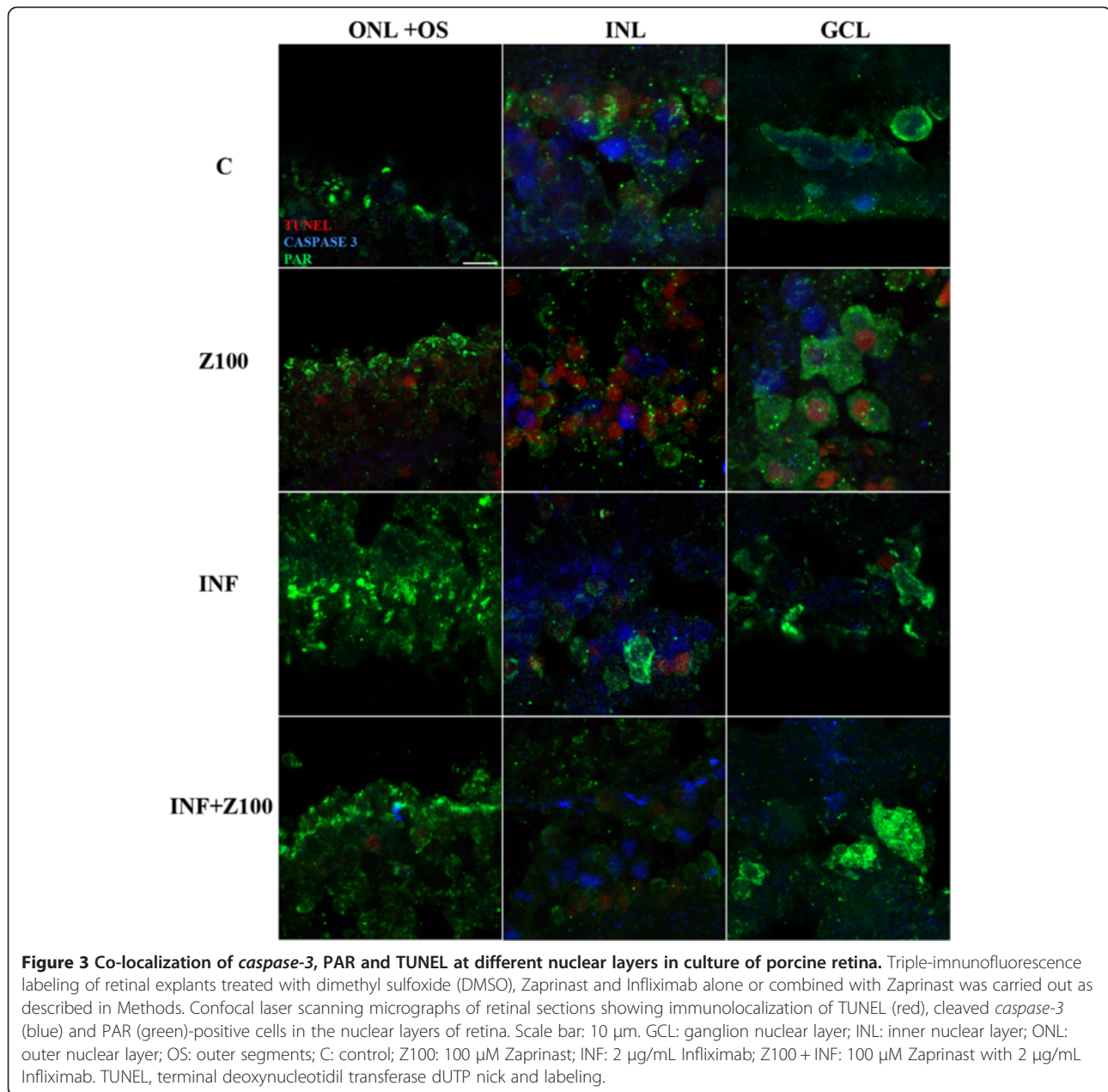
cGMP accumulation induces oxidative stress in murine models of retinal degeneration [34] as it does in our model of porcine retina treated with Zaprinast [25]. To explore whether Infliximab also prevented Zaprinast-induced oxidative damage in cultured porcine retina, we measured intracellular nitrite formation (iNOX), as stable NO metabolite, TBARS content as indicator of MDA and total antioxidant capacity (TAC).

As shown in Figure 5, Infliximab normalized TAC but did not prevent oxidative stress in Zaprinast-treated explants. Total antioxidant capacity returned to control level ( $230 \pm 15 \mu\text{mol/mg protein}$ , ANOVA Newman-Keuls post-test,  $P < 0.05$ ) (Figure 5A), but TBARS formation (Figure 5B) and intracellular NOX (Figure 5C) remained high after the blockade of TNF $\alpha$ .

#### **Discussion**

Abnormal pathological pathways such as oxidative stress and inflammation, including upregulation of TNF $\alpha$ , have been described in retinal neurodegenerative diseases both affecting the outer retina, such as RP and age-related macular degeneration (AMD), and the inner retina, such as glaucoma and ischemic retinopathy [35-39]. Low-grade inflammation is present in AMD and glaucoma. For instance, in AMD, many mediators of chronic low-grade inflammation such as C-reactive protein, immunoglobulins, and acute phase molecules, the complement-related proteins, autoantibodies, macrophage infiltration and microglial activation have been found [40]. In glaucoma, microglial activation and an inflammatory response involving Toll-like receptors (TLRs), complement molecules and cytokines, such as TNF $\alpha$  and IL-1 $\beta$ , is associated with secondary phase of the disease [41]. Much less is known about the inflammatory response to retinal ischemic-reperfusion (IR) injury. However, pro-inflammatory gene upregulation, accumulation of leukocytes, and microglial activation is found following IR in rodent retinas [42].

In RP, retinal degeneration is caused by various mutations that result in rod death followed by gradual death of cones [43]. Growing evidence suggests that, regardless of the causative mutation, neuroinflammation contributes to photoreceptor degeneration [44,45]. For instance, different animal models of RP (*rd*s mice, *rd1* mice, P23 rats, RCS rats) carrying mutations in different genes (*Prph2*, *PDE6*, *Rho*, *Mertk*) show signals of an inflammatory process [23,46-49]. In early stages of retinal degeneration the photoreceptor cells and surrounding cells, such as microglia, respond to unfavourable conditions with the production of

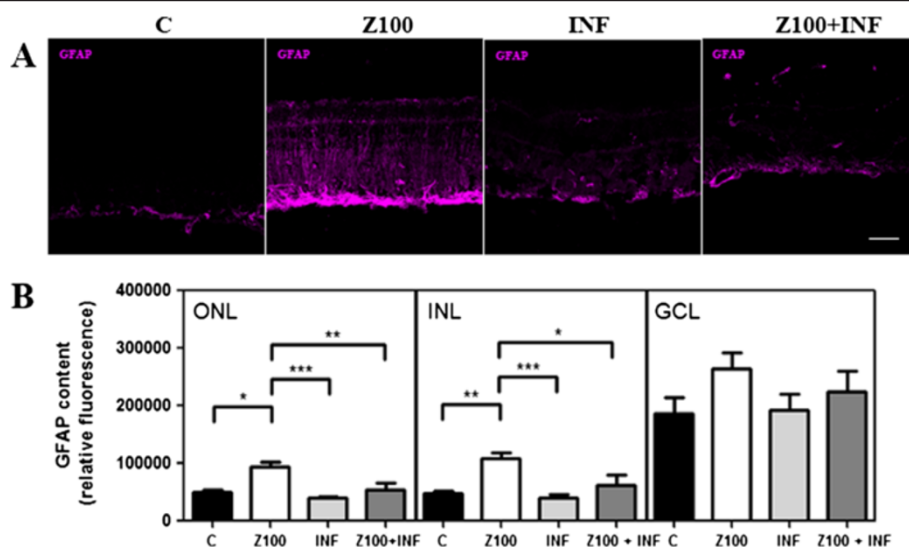


cytokines, chemokines, growth factors, and so on, in an attempt to protect neurons and to preserve retinal function. As disease progresses, sustained inflammatory mediators and others such as oxidative stress may exacerbate photoreceptor cell death and RP progression.

Early studies suggested the presence of immune reactivity in RP patients, including the presence of retinal autoantibodies in blood and lymphocytes in vitreous humor. However, these results were variable, maybe due to the inherent genetic heterogeneity of this disease [36]. Afterwards, microglial activation, a common hallmark of both inherited and induced retinal degeneration,

was described in RP patients and murine models of RP [12,45,50-53]. It has been shown that microglial activation leads to proliferation, followed by migration to damaged sites and release of cytokines (TNF $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ ) chemokines, neurotrophins, glutamate, NO, superoxide anions and prostaglandins to repair tissue damage. Although these events are triggered to prevent cell damage, sustained high levels of these molecules, especially cytokines, can cause progressive neurodegeneration. In models of RP, microglial activation coincides, or precedes, the peak of photoreceptor cell death and with high levels of TNF $\alpha$  [12,44,45,50,54,55] that seems to be





**Figure 4** Infliximab prevents Zaprinast-induced glial fibrillary acidic protein (GFAP) overexpression in cultured porcine retina. Retinal explants were incubated with dimethyl sulfoxide (DMSO), Zaprinast and Infliximab alone or combined with Zaprinast as described in Methods. **(A)** Confocal laser scanning micrographs of retinal sections showing GFAP content. Scale bar: 50  $\mu$ m. **(B)** Bar graphs showing the quantification of GFAP content. Values are the mean  $\pm$ SEM of six different cultures. Values that are significantly different are indicated by asterisks \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001 (Kruskal-Wallis, Dunn's post-test). C: control; Z100: 100  $\mu$ M Zaprinast; INF: 2  $\mu$ g/mL Infliximab; Z100 +INF: 100  $\mu$ M Zaprinast with 2  $\mu$ g/mL Infliximab.

toxic for photoreceptor cells *in vitro* [23]. Besides, microglial inhibition reduces photoreceptor cell death, TNF $\alpha$  content and improves visual function [46].

In our human study we confirmed (1) the presence of high levels of TNF $\alpha$  and IL-6 in aqueous humor in a larger population of RP patients than previously reported [11]; and we observed that (2) RP patients with higher TNF $\alpha$  values show better visual function (visual field). The conflictive positive correlation, between TNF $\alpha$  and better visual function, may be due to the different stage of the disease of the patients. It has been shown that an increase of proinflammatory markers, including TNF $\alpha$ , in mice models of RP occurs just before photoreceptor cell loss [12]. Therefore, it is tempting to speculate that at early onset of RP, when proinflammatory markers are elevated, visual function is better in patients, and after these stages patients lose visual function in parallel with TNF $\alpha$  decrease. In any case, these conflicting, and interesting, results strongly suggest that further studies are needed for clarification.

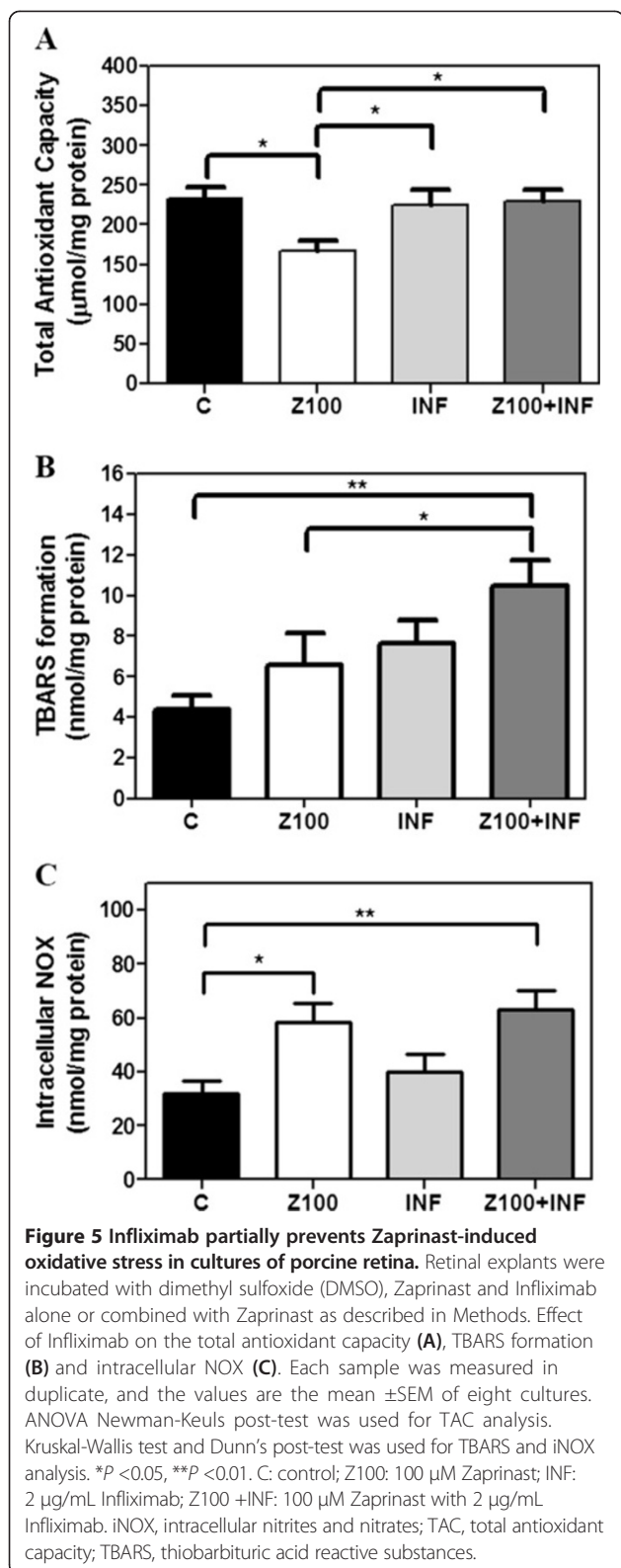
In the last few years, TNF $\alpha$  has been widely recognized as an attractive therapeutic target for the treatment of retinal diseases. Different types of monoclonal antibodies against TNF $\alpha$ , such as Infliximab, Adalimumab, Certolizumab pegol and Golimumab, or circulating receptor fusion protein, such as Etanercept, have been used to treat glaucoma [56,57], ischemic retinopathy [58] or AMD [59].

The role of TNF $\alpha$  in photoreceptor degeneration and the possible therapeutic use of antibodies against TNF $\alpha$

in the treatment of RP or other retinal degenerations remain quite unexplored. Based on previous studies we decided to evaluate the potential protective effect of the blockade of TNF $\alpha$  in an experimental porcine model of retinal degeneration. In a previous report we demonstrated that this porcine model recapitulated some aspects, especially those related to oxidative stress and inflammation, of the retinal degeneration observed in small animals after PDE inhibition [60,61] and RP patients [8,11]. Sustained elevation of intracellular cGMP in porcine retinal explants triggered different downstream effectors of cell death related to caspase-dependent mechanisms (*caspase-3*) and caspase-independent mechanisms (*calpain-2* and probably PARP activity) [25].

Our current study demonstrated that retinal degeneration accompanied by upregulation of TNF $\alpha$  and IL-6, GFAP and oxidative damage was ameliorated by blocking TNF $\alpha$  with Infliximab. Under our experimental conditions, Infliximab reduced retinal degeneration in all cell layers, mainly in the ONL, by decreasing the number of TUNEL-positive cells, supporting the idea that inflammation plays an important role in the processes of cell death.

We found that Infliximab reduced *caspase-3* activity and the number of cleaved *caspase-3* positive cells across the different cell layers, especially at the INL. Co-localization studies of *caspase-3* and PAR with TUNEL assay suggested that TNF $\alpha$  is promoting cell death through caspase-independent mechanisms in ONL and GCL and caspase-dependent mechanisms in INL.



TNF signaling can lead to cell death to two distinct outcomes, each of which is initiated by different signaling complexes: the apoptosis mode and the necrosis mode.

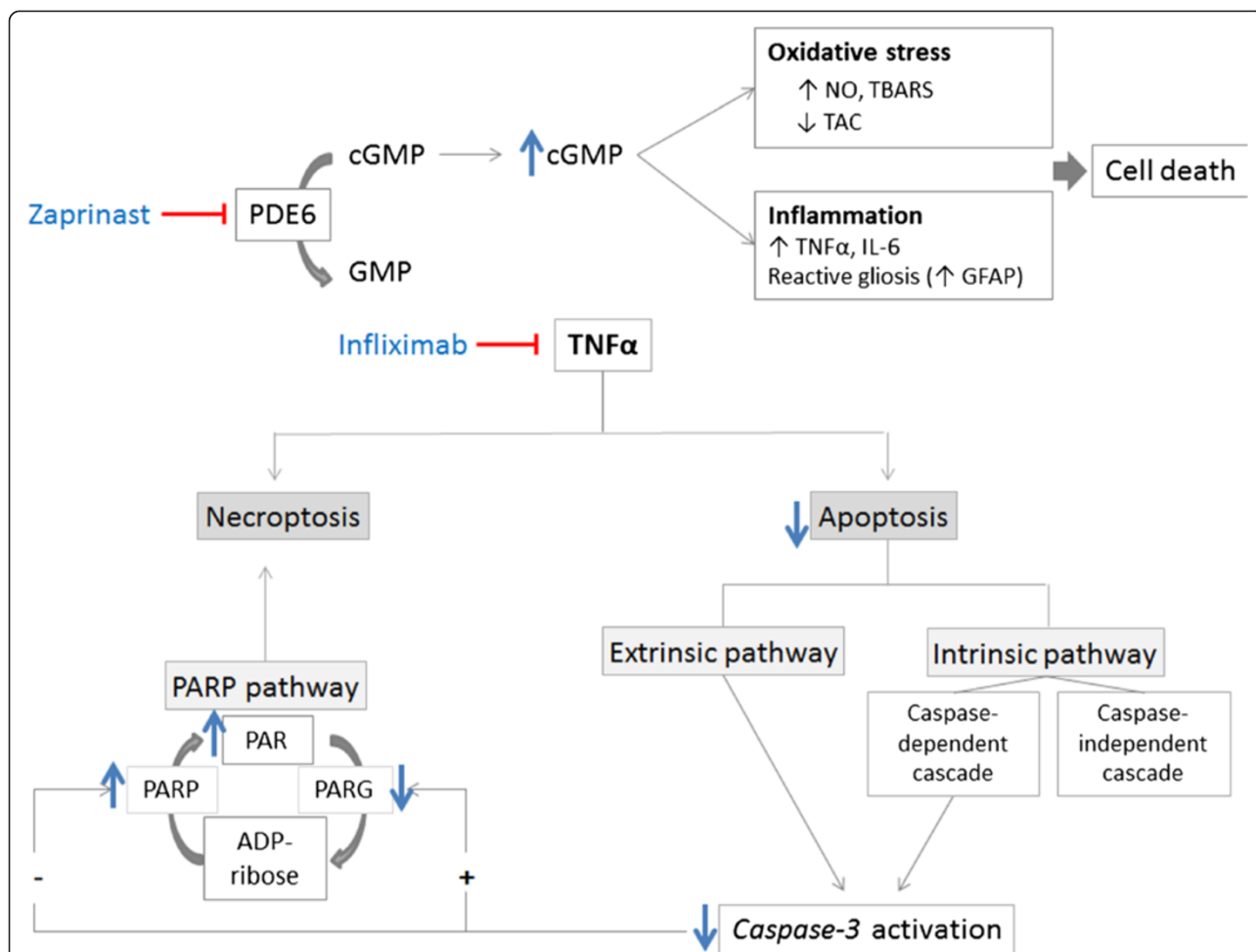
The apoptosis mode includes the extrinsic pathway, mainly mediated by caspases, and the intrinsic or mitochondrial pathway, that rely on the balance between the pro-apoptotic and the anti-apoptotic proteins from the Bcl-2 family. Both pathways converge on the same execution pathway. The execution pathway is initiated by the cleavage of *caspase-3* and results in DNA fragmentation and cell death.

We measured indirect activation of PARP through quantification of PAR accumulation. We found an upregulation of PAR due to PDE6 inhibition. However, blockade of TNFα did not prevent PAR accumulation but also increased it. PAR polymers are mainly degraded by poly (ADP-ribose) glycohydrolase (PARG) enzymes, some of them activated by *caspase-3* cleavage [62]. On the other hand, PARP can be inactivated by *caspase-3* cleavage [63]. Therefore, the inhibition of *caspase-3* induced by Infiximab could inhibit PARG activity and prevent PARP inactivation thus exacerbating PAR accumulation at all cell layers of retinal explants. These results support that PARP pathway is independent of TNFα-associated pathways in this experimental model of retinal degeneration (Figure 6).

These results were supported by previous reports in which reactive gliosis (GFAP overexpression) induced by exogenous TNFα was prevented by Adalimumab, other monoclonal anti-TNFα, in a similar model of organotypic culture of porcine neuroretina [64]. Activated Müller cells can release antioxidants, growth factors, and cytokines, including TNFα, contributing to retinal regeneration or to neurodegeneration. Müller cells are activated in models of RP [49,65-68] resulting in overexpression of GFAP, translocation of Müller cell bodies to the outer retina and thickening of their processes [69].

As previously shown, retinal degeneration induced by PDE inhibition was accompanied by oxidative stress in porcine retinas [25]. This is consistent with the idea that oxidative stress is also contributing to the progression of RP in animal models [70-72] and RP patients [8]. In the current study, we demonstrated that Infiximab partially prevented antioxidant defense depletion but not oxidative stress markers. Infiximab normalized the total antioxidant capacity in Zaprinst-treated explants, but it failed to return TBARS and NOX to control levels. In retinas of *rd10* mice antioxidant treatment reduced inflammatory mediators and photoreceptor cell loss [12]. Based on these data, it is tempting to speculate that the low Infiximab effect could be due to oxidative stress preceding upregulation of inflammatory mediators [12,73-75]. The other possibility is that Infiximab affects other oxidative stress markers that we did not measure in this study. Anyway, further studies will be needed to explore this issue in more depth.

In summary, our results corroborate that RP patients have ocular inflammation and that TNFα plays an important role



**Figure 6** Diagram showing the possible mechanism of Infliximab in the porcine retinal degeneration model. PDE6 inhibition induces cGMP accumulation and triggers retinal degeneration. The degeneration is accompanied by upregulation of inflammatory mediators, PARP pathway, reactive gliosis and oxidative stress markers. According to the current study, TNFα may be involved in the retinal degeneration by increasing *caspase-3* activation and reactive gliosis. Infliximab may prevent cell death by inhibiting caspase-dependent pathways that converge in *caspase-3* activation in the INL. Infliximab also may prevent cell death by caspase-independent pathways that remain unclear in the ONL and GCL. Moreover, Infliximab may exacerbate PARP over activation probably through the *caspase-3* inhibition. This over activation could contribute to the future cell death. cGMP: cyclic GMP; GCL, ganglion nuclear layer; GFAP: glial fibrillary acidic protein; INL, inner nuclear layer; NO: nitric oxide; ONL, outer nuclear layer; PAR: poly(ADP-ribose) polymers; PARG: poly(ADP-ribose) glycohydrolase; PARP: poly(ADP)ribose polymerase; PDE6: phosphodiesterase 6; TAC: total antioxidant capacity; TBARS: thiobarbituric acid reactive substances; TNFα: tumor necrosis factor alpha.

in the retinal degeneration induced by PDE6 inhibition in cultured porcine retinas. The mechanisms of cell death vary in the distinct cell layers. TNFα is involved in retinal degeneration through *caspase-3* activation, caspase-independent mechanisms and reactive gliosis. Our data suggest that other unknown molecules must be contributing to TNFα-mediated cell death in this model. On the other hand, PARP activation is independent of TNFα signaling and it is probably responsible for a future cell death in ONL. The existence of several distinct pathways that trigger programmed cell death implies that an efficient protection requires their simultaneous interruption via combined therapies.

The experimental model of organotypic culture has its own limitations because it involves transection of the optic

nerve and mechanical retinal detachment causing retrograde retinal ganglion cell degeneration. To minimize this problem, we have used detached retinas as controls. Moreover, the model cannot recapitulate the whole chronic nature of the degeneration, but we believe that it could be useful for studying some aspects related to the retinal degeneration. In our case, we believe that it may provide a helpful model to design and assay some treatments, such as Infliximab, thus replacing or reducing animal experiments. The use of this model allowed us to evaluate the effect of Infliximab faster and more cheaply than using the available *in vivo* models of RP.

The current model of retinal degeneration allowed us to describe an interesting and, in our opinion, neuroprotective

effect of Infliximab that strongly encourages further exploration using other experimental models. Due to the importance of the inflammatory process in the pathogenesis of several retinal degenerative conditions such as RP, AMD, ischemic retinopathy, or glaucoma, targeting inflammation could be a promising therapeutic strategy. In particular, TNF $\alpha$  blockers could be a new therapeutic strategy for the treatment of RP and other retinal degenerative conditions.

## Additional files

**Additional file 1: Table S1.** Individual data for each patient with retinitis pigmentosa (RP).

**Additional file 2: Table S2.** MANCOVA in aqueous humor from retinitis pigmentosa (RP) patients and healthy controls.

## Abbreviations

AIF: apoptosis inducing factor; AMD: age related macular degeneration; ANCOVA: analysis of variance; au: arbitrary units; BCVA: best-corrected visual acuity; BCA: biconchonic acid; BSA: bovine serum albumin; cGMP: cyclic guanosine monophosphate; DMSO: dimethyl sulfoxide; ELISA: enzyme-linked immunosorbent assay; GCL: ganglion nuclear layer; GFAP: glial fibrillary acidic protein; IL: interleukin; INL: inner nuclear layer; iNOX: intracellular nitrite; IR: ischemic-reperfusion; MANCOVA: multivariate analysis of covariance; MDA: malonyldialdehyde; NO: nitric oxide; NOX: intracellular nitrates and nitrites; ONL: outer nuclear layer; PAR: poly(ADP-ribose); PARP: poly(ADP-ribose) polymerase; PBS: phosphate-buffered saline; PDE6: phosphodiesterase 6; RP: retinitis pigmentosa; TAC: total antioxidant capacity; TBA: thiobarbituric acid; TBARS: thiobarbituric acid reactive substances; TLRs: Toll-like receptors; TNF $\alpha$ : tumor necrosis factor alpha; TUNEL: terminal deoxynucleotidyl transferase dUTP nick and labeling; VF: visual field.

## Competing interests

The authors declare that no competing financial or non-financial interests exist.

## Authors' contributions

CMFC carried out biochemical determinations, performed histological analysis and helped to write the draft. LOG carried out organotypic cell cultures and helped to biochemical and histological analysis. DH carried out statistical analysis and helped to revise the draft. DS obtained human samples and carried out ophthalmic examination. JMM participated in the design of the study and helped to revise the manuscript. RR conceived the study, designed and coordinated the study, performed cytokine determinations in human samples, analyzed data and wrote the draft. All authors read and approved the final manuscript.

## Acknowledgements

We are very grateful to the patients participating in the current study and to their relatives, to ONCE and to RETINA COMUNIDAD VALENCIANA. We thank Juan Martín (Local Slaughterhouse MercaValencia, Valencia, Spain) for providing pig eyes and the Microscopy Unit of IIS-La Fe. This work was supported by the European Regional Development Fund, Institute of Health Carlos III, P110/01825 and P112/0481 from the Spanish Ministry of Economy and Competitiveness (MEC). CIBERER is an initiative of the Institute of Health Carlos III from the MEC. Regina Rodrigo has a research-contract SNS Miguel Servet (CP09/118) from Institute of Health Carlos III.

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Received: 7 June 2014 Accepted: 25 September 2014

Published online: 10 October 2014

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doi:10.1186/s12974-014-0172-9

**Cite this article as:** Martínez-Fernández de la Cámara *et al.*: **Infliximab reduces Zaprinast-induced retinal degeneration in cultures of porcine retina.** *Journal of Neuroinflammation* 2014 **11**:172.

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