Intravitreal administration of adalimumab delays retinal degeneration in rd10 mice

Lorena Olivares-González1 | Sheyla Velasco1 | José María Millán2,3,4 | Regina Rodrigo1,2,3

1Pathophysiology and Therapies for Vision Disorders, Principe Felipe Research Center, Valencia, Spain
2Rare Diseases Networking Biomedical Research Centre (CIBERER), Madrid, Spain
3Joint Unit on Rare Diseases CIPF-La Fe, Valencia, Spain
4Molecular, Cellular and Genomic Biomedicine, Health Research Institute La Fe, Valencia, Spain

Correspondence
Regina Rodrigo, Pathophysiology and Therapies for Vision Disorders, Principe Felipe Research Center, Eduardo Primo Yúfera 3, 46012 Valencia, Spain. Email: rrodrigo@cipf.es

Funding information
European Regional Development Fund; MINECO; ISCIII, Grant/Award Number: PI15/00052 and PI18/00252; MEHUER Foundation; CIBERER, Grant/Award Number: CB06/07/1030; National Health System, Grant/Award Number: CP15/00019

Abstract
Retinitis pigmentosa (RP) is a group of inherited retinal dystrophies characterized by the progressive and irreversible loss of vision. We previously found that intraperitoneal administration of Adalimumab, a monoclonal anti-TNFα antibody, slowed down retinal degeneration in the murine model of RP, the rd10 mice. The aims of this study were to improve its neuroprotective effect and to deepen understanding of the molecular mechanisms involved in this effect. We analyzed (i) the in vitro effect of Adalimumab on the TNFα-mediated cell death in retinal cells; (ii) the effect of a single intravitreal injection of Adalimumab on retinal degeneration in rd10 mice at postnatal day (P) 23. In vitro studies showed that TNFα induced caspase and poly ADP ribose polymerase (PARP) activation, downregulation of (kinase receptor-interacting protein 1) RIPK1 and upregulation of RIPK3 in retinal cells. Adalimumab reduced cell death probably through the inhibition of caspase 3 activation. In vivo studies suggested that PARP and NLRP3 inflammasome are mainly activated and to a lesser extent caspase-dependent mechanisms in rd10 retinas at P23. Necroptosis seems to be inhibited by the downregulation of RIPK1. Adalimumab prevented from retinal degeneration without affecting caspase-dependent mechanisms but decreasing PARP activation, microglia activation as well as NLRP3 inflammasome.

KEYWORDS
NLRP3 inflammasome, PARP, photoreceptor degeneration, TNFα

Abbreviations: ADA, adalimumab; AIF, apoptosis-inducing factor; ASC, apoptosis-associated speck-like protein; BSA, bovine serum albumin; COX2, cyclooxygenase 2; DISC, death-induced signaling complex; ER stress, endoplasmic reticulum stress; FADD, fas-associated protein with death domain; GFAP, glial fibrillary acidic protein; INL, inner nuclear layer; LDH, lactate dehydrogenase; MLKL, mixed lineage kinase domain-like protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NLRP3, NLR family pyrin domain containing 3; ONL, outer nuclear layer; P, postnatal day; PAR, poly ADP ribose; PARP1, poly ADP ribose polymerase 1; RIPK1, kinase receptor-interacting protein 1; RIPK3, kinase receptor-interacting protein 3; RP, retinitis pigmentosa; TNFα, tumor necrosis factor alpha; TNFR1/TNFR2, tumor necrosis factor receptor 1 or 2; TRADD, death-domain containing adaptor protein; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2020 The Authors. The FASEB Journal published by Wiley Periodicals LLC on behalf of Federation of American Societies for Experimental Biology
1 | INTRODUCTION

Retinitis pigmentosa (RP) is a group of inherited retinal dystrophies. RP is considered a rare disease (prevalence 1 in 4000 worldwide). However, it is one of the most common forms of inherited retinal degeneration, constituting the largest single cause of inherited blindness in the developed world. RP causes a progressive and irreversible loss of vision that, in most studied models, parallels photoreceptor cell death (rods and cones). RP exhibits high genetic and clinical heterogeneity. To date, mutations in over 100 genes are known to cause different forms of both non-syndromic or syndromic RP (eg, Usher syndrome). RP is characterized by progressive rod photoreceptor degeneration in the initial stage and eventual cone photoreceptor degeneration in later stages. It is very likely that cone degeneration is influenced by the release of inflammatory molecules, oxidant radicals, etc from rods and other cells independently of gene defect.

Several studies suggest a key role of inflammation in the pathogenesis of RP. Microglial activation, a hallmark of neuroinflammation, and upregulation of inflammatory mediators such as tumor necrosis factor alpha (TNFα), interleukin 6 (IL-6), IL-1α or IL-1β are present in RP patients and murine models of RP. In particular, we have shown upregulation of TNF-α in aqueous humor of RP patients and retinas of rd10 mice, a mouse model of autosomal recessive RP. TNFα is a master cytokine of proinflammatory response, involved in several cellular processes such as proliferation, survival, differentiation, inflammation, and cell death through the binding to its receptors TNFR1 or TNFR2. In retina, TNFα is mainly secreted by macrophages and glial cells. TNFα regulates the production of chemokines and cytokines. It can lead cells to distinct outcomes depending on the activation of different signaling complexes: survival, apoptosis or necroptosis. TNFα binding to TNFR1 forms the complex I composed of TNFR1, TNFR-associated factor (TRAF)2, TRAF5, kinase-receptor interacting protein 1 (RIPK1), and cellular inhibitor of apoptosis (cIAP) proteins, which leads to NF-κB activation and the transcription of many inflammatory cytokines and anti-apoptotic proteins (survival signaling). TNFα can lead to a process of deubiquitination of RIPK1. This complex incorporates Fas-associated protein with death domain (FADD) instead of TRAFs and cIAPs, forming the complex II after recruitment and activation of caspase 8, the initiator of the extrinsic pathway of apoptosis. Activated caspase 8 triggers caspase 3 activation initiating apoptosis. However, when apoptosis is disrupted by caspase 8 inhibition, activated RIPK1 can associate with RIPK3 (necrosome) that phosphorylates mixed lineage kinase domain-like pseudokinase (MLKL) which results in the production of inflammatory cytokines and disruption of the plasma membrane (necroptosis). Moreover, RIPK3-MLKL signaling can promote activation of the nucleotide-binding oligomerization domain (NOD)-like receptor protein 3 (NLRP3) inflammasome to recruit the apoptosis-associated speck-like protein containing a CARD (ASC) and trigger caspase 1 processing of the proinflammatory cytokine IL-1β (pyroptosis). Therefore, TNFα seems to be a key molecule to modulate survival and cell death processes.

TNFα is considered a therapeutic target for several inflammatory diseases. Anti-TNFα biologics such as Adalimumab (ADA) or Infliximab are used to treat inflammatory disorders because of their effectiveness in blocking TNFα binding to its receptors TNFR1 and TNFR2. It is believed that this blockade reduces inflammation and cell death (in the form of apoptosis or other apoptosis-independent mechanisms). In this sense, we have previously observed that several intraperitoneal injections of 3 mg/kg ADA prevented retinal degeneration in rd10 mice at postnatal day (P) 18, P18, the time point of maximum photoreceptor cell death under our housing conditions. Intraperitoneal ADA reduced photoreceptor cell death, poly (ADP) ribose polymerase (PARP) activity, reactive gliosis, and improved antioxidant response, energetic and metabolic function in rd10 mice at this age. Excessive PARP activation can induce the nuclear translocation of apoptotic inducible factor (AIF) and subsequent cell death named PARthanatos. However, further studies are needed to better understand the molecular mechanisms involved in the neuroprotective effect of ADA. Besides, our previous study had some issues to address such as the possible side effects derived from an intraperitoneal administration (systemic effects) or the temporary effect of the treatment (only up to P18).

In the current study, we assess the molecular players involved in TNFα-induced cell death and its prevention by ADA in cultured retinal cells after exogenous TNFα or tunicamycin, an inducer of unfolded protein response, addition. Moreover, we study the effect of a single intravitreal dose of ADA on retinal degeneration, the cell death mechanisms involved, and neuroinflammation in rd10 mice at P23. We present that exogenous TNFα mainly induces caspase 1, 3, 8, and 9 activation, PAR accumulation, and an unbalanced expression of RIPK1 and RIPK3, major players of necroptosis. Similarly, rd10 mice show caspase 1 and 3 activation, PAR accumulation, unbalanced expression of RIPK1 and RIPK3, and possibly a NLRP3 inflammasome activation. ADA reduces PARP activation, microglial activation, and NLRP3 inflammasome activation (caspase 1, NLRP3, and IL1β) in retinas of rd10 mice at P23.

2 | MATERIAL AND METHODS

2.1 | Cell culture of 661W and BV2 cells

The 661W photoreceptor cells were provided by Dr Muayyad Al-Ubaidi (University of Oklahoma Health
 Cells were grown in Dulbecco’s Modified Eagle’s Medium/Nutrient Mixture F-12 Ham (#11320033, DMEM:F12; Gibco, Thermo Fisher Scientific, Madrid, Spain) supplemented with fetal bovine serum (#26140079, Gibco, Thermo Fisher Scientific, Madrid, Spain) and 100 units/mL of penicillin/100 µg/mL of streptomycin (#15140122, Gibco, Thermo Fisher Scientific, Madrid, Spain), 0.004% β-mercaptoethanol (#M7154, Sigma Aldrich, Madrid, Spain) and incubated at 37°C in 5% CO₂ humidified atmosphere (#3121, Thermo Electron Corporation, Waltham, Massachusetts, USA). The cells were passaged by trypsinization every 2 to 3 days. About 661 W cells were seeded in 96-well microliter plates (100,000 cells/mL) for cytotoxicity assays, in eight-well culture slides (#30108, SPL Life Sciences, Gyeonggi-do, Korea) (200,000 cells/mL) for immunocytochemistry, and in six-well plates (150,000 cells/mL) for gene expression, protein analysis, and caspase activities.

The 661W cells were exposed to different concentrations of the established cytotoxic agents TNFα (from 0.05 to 200 ng/mL) (#300-01A, PeproTech, Texas, UE) or Tunicamycin (from 0.01 to 10 µg/mL) (#2080A22420005, Barcelona, Spain) and were harvested after 24 hours. Different concentrations of ADA (from 0.1 to 10 µg/mL) (Humira, Abbvie, North Chicago, Illinois, UE), and a caspase 8 inhibitor, Z-IETD-FMK (10, 20 and 50 µM) (#1064-100, BioVision, Milpitas, CA, USA) were assayed before evaluating their possible protective effect against cytotoxic agents. For treatments, the 661W cells were pretreated with ADA (5 and 10 µg/mL), IETD-FMK (20 and 50 µM), a PARP inhibitor, Olaparib (1.5, 5, and 10 µM) (#S1060, Selleckchem, Deltaclon, Madrid, Spain) or vehicle for 2 hours before stimulation with tunicamycin (10 µg/mL) or with TNFα (100 ng/mL). Twenty-four hours later, the cells were collected or fixed for further analysis. At least five experiments were used for each determination. Each experiment was performed at least five times (five different cultures), with several replicas for each treatment.

BV2 cells were grown in Dulbecco’s Modified Eagle’s Medium (#11995-040, DMEM; Gibco, Thermo Fisher Scientific, Madrid, Spain) supplemented with fetal bovine serum (#26140079, Gibco, Thermo Fisher Scientific, Madrid, Spain) and 100 units/mL of penicillin/100 µg/mL of streptomycin (#15140122, Gibco, Thermo Fisher Scientific, Madrid, Spain) and incubated at 37°C in 5% CO₂ humidified atmosphere (#3121, Thermo Electron Corporation, Waltham, Massachusetts, US). The cells were passaged by trypsinization every 2 to 3 days. BV2 cells were seeded in six-well plates (150,000 cells/mL) for determination of IL-6 release and in eight-well culture slides (200,000 cells/mL) for immunocytochemistry.

The BV2 cells were pretreated with ADA (5 and 10 µg/mL) 2 hours before TNFα (15 ng/mL) stimulation. About 15, 30, 60 minutes and 24 hours later, cells were fixed and used for detection of PAR polymers. To detect IL-6 release supernatants were collected 24 hours after TNFα stimulation. Each experiment was performed four times (four different cultures), with three replicas for each treatment.

### 2.2 Animals and treatment

The rd10 mice are widely considered a model of autosomal recessive RP. The C57Bl6 mice are wild type animals with the same genetic background as the rd10 mice and therefore were used as a control group. Mice were kept under a 12-hour light/dark cycle, humidity and temperature-controlled, and with food and water supplied ad libitum. All cages were placed on the lower shelf of an IVC rack with light illumination of 115 ± 7 lux (95% CI: 98-131). The mice were housed in the Animal Facility of Health Research Institute La Fe (IIS-La Fe) of Valencia.

This study was carried out in accordance with the European Union Guidelines for the Care (European Union Directive (2010/63/EU) and the guidelines for the Use of Laboratory Animals. All animal procedures and protocols were approved (Reference number A1432043623509) and monitored by the Committee of Ethics in Research of IIS-La Fe. At least six animals for each group were used for each determination (histology, western blot, gene expression, and caspase activities). To detect ADA in retinal homogenates four animals were used for each time (0, 1, 3, and 6 days).

Intravitreal injections of 1, 1.5, and 2 mg of ADA in 4 mL of vitreous humour (0.2, 0.375, and 0.5 mg/mL) have been reported to be safe for humans.32,33 Besides, we applied the formula for dose conversion between humans and mice (HED = NOAEL * Km ratio (for mice)) to select the highest concentration of ADA (4.61 mg/mL = 0.375 mg/mL × 12.3).34 To select the best effective dose the eyes of rd10 mice received a single intravitreal (ivt) dose of the following ADA concentrations: 0.125, 0.25, 0.375, 4.61 mg/mL at P12. Intravitreal injection of ADA (HUMIRA, AbbVie, Madrid, Spain) was administered in one eye (left eye), while the contralateral eye was injected with 0.1 M phosphate-buffered saline (PBS) (#L0615, Dulbecco’s Phosphate Buffered Saline w/o Calcium w/o Magnesium, Bisowest, Nuaille, France) to serve as a control. To avoid artefactual effects due to possible contralateral eye effect untreated rd10 mice were also used as control. Mice were anesthetized with isoflurane (AbbVie, Madrid, Spain) at P12. After their pupils were dilated with a drop of topical tropicamide (Alcon, Geneva, Switzerland), 0.5 µL of PBS or ADA (final concentration in vitreous humor: 0.125, 0.25, 0.375 or 4.61 µg/µL) were injected into the vitreous humor with a syringe (33G; Hamilton, Bonaduz, Switzerland).
Switzerland) using a surgical microscope (#M320, Leica Microsystems SLU, L'Hospitalet de Llobregat, Spain). After intravitreal injection, a drop of tobramycin ophthalmic solution (Alcon, Geneva, Switzerland) was topically applied. Mice were euthanized by cervical dislocation at P23. The eyes were rapidly removed and processed as described below. The doses of ADA were chosen based on our previous studies with retinal explants (data not shown) and pharmacokinetic data from humans with other ocular diseases. No apparent side effects were detected in animals treated with ADA. Untreated rd10 mice and control mice were also euthanized at P23.

For biochemical and enzymatic determinations and gene expression analysis, retinas were isolated, placed immediately into the appropriate buffer, and stored at −80°C.

### 2.3 | PARP activity

The 661W cells were harvested in 100 μL of lysis buffer (0.5 M NaCl, 100 mM Tris-Cl [pH 8.0], 2% Triton-X-100, and 200 mM phenylmethylsulfonyl fluoride) and rested on ice for 5 minutes. A standard protein assay (#5000006, Bio-Rad, Herts, United Kingdom) was used to determine protein concentrations in cell lysates. PARP activity was measured using a 96-well PARP assay kit (#4677-096-K, Trevigen Inc, Gaithersburg, Maryland, USA) following the manufacturer’s protocol. This colorimetric assay measures at 450 nm the incorporation of biotinylated poly (ADP-ribose) onto histone proteins in a 96-well strip well format using a microtiter plate reader Halo LED 96 (Dynamica Scientific Ltd, Livingston, United Kingdom) overnight. PARP bands were detected using NZY Advanced ECL (supplementary material) shows whole membranes.

### 2.4 | Western Blotting

Cells or retinas were homogenized in 100 μL of lysis buffer (50 mM NaCl, 50 mM Tris-Cl [pH 7.5], 0.5 mM EGTA, 1 mM EDTA, 1% Triton-X-100, 0.1% SDS with protease cocktail inhibitor COMPLETE (Roche, Basel, Switzerland), rested on ice for 5 minutes and sonicated for 20 seconds with a Microson ultrasonic cell disruptor (Misonix Inc, New York, USA) before appropriate dilution in 1× SDS buffer. A standard protein assay (Bio-Rad protein assay, #5000006, Bio-Rad, Herts, United Kingdom) was used to determine protein concentrations in cell lysates. The Bio-Rad protein assay is a colorimetric assay for measuring protein concentration based on the Bradford dye-binding method. Protein was measured at 595-nm in a microtiter plate reader Halo LED 96 (Dynamica Scientific Ltd, Livingston, United Kingdom). β-mercaptoethanol (5%) (#M7154, Sigma Aldrich, Madrid, Spain) and bromophenol blue (1% wt/vol) (#B0126, Sigma Aldrich, Madrid, Spain) were added to samples. Then, samples were boiled for 5 minutes and electrophoresed on 6, 8 or 10% SDS polyacrylamide denaturing gel (depending on the molecular weight of the target protein) at 100 mV and 40 mA for 2 hours, followed by transfer to PVDF membranes (#10600023, Amersham TM, Little Chalfont, United Kingdom) at 1.3 A for 30 minutes using a Trans-Blot Turbo System (Bio-Rad, California, USA). Blots were blocked in 3% non-fat milk and incubated with primary antibody against RIPK1 (1:2000, #610458, Becton Dickinson, Madrid, España), RIPK3 (1:1000, #2283, Fort Collins, USA), NLRP3 (1:500, #ab91413, Abcam, Cambridge, United Kingdom), or β-actin (1:2000, #A2228, Sigma Aldrich, Madrid, Spain) overnight. Blots were washed in TBST (10 mM Tris-HCl [pH 8.0] 150 mM NaCl, 0.05% Tween) and incubated in 1.5% non-fat milk with the appropriated secondary peroxidase-conjugated antibody (#A6165-1ML, #A4416-1ML (rabbit or mouse), 1:10 000, Sigma-Aldrich, Madrid, Spain) one hour at room temperature and washed again three times with TBST. Protein bands were detected using NZY Advanced ECL (MB40201, Nzytech, Lisboa, Portugal) and imaged using the Alliance Q9 Advanced (Uvitec Cambridge, United Kingdom).

Western blots were quantified and normalized with β-actin using AlphaImager 2200 (alpha innotec, Germany). Figure 1 (supplementary material) shows whole membranes.

### 2.5 | Isolation of total RNA and cDNA synthesis

Total RNA was isolated from the 661W cells and frozen retinas using the RNeasy mini kit (#74104, Qiagen, Hilden, Germany) following the manufacturer’s protocol. RNA concentration was determined by spectrophotometry on the NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE). Then, cDNA was synthesized starting from 1 μg of RNA by reverse transcription using the GeneAmp Gold RNA PCR Reagent kit (#10783837, Applied Biosystems, Carlsbad, CA, USA) following manufacturer’s instructions. The cycling conditions consisted of primer annealing at 25°C for 15 minutes and reverse transcription at 42°C for 30 minutes.

For in vitro studies, each experiment was performed six times (six different cultures) with three replicates for each treatment. Eight frozen retinas for each group.

### 2.6 | Quantitative real-time PCR

The relative expression of RIPK1, RIPK3, TNFα, TNFR1, GFAP, COX2, IL-1β, IL-18, IL-6-atP23 was measured in murine retinas or in the 661W cells by real-time PCR using thermal-cycler (Applied Biosystems ViiATM 7 Real-Time PCR System; Life Technologies Corporation, Carlsbad, California, USA).
TaqMan gene expression assay, and specific TaqMan probes: Mm00436354_m1 (RIPK1), Mm00444947_m1 (RIPK3), Mm00443260_g1 (TNF-α), Mm00441883_g1 (TNFR1), Mm01253033_m1 (GFAP), Mm00434228_m1 (IL-1β), Mm00434226_m1 (IL-18), Mm00446190_m1 (IL-6), and TaqMan 2X PCR Master Mix (Applied Biosystems, Life Technologies Corporation, Carlsbad, California, USA). β2-microglobulin (β2m) gene (Mm00437762_m1) was used as the housekeeping gene.

Real-time PCR was performed with one cycle of 2 minutes at 50°C, followed by 1 cycle of denaturation of 10 minutes at 95°C, continued by 40 cycles of 15 seconds denaturation at 95°C and 60 seconds annealing at 60°C. Relative gene expression was normalized with the housekeeping gene. Then normalized values of control retinas/PBS-treated retinas or control 661W cells were normalized to 1 to determine the changes in the gene expression of rd10 retinas or of 661W cells treated with tunicamycin with/without ADA.

2.7 | Caspase activity assay

Caspase 1, 3, 8, and 9 activities were determined using colorimetric assay kits according to the manufacturer’s instructions (#K111, #K106, #K113, #K119 BioVision, Inc, Milpitas, CA, USA). Briefly, 661W cells or frozen retinas were lysed in sample lysis buffer (BioVision, Inc, Milpitas, CA, USA). The homogenates were then centrifuged at 10 000 xg for 1 minute at 4°C and the supernatant was collected for protein estimation using the bicinchoninic acid method (BCA, #23225, Pierce, Thermo Fisher Scientific, Madrid, Spain). The cells or retinal lysates were then incubated with the YVAD-pNA substrate (for caspase 1), the DEVD-pNA substrate (for caspase 3), IETD-pNA (for caspase 8) or LEHD-pNA (for caspase 9) for 2 hours at 37°C. Samples were measured at 405-nm in a microtiter plate reader Halo LED 96 (Dynamica Scientific Ltd, Livingston, United Kingdom). Fold-increase in caspase activity was determined by comparing the results of treated cells or eyes with the level of untreated control cells or eyes. For cell lysates, each experiment was performed six times in triplicate for each treatment. Eight retinas for each group for retinal lysates. Data represent the absorbance at 405 nm/milligram of protein (arbitrary units).

2.8 | Cell cytotoxicity assays

MTT assay: the 661W cells were grown to 80% confluency and then treated with different concentrations of TNFα,
tunicamycin, Z-IETD-FMK, Olaparib, and/or ADA for 24h. To test cell viability, 10 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (#M5655, MTT; Sigma-Aldrich, Madrid, Spain) from a 5 mg/mL of stock was added to the media, and the cells were incubated for two hours at 37°C. Mitochondrial succinate dehydrogenase (EC1.3.9.9) from viable cells reduces MTT to form a dark blue formazan crystal. Then, the media were removed the formazan precipitate was dissolved in 100 µL dimethyl sulfoxide (DMSO) (#D2650, Sigma-Aldrich, Madrid, Spain), and the absorbance was read at 550-nm in a microtiter plate reader Halo LED 96 (Dynamica Scientific Ltd, Livingston, United Kingdom). The viability of the cells was expressed as the percentage of untreated cells (100% viability). Each experiment was performed as follows: four times for ADA, eight times for TNFα, five times for TNFα + Z-IETD-FMK, eight times for TNFα + ADA, four times for TNFα + Olaparib, six times for tunicamycin, and five times for tunicamycin + ADA, with 24 replicas for each treatment.

Lactate dehydrogenase (LDH) release: LDH is a stable cytosolic enzyme that is released to the culture medium upon cell damage. LDH release into the culture medium of the 661W cells was assessed using the CyQUANT LDH cytotoxicity Assay Kit (#C20300, Invitrogen, Thermo Fisher Scientific, Madrid, Spain) according to the manufacturer’s instructions. The lysis buffer was used to determine the maximum amount of LDH present. The absorbance was measured at wavelengths of 490 nm and 690 nm (background absorbance) using a microplate reader Halo LED 96 (Dynamica Scientific Ltd, Livingston, United Kingdom). Subsequently, the percentage of cytotoxicity was calculated by the following formula: Cytotoxicity (%) = 100 [(compound-treated LDH activity-spontaneous LDH activity (untreated cells)/ (maximum LDH activity-spontaneous LDH activity (untreated cells)). Each experiment was performed as follows: four times for TNFα + Olaparib, and four times for TNFα + ADA, with 24 replicas for each treatment.

Flow Cytometry: Annexin V-fluorescein isothiocyanate (FITC) (AV) (#130-093-060, Miltenyi Biotec GmbH, Gladbach, Germany) and propidium iodide (PI) (#P470, Sigma Aldrich, St. Louis, MO, USA) were used to detect cell death. The 661W cells were harvested and centrifuged at 300 g for 5 minutes. After cold-PBS washing three times, the cells were re-suspended in 100 µL of binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2). Then, the cells were mixed gently with 0.5 µL of Annexin V-FITC and 0.5 µL of PI (5 µg/mL) and incubated for 15 minutes at room temperature in dark. After that, 200 µL of binding buffer was added. Three controls were included for each experiment: (1) unstained—without any stain (Annexin V-FITC, PI); (2) Annexin V only; (3) PI only. Flow cytometry of samples was performed using a CytoFLEX S (Beckman Coulter Life Sciences, Indianapolis, IN, USA). Annexin-FITC fluorescence was collected through a 525/40 band-pass filter, whereas PI fluorescence was collected through a 610/20 band-pass filter. Data acquisition was performed using the CytExpert 2.1 software (Beckman Coulter Life Sciences, Indianapolis, IN, USA). Four types of cell populations (%) were detected in a dot plot of Annexin V-FITC/PI staining: live cells (Annexin V−/PI−), early apoptotic cells (Annexin V+/PI−), late apoptotic or necrotic cells (Annexin V+/PI+), and necrotic cells (Annexin V−/PI+). Each experiment was performed four times with 24 replicas for each treatment.

2.9 | Cytokines and adalimumab enzyme-linked immunosorbenent assay (ELISA)

Extracellular TNFα concentration was quantified in the culture medium of 661W cells exposed to tunicamycin, and tunicamycin + ADA for 24 hours using an ELISA kit from Diaclone (#860.040.096, Diaclone, Besançon, France), according to manufacturer’s instructions. Extracellular IL6 concentration was quantified in the culture medium of BV2 cells exposed to TNFα, and TNFα + ADA for 24 hours using an ELISA kit from abfrontier (#LF-EK0270, abfrontier AdipoGen Life Sciences, Liestal, Switzerland) according to manufacturer’s instructions. Readings were performed at 450-nm in a microtiter plate reader Halo LED 96 (Dynamica Scientific Ltd, Livingston, United Kingdom). TNFα and IL-6 concentrations were expressed as pg/mL. Six (for TNFα) and four (for IL-6) different cell cultures were used per treatment, with three replicas for each treatment. The minimum detectable dose of TNFα was 9.2 pg/mL.

ADA concentration in retinal tissue was quantified using an ELISA kit from AffinityImmuno (#EL-1611-011, AffinityImmuno Inc, Charlottetown, PE, Canada) according to manufacturer’s instructions. Readings were performed at 450-nm in a microtiter plate reader Halo LED 96 (Dynamica Scientific Ltd, Livingston, United Kingdom). Briefly, retinas were collected and homogenized in 100 µL of 0.1M phosphate buffer, sonicated for 20 seconds with a Microson ultrasonic cell disruptor (Mixon Inc, New York, USA), and centrifuged at 300 ×g for 1 minutes at 4°C. Supernatants were collected for ADA detection and protein estimation using the BCA method (#23225, Pierce, Thermo Fisher Scientific, Madrid, Spain). ADA concentration was expressed as ng/mg protein.

2.10 | Immunocytochemistry

Both the 661W and the BV2 cells were fixed in 4% filtered paraformaldehyde for ten minutes at room temperature.
Slides were incubated in the blocking solution containing 5% normal goat serum, 1% bovine serum albumin, and 0.25% Triton X-100 (#A1388, PanReac AppliChem, Darmstadt, Germany) for one hour. Slides were incubated with primary antibody against PAR (1:100, #ALX-804-220, Enzo Life Science, Madrid, Spain), as a marker of PARP activity, and/or against cleaved-caspase 3 (Asp 175) (1:400, #9661, Cell Signalling Technology, Leiden, The Netherlands) overnight at 4°C. Then the samples were incubated with the fluorescence-conjugated secondary antibodies Alexa Fluor 488 or 647 (1:400, #A-11001, #A-21235, Invitrogen, Life Technologies, Madrid, Spain) for one hour at room temperature. After labeling and counterstaining with DAPI, the slides were mounted in Fluoromount-G (#0100-01, Southern Biotechnology, Birmingham, AL, USA), and observed under an SP5 confocal microscope at 22°C. Four different experiments were analyzed. Slides without primary antibody were used as negative controls.

2.11 TUNEL assay and retinal histology

To obtain retinal sections, the eyes were rapidly removed and fixed in 4% filtered paraformaldehyde for two hours at room temperature and cryoprotected in a sucrose gradient (15-20%-30%). The eyes were frozen, embedded in OCT and 10 μm sections were cut in a cryostat (Leica CM1900, Nussloch, Germany). To evaluate cell death, the terminal deoxynucleotidyl transferase dUTP nick and labeling (TUNEL) assay (#S7165, Merk, Darmstadt, Germany) was used for one hour at room temperature. After labeling and counterstaining with DAPI, the slides were mounted in Fluoromount-G (#0100-01, Southern Biotechnology, Birmingham, AL, USA), and observed under an SP5 confocal microscope at 22°C. Eight left and right retinas were analyzed for each group. Slides without primary antibody served as a negative control.

To analyze retinal structure fixed eyes were embedded in paraffin. Five μm paraffin (#115161, Sigma-Aldrich, Madrid, Spain) sections were baked for 1 hour at 60°C, dewaxed with xylene, and rehydrated through graded ethanol. Haematoxylin (#2569911610, PanReac AppliChem, Darmstadt, Germany) and eosin (#115935, Sigma-Aldrich, Madrid, Spain) staining was performed using a Leica ST5010 autostainer (Leica, Nussloch, Germany).

2.12 Microscopy and quantification

The 661W cells, the BV2 cells, and the retinal sections were examined under a confocal microscope (40X magnification, Leica TCS SP5 Confocal microscope, Leica Microsystems CMS GmbH, Mannheim, Germany) from the Microscopy Unit of the IIS-La Fe (Valencia, Spain) using a spatial resolution of 1024 × 1024. The pinhole was set at 1 airy unit and z-stacks were made of 10 pictures (1.0 μm steps) for retinal sections and of three pictures for cells. All stacks were taken under 40× magnification, with an acquisition rate of 16 frames per second. A zoom factor of 3.0 was employed in some retinal sections or cell preparations. Leica LAS AF was used as microscope imaging software (Leica Microsystems CMS GmbH, Mannheim, Germany). Negative controls (without primary antibody) were used to detect autofluorescence or noise. The acquisition parameters for each fluorophore were adjusted (eg, gain, smart offset, and excitation energy) to get a proper image. In case of double immunostaining plus DAPI counterstain sequential acquisition was used to avoid crosstalk between fluorophore. Direct counting of photoreceptor nuclei in the outer nuclear layer (ONL), the number of TUNEL-, PAR- and Iba1-positive cells (manual cell counting), and the integrated density of GFAP was carried out using the ImageJ open-source Software. Adobe Photoshop 10 software (Adobe Systems Inc, San Jose, CA, United States) was used to process the images. We measured the ONL thickness of the entire retina normalized to the thickness of the inner nuclear layer (INL) to avoid the bias derived from the angle of the sectioning plane. Because the ONL thickness and the degenerative process in the rd10 model vary in different retinal locations, we performed several measurements across the entire retina (from the nasal to the temporal retina) for each mouse. The normalized ONL thickness ratio is defined as the ONL thickness/INL thickness. At least seven entire retinas were analyzed per experimental group.
The number of TUNEL, PAR-positive cells was represented as the ratio between the number of TUNEL, or PAR-positive cells in the ONL and the normalized ONL thickness ratio of each section.

Microglial activation was measured as previously described. Corrected fluorescence of GFAP was quantified as previously described. TUNEL, cleaved caspase 3- and PAR-positive cells, microglial migration index, and the corrected fluorescence of GFAP were quantified from four non-adjacent sections of at least seven retinas for each experimental group.

### 2.13 Statistical analyses

Statistical analyses were performed using GraphPad Software (Prism; GraphPad Software, Inc, San Diego, CA). Normal distribution of data was analyzed by Shapiro-Wilk and Kolmogorov-Smirnov tests. Cell viability was performed using ANOVA and post hoc Dunnett’s multiple comparisons test. Comparisons between control and rd10 mice were performed using unpaired Student’s t- or Mann-Whitney U tests depending on data distribution (parametric or nonparametric analysis). Comparisons between Adalimumab-treated and PBS-treated eyes were performed using paired Student’s t-test or Wilcoxon matched-pairs signed-rank tests. From six to eight animals/group were used for each analysis. A P-value < .05 was considered statistically significant. The data were plotted using the GraphPad Software. The data are presented as mean ± standard error of the mean (SEM).

### 3 RESULTS

#### 3.1 TNFα induced cell death through activation of caspases (3, 8, and 9) in cone-derived 661W cells. Adalimumab prevented in vitro activation of caspase-dependent cell death

Upregulation of TNFα seems to contribute to photoreceptor cell death in rd10 mice. We previously described that intraperitoneal administration of ADA, an antibody against TNFα, reduced retinal degeneration at P18. We decided to evaluate the effect of ADA in cone-derived 661W cells treated with exogenous TNFα or tunicamycin. This substrate is a known potent activator of the unfolded protein response (UPR) that also upregulates TNFα production. First, we incubated the cone-derived 661W cells with a series of increasing the concentration of ADA (0-10 µg/mL), or TNFα (0-200 ng/mL) for 24 hours. ADA concentrations did not have any effect on cell metabolic activity (Figure 1A). We selected the two highest concentrations of ADA (5 and 10 µg/mL). As shown in Figure 1A, cell metabolic activity was significantly decreased by TNFα treatment in a dose-dependent manner (One-way ANOVA and Dunnett’s multiple comparisons test). We selected 100 ng/mL for exogenous TNFα to evaluate the effect of ADA on TNFα-mediated cell death mechanisms. We assumed that the reduced cell metabolic activity was a consequence of a reduced cell viability, as it has been observed in other studies. LDH release corroborated it (Figure 1B). Besides, these observations were validated by flow cytometric analysis (Figure 1C). As observed, exogenous TNFα resulted in a significant decrease in live cells (Annexin V−/PI−), an increase in early apoptotic cells (Annexin V+/PI−), and especially in late apoptotic and necrotic cells (Annexin V+/PI+) (One-way ANOVA, Tukey’s multiple comparisons test). Two hours of pre-incubation with 5 or 10 µg/mL of ADA (ADA5 or ADA10), before adding exogenous TNFα (100 ng/mL) significantly prevented cell death (MTT assay and LDH release) (One-way ANOVA, Tukey’s multiple comparisons test and Kruskal-Wallis test, Dunn’s multiple comparisons test, respectively) (Figure 1B). Both ADA5 and ADA10 reduced the percentage of early apoptotic (Annexin V+/PI−), late apoptotic cells and necrotic cells (Annexin V+/PI+) but slightly increased necrotic cells (Annexin V−/PI+) (Figure 1C).

We analyzed activation of caspase 3, caspase 8 (extrinsic apoptotic pathway), and caspase 9 (intrinsic apoptotic pathway) in cell lysates of TNFα-treated cells (Kruskal-Wallis test, Dunn’s multiple comparisons test for caspases 3, and 8; One-way ANOVA, Tukey’s multiple comparisons test for caspase 9) (Figure 2A-C). ADA significantly reduced caspase 3 and 9 activities (Kruskal-Wallis test, Dunn’s multiple comparisons test and One-way ANOVA, Tukey’s multiple comparisons test) (Figure 2B) in TNFα-treated cells. To evaluate the direct involvement of caspase 8 activation on TNFα-induced cell death, the 661W cells were pre-incubated with the caspase 8 inhibitor, Z-IETD-YMK, (20 µM or 50 µM) for two hours before adding TNFα (100 ng/mL). Caspase 8 inhibition did not prevent TNFα-induced cell death (Figure 2D). Z-IETD-YMK did not normalize caspase 3 activity in TNFα-treated cells (Figure 2E).

In addition to apoptosis, cone cell death was also accompanied by activation of caspase 1, 1.5-fold up-regulation of RIPK3 protein, and 0.5-fold decrease of RIPK1 protein (Kruskal-Wallis test, Dunn’s multiple comparisons tests) (Figure 2F-I). ADA treatment reduced caspase 1 activity (Kruskal-Wallis test, Dunn’s multiple comparisons tests), restored RIPK1 protein content but did not have an effect on RIPK3 protein (Kruskal-Wallis test, Dunn’s multiple comparisons tests) (Figure 2F-I).

PARP activation is considered to be a hallmark of oxidative stress-induced cell death. To determine the effect of exogenous TNFα on PARP enzymes, we measured the...
PARP activity and poly-ADP (ribose) (PAR) polymers formation by immunofluorescence. PARP activity tended to decrease in TNFα-treated cells compared with untreated 661W cells ($P = .08$, unpaired t test) in cell lysates (Figure 2J). However, we observed PAR accumulation in TNFα-treated cells (Figure 2K). PARP activity tended to normalize in cell lysates after ADA treatment. Besides, PAR accumulation decreased especially after ADA10 in intact cells (Figure 2K). In order to assess whether PARP activation was responsible for TNFα-induced cell death, we pretreated cells with olaparib
ADA did not prevent tunicamycin-induced cell death or caspase 3 activation (Kruskal-Wallis test, Dunn test multiple comparisons) (Figure 3F-H). However, ADA seemed to reduce PAR accumulation in intact in TNFα-treated cells (Figure 3H). These results were similar to those obtained in MEF cells treated with anti-TNFα, suggesting that under ER stress conditions (tunicamycin treatment) retinal cells die by TNFα/TNFR1 independent-cell death mechanisms.41

Therefore, exogenous TNFα molecules probably promoted in vitro photoreceptor cell death through caspase-dependent mechanisms. TNFα also induced PAR accumulation, caspase 1 activation, and overexpression of RIPK3 in these cells. Addition of ADA to these cells protected them from TNFα-induced cell death through inhibition of caspase 3 and caspase 9 but not through PARP inhibition. As previously described, tunicamycin induced ER stress and TNFα upregulation. However, ADA did not protect from tunicamycin-induced cell death (ES stress).

3.2 | Key players of apoptosis, necroptosis and NLRP3 inflammasome are altered in rd10 retinas at P23

We previously characterized the time course of retinal degeneration in rd10 mice from P15 to P60.13 We described that repeated intraperitoneal administrations of ADA prevented cell death at P18, which is when there is a peak of photoreceptor degeneration under our housing conditions.14 However, the protective effect disappeared at P20. Now we selected a later age, P23, to assess whether a single intravitreal injection of ADA could delay retinal degeneration until this age. Before assessing ADA treatment, we analyzed some cell death markers to discover possible cell death mechanisms involved at this age.

We incubated the 661W cells with increasing concentration of tunicamycin (0-10 µg/mL) for 24 hours. As shown in Figure 3A, cell metabolic activity was significantly decreased by tunicamycin treatment in a dose-dependent manner (One-way ANOVA and Dunnett’s multiple comparisons test). Tunicamycin toxicity is well established.39 We selected 10 µg/µL for tunicamycin to evaluate the effect of ADA on the mechanism of the tunicamycin-mediated cell death. After 24 hours, the treatment with tunicamycin (10 µg/µL) upregulated TNFα (TNFα release and TNFα gene expression) and downregulated TNFR1 (gene expression) in a dose-dependent manner (Kruskal-Wallis test, Dunn test multiple comparisons). Addition of tunicamycin (10 µg/mL) increased TNFα release up to 28 ± 4 pg/mL (untreated cells 11 ± 1 pg/mL) and 1.8-fold up-regulation of gene expression (Figure 3B,C). Tunicamycin induced a lower increase of TNFα protein (0.028 ng/mL) than exogenous administration of TNFα (100 ng/mL). As we hypothesized, treatment with ADA10 downregulated TNFα (TNFα release and TNFα gene expression) (Kruskal-Wallis test, Dunn test multiple comparisons) and had no effect on TNFR1 expression (Figure 3D, E). As shown in Figure 3F-H, cell death was accompanied by caspase 3 activation and PAR accumulation in tunicamycin-treated cells as previously described for mouse embryonic fibroblast (MEF) cells.41 Incubation of the cells with ADA did not prevent tunicamycin-induced cell death or caspase 3 activation (Kruskal-Wallis test, Dunn test multiple comparisons) (Figure 3F-H). However, ADA seemed to reduce PAR accumulation in intact in TNFα-treated cells (Figure 3H). These results were similar to those obtained in MEF cells treated with anti-TNFα, suggesting that under ER stress conditions (tunicamycin treatment) retinal cells die by TNFα/TNFR1 independent-cell death mechanisms.41

We confirmed the upregulation of RIPK3 protein content in rd10 retinas (Mann-Whitney test) (Figure 4A). Opposite effect was found for RIPK1 and RIPK3 gene expression in the rd10 retinas. While RIPK1 expression was downregulated, RIPK3 expression was significantly increased (unpaired t test) (Figure 4B). We confirmed the upregulation of RIPK3 protein content...
by western blotting (272 ± 46% of control retinas, unpaired t test) (data not shown). PAR formation was also increased at the ONL of rd10 retinas (Figure 2 suppl). At P23 retinal degeneration was also accompanied by neuroinflammation including TNFα upregulation (unpaired t test) (Figure 4C), reactive gliosis (GFAP upregulation, Figure 4C) and microglia activation. However, at this age, other inflammatory mediators were downregulated (IL-6, COX2) (unpaired t test) (Figure 4C). Upregulation of RIPK3 and caspase 1 activation could indicate activation of NLRP3 inflammasome. Thus, we quantified some players of the NLRP3 inflammasome: IL-18 and IL-1β gene expression and NLRP3 protein content. IL-1β gene expression and NLRP3 protein were upregulated (unpaired t test) in retinas of rd10 mice, suggesting the activation of NLRP3 inflammasome (Figure 4C, D). However, IL-18 gene expression was significantly downregulated at this age (unpaired t test) (Figure 4C). Further analysis of the active forms of these cytokines should be addressed.

3.3 A single dose of intravitreal adalimumab slowed down retinal degeneration in rd10 mice at P23

It has been described that TNFα is upregulated in rd10 retinas before the photoreceptor cell death peak. As commented
above, intraperitoneal injections of ADA slowed down retinal degeneration reducing PAR accumulation and neuroinflammation at P18 in rd10 mice. In this study, we assessed whether a single dose of intravitreal ADA was able to prevent retinal degeneration for a longer time (P23). Moreover, we assessed the putative molecular mechanisms involved in cell death at this age (caspase-dependent or independent mechanisms).

To confirm that ADA reached the retina after intravitreal injection, we measured ADA content in rd10 retinas 1, 2, and 6 days after ADA (4.61 µg/µL) injection (Figure 5A). Detectable amounts of ADA were only found 24 hours after injection. A small increase (by 15% of treated eyes) was also detected in the contralateral eye (vehicle, PBS) at this time.

Different doses of ADA were intravitreally injected into the left eye of each mouse at P12; the right eye was injected with PBS (vehicle) and, it was used as control. Besides, we used rd10 mice to be sure that findings in PBS-treated eyes were similar to those obtained from untreated eyes. To determine whether intravitreal ADA administration protects against photoreceptor cell death, we quantified the number of rows in the ONL and the normalized ONL thickness ratio in retinas of rd10 mice at P23. As shown in Figure 5B-C, photoreceptor quantification (nuclei and ONL thickness) showed a significant rescue by intravitreal ADA at 0.375 and 4.61 µg/µL with a 1.4- and 1.6-fold increase in cell number compared to vehicle or non-treated eyes (paired t test). The architecture of the retina was not affected by ADA treatment (Figure 5D). Based on these results we selected 0.375 and 4.61 µg/µL of ADA (ADA0.375 and ADA4.61) for further studies.

We previously described that intraperitoneal ADA significantly reduced the number of TUNEL-positive cells in the ONL at P18. In the current study, we sought to further investigate these events so we could better explain this point. First, we performed TUNEL assay to detect DNA fragmentation (a late event of cell death) at P23. The number of TUNEL-positive cells detected in the ONL was not significantly different between ADA-treated retinas and PBS-treated retinas (paired t test and Wilcoxon matched-pairs
signed-rank test for ADA0.375 and ADA4.61, respectively). However, these data suggested that ADA0.375-treated retinas tended to present more TUNEL-positive cells than PBS-treated retinas (Figure 6A). To co-localize active caspase 3 (an early event in apoptosis) and DNA fragmentation in degenerating photoreceptors, we performed sequential immunostaining for cleaved caspase 3 and TUNEL staining. As shown in Figure 6B, TUNEL-positive cells did not show active caspase 3 at ONL (with or without ADA). As shown in Figure 6C, ADA0.375 did not significantly affect caspase 3 activity (paired t test). But, retinas treated with ADA4.61 showed a significant increase in caspase 3 activity.

The treatment with ADA0.375 did not affect RIPK1 expression but downregulated RIPK3 gene expression compared to PBS-treated retinas (paired t test) (Figure 6D). ADA4.61 also decreased RIPK3 expression compared to PBS-treated retinas (paired t test). At the highest ADA concentration RIPK1 expression tended to increase ($P = .07$, paired t test) (Figure 6D).

Both ADA concentrations reduced nuclear PAR accumulation compared to PBS-treated retinas (paired t test) (Figure 6E). These data suggest that intravitreal ADA could reduce, at least partially, retinal degeneration through PARP inhibition.

### 3.4 A single dose of intravitreal adalimumab reduced NLRP3 inflammasome activation and microglial activation in rd10 mice at P23

We assessed whether ADA had any effect on some components of NLRP3 inflammasome: caspase 1 activity (Figure 6A), IL-1β, and IL-18 gene expression (Figure 7A), or NLRP3 protein content (Figure 7B). ADA0.375-treated retinas showed downregulation of caspase 1 activity and protein content of NLRP3 compared to PBS-treated retinas (paired t test). ADA4.61 had a similar effect on NLRP3 protein but this concentration did not only decrease caspase 1 activity but also increased it. In addition, both concentrations of ADA significantly reduced IL-1β gene expression without affecting IL-18 gene expression (paired t test) (Figure 7A).

We observed the downregulation of TNFα gene expression after the intravitreal injection of ADA0.375. Surprisingly, ADA4.61 increased TNFR1 gene expression (Figure 7A).
Microglial activation and upregulation of GFAP in Müller glial cells, which commonly indicates gliosis, were previously found in rd10 mice. Activated glial cells can release cytokines such as TNFα that could exacerbate and propagate neuroinflammation. In this study, intravitreal ADA0.375 and ADA4.61 did not reduce GFAP upregulation (paired t-test) but ameliorated microglial activation (Figure 7C, D). Conversely, ADA4.61 enhanced GFAP upregulation in these retinas (Figure 7A, C). As shown in Figure 7D, PBS-treated retinas showed the presence of Iba-1 positive cells (microglia) with ameboid shape and migration to the outer retina (ONL). However, ADA-treated retinas showed Iba-1 positive cells with ramified shape that resided mostly in the inner retina (Figure 7D). Double staining of PAR and Iba1 revealed that some microglial cells located at the ONL also presented PAR polymers at ONL (Figure 7D). We assessed whether exogenous TNFα induced inflammatory markers and PAR polymers in microglia cells in culture. After 24h TNFα (15 ng/mL) upregulated IL-6 (Kruskal-Wallis test, Dunn test multiple comparisons) in BV2 cells. We confirmed PAR accumulation after 15 minutes, 30, 60 minutes, and 24 hours of TNFα (15 ng/mL). ADA10 decreased its accumulation (Figure 7E). No significant changes were observed for the inflammatory markers COX2, iNOS, and IL6 (Figure 7A).
FIGURE 7  Effect of a single intravitreal injection of ADA on inflammation, microglia activation, and NLRP3 inflammasome in rd10 retinas at postnatal day 23. Gene expression of inflammatory mediators in rd10 retinas treated with PBS or with 0.375 µg/µL or 4.61 µg/µL of ADA (ADA0.375 or ADA4.61) (A) Representative western blot and quantification of NLRP3 (B) Corrected fluorescence of GFAP and representative photomicrographs of retinal sections showing GFAP-labeling (Müller cells) in DAPI-counterstained sections (C) Index of microglial migration and representative photomicrographs of retinal sections showing Iba1- a PAR-labeling (microglial cells) in DAPI-counterstained section D, in PBS-, ADA0.375-, ADA4.61-treated retinas; IL-6 release and poly ADP-ribose polymers (PAR) accumulation in microglial cells (BV2 cells) after 100 ng/mL (IL-6 release) or 15 ng/mL (PAR accumulation) of TNFα and 5 µg/mL of Adalimumab (ADA5) or 10 µg/mL (ADA10) for 24 hours (IL-6 release) or short periods (PAR accumulation) (E) ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion nuclear layer. Scale bar: 50 µm, 10 µm for optical. *Paired t-test for left rd10 retina (PBS) vs right rd10 retina (ADA treatments), Kruskal-Wallis test, Dunn test multiple comparisons for BV2 cells. *P < .05; **P < .01. Data are presented as mean ± standard error of the mean (SEM). Eight retinas were used for each group. Each experiment of BV2 cells was performed four times.


4 | DISCUSSION

Retinitis pigmentosa is a group of inherited retinal degenerations. However, there are cellular events such as inflammation or oxidative stress that can exacerbate or propagate photoreceptor cell death. \(^{5,46}\) We previously focused on the pro-inflammatory cytokine TNFα as a putative therapeutic target. \(^{7,14,37}\) We demonstrated that several intraperitoneal injections of ADA, a recombinant human monoclonal antibody against TNFα, reduced retinal degeneration decreasing photoreceptor cell death and reactive gliosis at early stages of RP in rd10 mice. However, the temporal effect of the treatment, which only lasted until P18, and the possible systemic side effect of long-term treatment for future human studies, encouraged us to explore intravitreal treatment. It remained unclear what cell death mechanisms were involved during retinal degeneration and, how ADA protected cells from degeneration. Apoptosis and necroptosis have been proposed mechanisms responsible for the sequential death of rods and cones, but, more than one biological pathway could influence the course of RP, as occurs in other retinal degenerative diseases such as diabetic retinopathy or age-related macular degeneration.

Until some years ago, two classical forms of cell death were described: apoptosis and necrosis. Apoptosis relies on an intracellular proteolytic cascade, which is mediated by initiator caspases (caspase 8 and caspase 9) and executioner caspases (caspase 3, caspase 6 and caspase 7). Initiator caspases can be activated by the extrinsic or the intrinsic pathway. \(^{48}\) The extrinsic pathway is activated by binding to death receptors of members of TNF superfamily of cytokines, such as TNFα, among others. Binding of these molecules induces the formation of the death-induced signaling complex (DISC), and subsequent activation of the initiator caspase 8. Activated caspase 8 triggers the executioner caspase 3, which executes apoptosis. The intrinsic pathway, which is dependent on mitochondrial activity, can be activated following oxidative damage to mitochondrial proteins, DNA damage driven by excessive levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS). During the intrinsic pathway, mitochondrial dysfunction results in the release of cytochrome c and activation of caspase 9 that cleaves and turns on caspase 3. In addition, the extrinsic pathway can trigger intrinsic apoptosis through the generation of truncated BID (tBID) by activated caspase 8. tBID translocates to mitochondria inducing cytochrome c release, sequentially activating caspases 9. Several studies have demonstrated that elevated TNFα, ROS, and RNS in RP accompany retinal degeneration. \(^{5,49}\) TNF-α can activate both the extrinsic and the intrinsic apoptotic pathways. \(^{50}\)

In recent years, the existence of necroptosis, ferroptosis, PARthanatos, and pyroptosis has been demonstrated as drivers of necrotic cell death during retinal degenerations in age-related macular degeneration or RP. \(^{51-54}\) Necroptosis is regulated necrosis mediated by death receptors. Typically, necroptosis starts by stimuli such as TNFα, which binds to its receptors leading to RIPK1 activation. As commented in the Introduction, under apoptotic conditions, active caspase 8 prevents further necrotic signaling by cleaving and inactivating RIPK1 and RIPK3. However, when caspase 8 is not active, RIPK1 recruits RIPK3 to form the necroosome complex. RIPK3 phosphorylates MLKL leading to its oligomerization. Thus, MLKL recruitment to the plasma membrane induces necroptosis by triggering Ca\(^{2+}\) and Na\(^{+}\) influx into the cell. \(^{55}\) Hence, TNFα can induce both apoptosis and necroptosis. Besides, high levels of TNFα or oxidative stress could activate PARP1. PARthanatos is considered a form of cell death resulting from overactivation PARP. \(^{56}\) PARPs are multidomain enzymes that bind and cleave NAD\(^+\) to nicotinamide and ADP-ribose. PARPs couple one or more PAR to acceptor proteins (PARylation) and components of the DNA repair machinery. PARP1 is the most characterized and abundant (85%-90%) chromatin-associated enzyme. \(^{57}\) Apart from its role in DNA damage repair, PARP1 modulates neuroinflammatory processes in glial cells. \(^{58}\) For instance, PARP1 is indispensable for the NF-κB-mediated activation of microglia and the transcription of IL-1β or TNFα. \(^{58,59}\)

Overactivation of PARP1 can deplete cellular ATP and NAD\(^+\) storage in an attempt to repair the damaged DNA. This depletion would lead to bioenergetic collapse and necrotic cell death. PARP1 could also induce programmed cell death, via the production of PAR polymers, which would stimulate the mitochondria to release AIF, which in turn induces cell death. \(^{60-62}\) This mechanism appears to be caspase-independent.

In the current study, we observed that in vitro TNFα induced cell death, activation of caspases 3, 8, and 9, dysregulation of RIPK1 and RIPK3 proteins and changes in PARP activity in cone-derived 661W cells. Caspase 3 activation was also found by Rana et al., \(^{38}\) suggesting that TNFα induces cell death, at least partly, by caspase-dependent mechanisms in these retinal cells. \(^{38}\) ADA prevented cell death by reducing caspase 3 and 9 activities. However, ADA did not reduce significantly caspase 8 activity. Inhibition of caspase 8 was not able to prevent cell death nor caspase 3 activation after 24 hours. Taking together these results, we suggest that TNFα would activate the extrinsic and the intrinsic apoptotic pathways. Maybe TNFα directly activates the intrinsic apoptotic pathway promoting caspase 9 activation as described for neuroblastoma cells. \(^{50}\)

Moreover, TNFα increased the accumulation of PAR polymers in intact cells and reduced PARP activity in cell lysates. PARP1 is the most prominent target protein for PARylation. \(^{63}\) This autoPARylation inhibits PARP1. \(^{64}\) Therefore, it is possible that the reduced activity of PARP1
is a consequence of this autoPARylation. Reduced PARP1 activity could be due to its cleavage by caspase 3, 7 or even 1.\textsuperscript{55-60} It is also believed that PARP1 cleavage prevents the repair of DNA strand breaks during the apoptotic program and the energy depletion (NAD\textsuperscript{+} and ATP).\textsuperscript{66,69-71} In this study, we observed that caspase 3 did not colocalize with PAR polymers in TNF\textalpha-treated cells (661W). Besides, Olaparib, a PARP inhibitor, did not prevent TNF\textalpha-induced cell death. TNF\textalpha-induced PARP activation has been described in other cultured cells like L929, Jurkat, HT-29, and NIH3T3 (fibroblasts) and the use of PARP inhibitors in these cells corroborate our results.\textsuperscript{72}

TNF\textalpha increased caspase 1 activity and RIPK3 protein and downregulated RIPK1 protein in 661W cells. ADA restored the RIPK1 level and tended to normalize PARP activity. In cone-derived 661W cells, exogenous TNF\textalpha seems to induce cell death through caspase-dependent mechanisms although it also alters other components related to necroptosis and PARthanatos.

Tunicamycin, an inducer of unfolded protein response, induced cell death by promoting caspase 3 activation as occurs in other retinal cells such as ARPE-19, or primary culture of retinal ganglion cells.\textsuperscript{39,73} In this case, ADA did not prevent from cell death suggesting other key players responsible for cell death. Besides, tunicamycin induced TNF\textalpha upregulation, downregulation of TNFR1, and perinuclear accumulation of PAR polymers. Several types of stress situations including tunicamycin treatment can induce the assembly of stress granules, which are organized around the perinuclear region.\textsuperscript{74} PAR polymers are required for stress granule assembly and disassembly.\textsuperscript{75} Therefore, perinuclear PAR accumulation could contribute to the stress granule formation induced by tunicamycin.

We observed that retinas of rd10 mice also showed activation of caspase 3, caspase 1, PAR accumulation, upregulation of RIPK3 protein, and downregulation of RIPK1 protein at P23. At this postnatal age, these results could suggest that photoreceptor cells are degenerating by caspase-dependent or PAR-dependent (PARthanatos) mechanisms. However, the role of caspases is controversial in RP. Several authors have proposed the upregulation of caspase 3/8 activities and mitochondrial cytochrome C release in RP models like transgenic S334ter rats, tubby mice, rd1 mice, etc.\textsuperscript{76-79} However, other caspase 3-independent mechanisms have been reported for rod cell death in rd mice or P23H rats.\textsuperscript{80,81} Double labeling with TUNEL and cleaved caspase 3 indicated that degenerating photoreceptors (TUNEL positive cells) were not cleaved-caspase 3 positive cells (an early event of apoptosis) at this age. A single dose of intravitreal ADA0.375 did not affect caspase 3 activity but increased it at ADA4.61. At P23 degenerating photoreceptors would be dying by caspase 3-independent mechanisms. Caspase-dependent mechanisms could contribute to cell death at later stages. Furthermore, TNF\textalpha seems not to be involved in the caspase 3 activation at this age.

At P23 we observed overactivation of PARP by detecting accumulation of PAR polymers at the ONL of retinas of rd10 mice corroborating results from other authors.\textsuperscript{82-84} Double staining of PAR polymers and Iba1, a marker of microglia cells, suggests that some activated microglia cells accumulated PAR polymers at the ONL of rd10 retinas. Exogenous TNF\textalpha also induced PAR polymers accumulation in BV2 cells. Many authors have published that migration and invasion of the outer retina by microglial cells happens during retinal degeneration.\textsuperscript{85-88} ADA prevented PAR accumulation both in vitro (BV2 cells) and in vivo (rd10 retinas) and microglial activation (migration and morphology changes) corroborating showed that PARP activation is important for microglial activation in experimental models of retinal degeneration and neuronal damage.\textsuperscript{89-92} PARP activation is an essential step for activating necroptosis through NF-κB.\textsuperscript{93,94} ADA could prevent microglial activation through PARP inhibition. Unlike in vitro studies where apoptotic mechanisms mediated by caspases are major effectors of cell death, PARthanatos seems to be a major contributor to cell death in retinas of rd10.

As previously mentioned, retinal degeneration is accompanied by reactive gliosis at different postnatal days including at P23. ADA did not prevent the upregulation of GFAP immunoreactivity, a hallmark of gliosis.

An important finding of our study is the imbalance between RIPK1 and RIPK3. We observed the downregulation of RIPK1 protein or gene expression in retinal cells exposed to TNF\textalpha and rd10 retinas. RIPK1 can mediate necroptosis, apoptosis, and inflammation.\textsuperscript{95} RIPK1 is recruited to the TNFR1 complex to mediate pro-inflammatory signaling and to regulate TNF\textalpha-induced cell death.\textsuperscript{96} RIPK1/RIPK3 interaction is an essential step for activating necroptosis. Activated RIPK1 can associate with RIPK3 to induce MLKL-dependent necroptosis and production of inflammatory cytokines or recruit FADD (Fas-associated protein with death domain) and activate caspase 8 to induce apoptosis. Polykratis et al demonstrated that RIPK1 deficiency results in sensitivity to TNF\textalpha-induced apoptosis instead of necroptosis.\textsuperscript{96,97} Other studies of RIPK1 deficiency found that RIPK1 suppresses caspase 8-dependent apoptosis in some cell types and RIPK3/MLKL-dependent necroptosis in others. Qiu et al recently showed that short-term RIPK1 knockdown increases inflammatory cytokines, while long-term RIPK1 knockdown led to caspase 3-mediated apoptosis in primary chondrocyte cells.\textsuperscript{98} In rd10 retinas, we showed RIPK1 downregulation that could be partly responsible for apoptosis activation instead of necroptosis activation and even inflammation at P23.

Moreover, we observed the upregulation of RIPK3 in retinal cells exposed to TNF\textalpha and rd10 retinas. RIPK3 can also
FIGURE 8  A scheme of possible cell death mechanisms during TNFα-induced signaling in rd10 retinas at postnatal day 23 (P23) (A) and effect of a single intravitreal administration of ADA in cell death mechanisms (B) TNFα can simultaneously activate multiple signaling pathways in cells. In the TNFα/TNFR1 pathway, TNFα binds to TNFR1 triggering its trimerization. The intracellular domain of TNFR1 recruits a death-domain containing adaptor protein (TRADD). TRADD recruits TRAF2 and RIPK1 to form the complex 1. Complex 1 seems to be important for NF-κB activation. Complex 1 dissociates from TNFR1 and integrates FADD and pro-caspase 8 to form the complex 2. FADD/caspase 8 association depends on complexes containing unubiquitinated RIPK1 as a scaffold. Activated caspase 8 induces caspase 3 and apoptosis. On complex 1 formation, NF-κB regulates anti-apoptotic genes to block initiation of apoptosis by complex 2. Necroptosis is also mediated through TNFα signaling. Under apoptotic conditions, active caspase 8 prevents further necroptotic signaling by cleaving and inactivating RIPK1 and RIPK3. However, when caspase 8 is not active, RIPK1 recruits RIPK3 to form the necosome complex. RIPK3 phosphorylates the mixed lineage kinase domain-like protein (MLKL) leading to its oligomerization. Thus, MLKL recruitment to the plasma membrane induces necroptosis by triggering Ca2+ and Na+ influx into the cell. RIPK3 can also promote NLRP3 inflammasome and IL-1β inflammatory responses. TNFα or oxidative stress could activate PARthanatos through overactivation of PARP1. PARP1 cleaves NAD+ to nicotinamide and ADP-ribose. PARPs couple one or more ADP-ribose (PAR) to acceptor proteins (PARylation) and components of the DNA repair machinery. Overactivation of PARP1 can deplete cellular ATP and NAD+ storage and lead to a bioenergetic collapse and to a necrotic cell death. At postnatal day 23, rd10 retinas present caspase 3 activation, PARP activation, downregulation of RIPK1, upregulation of RIPK3, and some players of NLRP3 inflammasome (IL1β, caspase 1 activity, and NLRP3) A. Blockade of TNFα with 0.375 µg/µL ADA reduces RIPK3, PARP overactivation, IL1β, NLRP3, and caspase 1 activation (1). But it has no effect on caspase 3 activation, RIPK1 nor IL18 (X). Blockade of TNFα with 4.61 µg/µL ADA reduces RIPK3, PARP overactivation, IL1β, and NLRP3 (4). However, this concentration increases caspase 1 and 3 activities and to a lesser extent RIPK1 (1). It has no effect on IL18 (X) (B).
drive inflammation without necroptotic cell death regulating NF-κB, inflammasome activation, and kinase-independent apoptosis. The involvement of necroptosis in photoreceptor cell death during RP remains controversial. For instance, Viringipurampeer et al proposed that the primary driver of early rod cell death was a caspase-dependent process, whereas cone cell death occurred through RIPK3-dependent necroptosis in the transgenic S334ter rhodopsin. The same authors described that primary necroptosis was involved in early rod cell death, whereas cone cell loss occurred through inflammasome activation in P23H rats. Murakami et al suggested that apoptosis occurred early, during rod degeneration but, RIPK3-mediated necroptosis was responsible for cone degeneration at later stages (at P35 or later) in rd10 mice. Upregulation of RIPK1 and RIPK3 was found at the later stages of RP (P35) in rd10 retinas. These authors showed that RIPK1 and RIPK3 gene expression/protein did not change at P21 in rd10 mice. However, in our study, we found an imbalanced expression of these two main players of necroptosis in rd10 retinas at P23 (early intermediate stage of RP). We believe that RIPK3 is playing a key role on NLRP3 inflammasome activation that, in turn, could promote other types of cell death. The blockade of TNFα signaling does affect gene expression of RIPK1 but downregulates RIPK3 in rd10 retinas.

As mentioned above, we found an upregulation of some components of the NLRP3 inflammasome (caspase 1, NLRP3, and IL-1β) in the rd10 retinas. High caspase 1 activity, NLRP3 protein, and IL-β in rd10 retinas suggested that retinal degeneration is accompanied by NLRP3 inflammasome activation at this postnatal age. High levels of TNFα, oxidative, and nitrosative stress can activate PARP and NLRP3 inflammasome, precipitating necroptosis, ferroptosis or pyroptosis. The inflammasome is a major intracellular multiprotein that induces inflammatory responses by mediating immune cell infiltration, thus exacerbating cell injury. One of the most important inflammasomes consists of NLRP3, ASC (apoptosis-associated speck-like protein), and caspase 1, which processes pro-IL-1β, pro-IL-18 into their mature forms. RIPK3 can activate NLRP3 inflammasome and induce increased expression of ASC and cleaved caspase 1 with the secretion of the proinflammatory cytokines IL-1β and IL-18, resulting in pyroptosis, a type of necrosis-like programmed cell death. Some authors have described inflammasome activation in murine and canine models of RP like P23H, rd1, rcd1 or xlpra2. Besides, NLRP3 inflammasome has been involved in the progression of other retinal degenerations like age-related macular degeneration or diabetic retinopathy disease. A single dose of intravitreal ADA not only downregulated PARP overactivation but also downregulated RIPK3, NLRP3, and IL-1β, suggesting that the blockade of TNFα signaling inhibits NLRP3 inflammasome activation. However, further studies are needed to clarify this point.

To summarize, we present several key players that could contribute to cell death in rd10 retinas at postnatal day 23 (Figure 8). We proposed that PARP activation (PARthanatos) seems to be an important contributor to cell death at this age. Nevertheless, we cannot exclude that other contributors such as calpains, may play a role in these events. RIPK3 could promote NLRP3 inflammasome activation and in turn cell death through non-necroptotic mechanisms (eg, pyroptosis). Cell death processes and the relationship between each other during retinal degeneration in RP are very complex. Some authors suggest predominant apoptosis at the early stages of RP (rod degeneration) and necroptotic or even pyroptotic processes at the later stages of RP (cone degeneration). However, the impact of PARP activation is also very important during retinal degeneration. Therefore, we believe that there is a cross-talk between all these processes along the degenerative process. A single intravitreal injection of ADA at P12 was capable to delay retinal degeneration at P23, in spite of its “rapid” clearance (only detectable the first 24 hours after intravitreal injection). These results suggest that TNFα is involved in some cell death mechanisms during RP. In any case, it is important to clarify the different cell death mechanisms involved during retinal degeneration in RP in order to develop more specific and useful therapies.

ACKNOWLEDGMENTS

The authors would like to thank Isabel Campillo (Pathophysiology and Therapies for Vision Disorders, CIPF, Valencia, Spain) for her technical support, and Prof. Consuelo Guerrri (Cellular Pathology, CIPF, Valencia, Spain) for kindly providing BV2 cells. The authors thank the Health Research Institute Hospital La Fe (IIS-La Fe, Valencia, Spain) and its staff for providing animal facilities; Dr Pilar Marin (Microscopy Unit, IIS-La Fe, Valencia, Spain) for her kind help and the Cytomics Core Facility of the Research Center Principe Felipe (CIPF) for helping us with flow cytometry studies. This work was supported by the European Regional Development Fund; Spanish Ministry of Economy, Industry, and Competitiveness (MINECO) Carlos III Health Institute (ISCIII) Grants PI15/00052 and PI18/00252 and by the MEHUER Foundation and Ilustre Colegio Oficial de Farmacéuticos de Sevilla Grant Moises Abascal Alonso. LOG had an ISCIII CIBERER research contract CB06/07/1030; RR had an ISCIII Spanish National Health System (SNS) Miguel Servet Type II research contract CP15/00019. IC has a contract supported by the Grant PI18/00252. The surgical microscope M320 from Leica Microsystems was provided by Conselleria de Sanitat Universal i Salut Pública, Generalitat Valenciana through the European strategy: “Improvement of research infrastructures for rare diseases” CV FEDER 2014-2020 (Co-funded by European Regional Development Fund (ERDF) from the European Commission “A way to make Europe”/“Investing in your future”).
CONFLICTS OF INTEREST
The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS
L. Olivares helped to design the study. She performed animal studies (rd10 management, histological analysis, qPCR, western blot, etc) and contributed to write the manuscript. S. Velasco performed in vitro studies (cell cultures, MTT assays, western blot, qPCR, etc) and helped to analyze the data. J. M. Millán provided advice, helped to design, and revised the manuscript. R. Rodrigo designed and supervised the study, carried out statistical analysis, and wrote the manuscript. All authors read and approved the final manuscript.

REFERENCES
1. Corton M, Blanco MJ, Torres M, Sanchez-Salorio M, Carracedo A, Brion M. Identification of a novel mutation in the human PDE6A gene in autosomal recessive retinitis pigmentosa: homology with the nmt28/nmt28 mice model. *Clin Genet*. 2010;78:495-498.
2. Portera-Cailliau C, Sung CH, Nathans J, Adler R. Apoptotic photoreceptor cell death in mouse models of retinitis pigmentosa. *Proc Natl Acad Sci USA*. 1994;91:974-978.
3. Rivas MA, Vecino E. Animal models and different therapies for treatment of retinitis pigmentosa. *Histol Histopathol*. 2009;24:1295-1322.
4. Daiger S, Sullivan L, Bowne S. (1996-2014) Retinal Information Network. Houston: University of Texas-Houston Health Science Center.
5. Campochiaro PA, Mir TA. The mechanism of cone cell death in retinitis pigmentosa. *Prog Retin Eye Res*. 2018;62:24-37.
6. Yoshiha N, Ikeda Y, Notomi S, et al. Laboratory evidence of sustained chronic inflammatory reaction in retinitis pigmentosa. *Ophthalmology*. 2013;120:e5-e12.
7. Zeng HY, Zhu XA, Zhang C, Yang LP, Wu LM, Tso MO. Identification of sequential events and factors associated with microglial activation, migration, and cytotoxicity in retinal degeneration in rd mice. *Invest Ophthalmol Vis Sci*. 2005;46:2992-2999.
8. Gupta N, Brown KE, Milam AH. Activated microglia in human retinitis pigmentosa, late-onset retinal degeneration, and age-related macular degeneration. *Exp Eye Res*. 2003;76:463-471.
9. Sasahara M, Otani A, Oishi A, et al. Activation of bone marrow-derived microglia promotes photoreceptor survival in inherited retinal degeneration. *Am J Pathol*. 2008;172:1693-1703.
10. Ebert S, Weigelt K, Walczak Y, et al. Docosahexaenoic acid attenuates microglial activation and delays early retinal degeneration. *J Neurochem*. 2009;110:1863-1875.
11. Sheets KG, Jun B, Zhou Y, et al. Microglial ramification and redistribution concomitant with the attenuation of choroidal neovascularization by neuroprotectin D1. *Mol Vis*. 2013;19:1747-1759.
12. Martinez-Fernandez de la Camara C, Olivares-Gonzalez L, Hervas D, Salom D, Millan JM, Rodrigo R. Inflimixim reduces Zaprinast-induced retinal degeneration in cultures of porcine retina. *J Neuroinflammation*. 2014;11:172.
13. Olivares-Gonzalez L, Martinez-Fernandez de la Camara C, Hervas D, Millan JM, Rodrigo R. HIF-lalpha stabilization reduces retinal degeneration in a mouse model of retinitis pigmentosa. *FASEB J*. 2018;32:2438-2451.
33. Leal I, Rodrigues FB, Sousa DC, et al. Efficacy and safety of intravitreal anti-tumour necrosis factor drugs in adults with non-infectious uveitis – a systematic review. Acta Ophthalmol. 2018;96:e665-e675.

34. Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. J Basic Clin Pharm. 2016;7:27-31.

35. Sears JE, Hoppe G, Ebrahem Q, Anand-Apte B. Prolyl hydroxylase inhibition during hypoxia prevents oxygen-induced retinopathy. Proc Natl Acad Sci USA. 2008;105:19989-19993.

36. Trichonas G, Lee TJ, Hoppe G, Au J, Sears JE. Prolyl hydroxylase inhibition during hypoxia prevents oxygen-induced retinopathy in the rat 50/10 model. Invest Ophthalmol Vis Sci. 2013;54:4919-4926.

37. Martinez-Fernandez de la Camara C, Sequedo MD, Gomez-Pinedo U, et al. Phosphodiesterase inhibition induces retinal degeneration, oxidative stress and inflammation in cone-enriched cultures of porcine retina. Exp Eye Res. 2013;111:122-133.

38. Rana T, Kotla P, Fullhard R, Gorbatyuk M. TNFα knockdown in the retina promotes cone survival in a mouse model of autosomal dominant retinitis pigmentosa. Biochimica et biophysica acta. Mol Basis Dis. 2017;1863:92-102.

39. Hwang N, Kwon MY, Cha JB, Chung SW, Woo JM. Tunicamycin-induced endoplasmic reticulum stress upregulates the expression of pentraxin 3 in human retinal pigment epithelial cells. Korean J Ophthalmol: KJO. 2016;30:468-478.

40. Shen HM, Codogno P. Autophagy is a survival force via suppression of necrotic cell death. Exp Cell Res. 2012;318:1304-1308.

41. Estornes Y, Aguilera MA, Dubuisson C, et al. RIPK1 promotes death receptor-independent caspase-8-mediated apoptosis under unresolved ER stress conditions. Cell Death Dis. 2015;6:e1798.

42. Genni S, Beltran WA, Aguirre GD. Up-regulation of tumor necrosis factor superfamily genes in early phases of photoreceptor degeneration. PLoS One. 2013;8:e85408.

43. Roche SL, Ruiz-Lopez AM, Moloney JN, Byrne AM, Cotter TG. Microglial-induced Müller cell gliosis is attenuated by progesterone in a mouse model of retinitis pigmentosa. Glia. 2018;66:295-310.

44. Benlloch-Navarro S, Trachsel-Moncho L, Fernández-Carbonell Á, et al. Progesterone anti-inflammatory properties in hereditary retinal degeneration. J Steroid Biochem Mol Biol. 2019;189:291-301.

45. Viringipurampeer IA, Bashar AE, Gregory-Evans CY, Moritz OL, Gregory-Evans K. Targeting inflammation in emerging therapies for genetic retinal disease. Int J Inflam. 2013;2013:581751.

46. Kruk J, Kubasik-Kladna K, Aboul-Enein HY. The role oxidative stress in the pathogenesis of eye diseases: current status and a dual role of physical activity. Mini Rev Med Chem. 2015;16:241-257.

47. Murakami Y, Matsumoto H, Roh M, et al. Receptor interacting protein kinase mediates necrotic cone but not rod cell death in a mouse model of inherited degeneration. Proc Natl Acad Sci USA. 2012;109:14598-14603.

48. Circu ML, Aw TY. Reactive oxygen species, cellular redox systems, and apoptosis. Free Radic Biol Med. 2010;48:749-762.

49. Yoshida N, Ikeda Y, Notomi S, et al. Clinical evidence of sustained chronic inflammatory reaction in retinitis pigmentosa. Ophthalmology. 2013;120:100-105.

50. Alvarez S, Blanco A, Fresno M, Muñoz-Fernández M. TNF-α contributes to caspase-3 independent apoptosis in neuroblastoma cells: role of NFAT. PLoS One. 2011;6:e16100.

51. Liao Y, Zhang H, He D, et al. Retinal pigment epithelium cell death is associated with NLRP3 inflammasome activation by all-trans retinal. Invest Ophthalmol Vis Sci. 2019;60:3034-3045.

52. Gao J, Cui ZZ, To E, Cao S, Matsubara JA. Evidence for the activation of pyroptotic and apoptotic pathways in RPE cells associated with NLRP3 inflammasome in the rodent eye. J Neuroinflammation. 2018;15:15.

53. Totsuka K, Ueta T, Uchida T, et al. Oxidative stress induces ferroptotic cell death in retinal pigment epithelial cells. Exp Eye Res. 2019;181:316-324.

54. Power M, Das S, Schutze K, Marigo V, Ekstrom P, Paquet-Durand F. Cellular mechanisms of hereditary photoreceptor degeneration - Focus on cGMP. Prog Retin Eye Res. 2019;100772.

55. Vanden Berghe T, Kaiser WJ, Bertrand MJ, Vandenabeele P. Molecular crosstalk between apoptosis, necroptosis, and survival signaling. Mol Cell Oncol. 2015;2:e975093.

56. Galluzzi L, Vitale I, Aaronson SA, et al. Molecular mechanisms of cell death: recommendations of the nomenclature committee on cell death 2018. Cell Death Differ. 2018;25:486-541.

57. Lindahl T, Satoh MS, Poirier GG, Klungland A. Post-translational modification of poly(ADP-ribose) polymerase induced by DNA strand breaks. Trends Biochem Sci. 1995;20:405-411.

58. Chiarugi A, Moskowitz MA. Poly(ADP-ribose) polymerase-1 activity promotes NF-kappaB-driven transcription and microglial activation: implication for neurodegenerative disorders. J Neurochem. 2003;85:306-317.

59. Nakajima H, Nagaso H, Kakui N, Ishikawa M, Hiranuma T, Hoshiko S. Critical role of the automodification of poly(ADP-ribose) polymerase-1 in nuclear factor-kappaB-dependent gene expression in primary cultured mouse glial cells. J Biol Chem. 2004;279:42774-42786.

60. Cregan SP, Fortin A, MacLaurin JG, et al. Apoptosis-inducing factor is involved in the regulation of caspase-independent neuronal cell death. J Cell Biol. 2002;158:507-517.

61. Hong SJ, Dawson TM, Dawson VL. Nuclear and mitochondrial conversations in cell death: PARP-1 and AIF signaling. Trends Pharmacol Sci. 2004;25:259-264.

62. Yu SW, Andrabi SA, Wang H, et al. Apoptosis-inducing factor mediates poly(ADP-ribose) (PAR) polymerase-induced cell death. Proc Natl Acad Sci USA. 2006;103:18314-18319.

63. Rouleau M, Patel A, Hendzel MJ, Kaufmann SH, Poirier GG. Specific proteolytic cleavage of poly(ADP-ribose) polymerase-1 in nuclear factor-kappaB-dependent gene expression in primary cultured mouse glial cells. J Biol Chem. 2004;279:42774-42786.

64. Bai P. Biology of poly(ADP-Ribose) polymerases: the factotums of cell maintenance. Mol Cell. 2015;58:947-958.

65. Fischer U, Janicke RU, Schulze-Osthoff K. Many cuts to ruin: a comprehensive update of caspase substrates. Cell Death Differ. 2003;10:76-100.

66. Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE, Poirier GG. Specfic proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. Cancer Res. 1993;53:3976-3985.

67. Tewari M, Quan LT, O'Rourke K, et al. Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell. 1995;81:801-809.

68. Margolin N, Raybuck SA, Wilson KP, et al. Substrate and inhibitor specificity of interleukin-1 beta-converting enzyme and related caspases. J Biol Chem. 1997;272:7223-7228.
1. Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, Earnshaw WC. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature*. 1994;371:346-347.

2. Wang ZQ, Stengl L, Morrison C, et al. PARP is important for genomic stability but dispensable in apoptosis. *Genes Dev*. 1997;11:2347-2358.

3. Schlegel J, Peters I, Orrenius S, et al. CPP32/apopain is a key interleukin 1 beta converting enzyme-like protease involved in Fas-mediated apoptosis. *J Biol Chem*. 1996;271:1841-1844.

4. Sosna J, Voigt S, Mathieu S, et al. TNF-induced necroptosis and PARP-1-mediated necrosis represent distinct routes to programmed necrotic cell death. *Cell Mol Life Sci*. 2014;71:331-348.

5. McLaughlin T, Dhimal N, Li J, Wang JJ, Zhang SX, p58(IPK) is an endogenous neuroprotectant for retinal ganglion cells. *Front Aging Neurosci*. 2018;10:267.

6. Lane DJ, Saletta F, Rahmann YS, Kovacevic Z, Richardson DR. Correction: N-myc downstream regulated 1 (NDRG1) is regulated by eukaryotic initiation factor 3a (eIF3a) during cellular stress caused by iron depletion. *PLoS One*. 2016;11:e0149922.

7. Catara G, Grimaldi G, Schembri L, et al. PARP1-produced poly-ADP-ribose causes the PARP12 translocation to stress granules and impairment of Golgi complex functions. *Sci Rep*. 2017;7:14035.

8. Kaur H, Chauhan S, Sandhir R. Protective effect of lycopene on oxidative stress and cognitive decline in rotenone induced model of Parkinson’s disease. *Neurochem Res*. 2011;36:1435-1443.

9. Viringiparampeea IA, Gregory-Evans CY, Metcalfe AL, Bashar E, Moritz OL, Gregory-Evans K. Cell death pathways in mutant rhodopsin rat models identifies genotype-specific targets controlling retinal degeneration. *Mol Neurobiol*. 2019;56:1637-1652.

10. Bode C, Wolfrum U. Caspase-3 inhibitor reduces apoptotic photoreceptor cell death during inherited retinal degeneration in tubby mice. *Mol Vis*. 2003;9:144-150.

11. Lohr HR, Kuntschithapauktham K, Sharma AK, Rohrer B. Multiple, parallel cellular suicide mechanisms participate in photoreceptor cell death. *Exp Eye Res*. 2006;83:380-389.

12. Cottet S, Schorderet DF. Mechanisms of apoptosis in retinitis pigmentosa. *Curr Mol Med*. 2009;9:375-383.

13. Kaur J, Mencel S, Sahaboglu A, et al. Calpain and PARP activation during photoreceptor cell death in P23H and S334ter rhodopsin mutant rats. *PLoS One*. 2011;6:e22181.

14. Paquet-Durand F, Silva J, Talukdar T, et al. Excessive activation of poly(ADP-ribose) polymerase contributes to inherited photoreceptor degeneration in the retinopathy 1 mouse. *J Neurosci*. 2007;27:10311-10319.

15. Sahaboglu A, Sharif A, Feng L, Secer E, Zrenner E, Paquet-Durand F. Temporal progression of PARP activity in the Prph2 mutant rd2 mouse: neuroprotective effects of the PARP inhibitor PJ34. *PLoS One*. 2017;12:e0181374.

16. Jiao K, Sahaboglu A, Zrenner E, Ueffing M, Ekstrom PA, Paquet-Durand F. Efficacy of PARP inhibition in Pde6a mutant mouse models for retinitis pigmentosa depends on the quality and composition of individual human mutations. *Cell Death Discov*. 2016;2:16040.

17. Vecino E, Rodriguez FD, Ruzafa N, Pereiro X, Sharma SC. Glia-neuron interactions in the mammalian retina. *Prog Retin Eye Res*. 2016;51:1-40.

18. Di Pridorganic J, García-Ayuso D, Agudo-Barriuso M, Vidal-Sanz M, Villegas-Pérez MP. Role of microglial cells in photoreceptor degeneration. *Neural Regen Res*. 2019;14:1186-1190.

19. Noailles A, Maneu V, Campello L, Gómez-Vicente V, Lax P, Cuenca N. Persistent inflammatory state after photoreceptor loss in an animal model of retinal degeneration. *Sci Rep*. 2016;6:33356.

20. Zhao L, Zabel MK, Wang X, et al. Microglial phagocytosis of living photoreceptors contributes to inherited retinal degeneration. *EMBO Mol Med*. 2015;7:1179-1197.

21. Nakatake S, Murakami Y, Ikeda Y, et al. MUTYH promotes oxidative microglial activation and inherited retinal degeneration. *JCI Insight*. 2016;1:e87781.

22. Ullrich O, Dietzel A, Eyüpoglu IY, Nitsch R. Regulation of microglial expression of integrins by poly(ADP-ribose) polymerase-1. *Nat Cell Biol*. 2001;3:1035-1042.

23. Kauppinen TM, Swanson RA. Poly(ADP-ribose) polymerase-1 promotes microglial activation, proliferation, and matrix metalloproteinase-9-mediated neuron death. *J Immunol*. 2005;174:2288-2296.

24. d’Avila JC, Lam TI, Bingham D, et al. Microglial activation induced by brain trauma is suppressed by post-injury treatment with a PARP inhibitor. *J Neuroinflammation*. 2012;9:31.

25. Stoica BA, Loane DJ, Zhao Z, et al. PARP-1 inhibition attenuates neuronal loss, microglia activation and neurological deficits after traumatic brain injury. *J Neurotrauma*. 2014;31:758-772.

26. Xu J, Wang H, Won SJ, Basu J, Kapfhammer D, Swanson RA. Microglial activation induced by the alarmin S100B is regulated by poly(ADP-ribose) polymerase-1. *Glia*. 2016;64:1869-1878.

27. Wegner KW, Saleh D, Desterev A. Complex pathologic roles of RIPK1 and RIPK3: moving beyond necroptosis. *Trends Pharmacol Sci*. 2017;38:202-225.

28. Polykratis A, Hermance N, Zelic M, et al. Cutting edge: RIPK1 Kinase inactive mice are viable and protected from TNF-induced necroptosis in vivo. *J Immunol*. 2014;193:1539-1543.

29. Wegner A, Benson S, Rebernik L, et al. Sex differences in the pro-inflammatory cytokine response to endotoxin unfold in vivo but not ex vivo in healthy humans. *Innate Immunity*. 2017;23:432-439.

30. Qiu X, Zhuang M, Lu Z, et al. RIPK1 suppresses apoptosis mediated by TNF and caspase-3 in intervertebral discs. *J Transl Med*. 2019;17:135.

31. Moriwaki K, Chan FK. The inflammatory signal adaptor RIPK3: functions beyond necroptosis. *Int Rev Cell Mol Biol*. 2017;328:253-275.

32. Yang H, Fu Y, Liu X, et al. Role of the sigma-1 receptor chaperone in rod and cone photoreceptor degenerations in a mouse model of retinitis pigmentosa. *Mol Neurodegener*. 2017;12:68.

33. Rodriguez-Vargas JM, Ruiz-Magana MJ, Ruiz-Ruiz C, et al. ROS-induced DNA damage and PARP-1 are required for optimal induction of starvation-induced autophagy. *Cell Res*. 2012;22:1181-1198.

34. Abais JM, Xia M, Zhang Y, Boini KM, Li PL. Redox regulation of NLRP3 inflammasomes: ROS as trigger or effector? *Antioxid Redox Signal*. 2015;22:1111-1129.

35. Cao JY, Dixon SJ. Mechanisms of ferroptosis. *Cell Mol Life Sci: CMLS*. 2016;73:2195-2209.

36. Malik A, Kanneganti TD. Inflammasome activation and assembly at a glance. *J Cell Sci*. 2017;130:3955-3963.

37. Appelbaum T, Santana E, Aguirre GD. Strong upregulation of inflammatory genes accompanies photoreceptor demise in canine models of retinal degeneration. *PLoS One*. 2017;12:e0177224.

38. Viringiparampeea IA, Metcalfe AL, Bashar AE, et al. NLRP3 inflammasome activation drives bystander cone photoreceptor cell death in a P23H rhodopsin model of retinal degeneration. *Hum Mol Genet*. 2016;25:1501-1516.
in mice lacking innate immunity adaptor protein MyD88. *Exp Neurol.* 2015;267:1-12.

108. Kauppinen A, Paterno JJ, Blasiak J, Salminen A, Kaarniranta K. Inflammation and its role in age-related macular degeneration. *Cell Mol Life Sci: CMLS.* 2016;73:1765-1786.

109. Akhtar-Schafer I, Wang L, Krohne TU, Xu H, Langmann T. Modulation of three key innate immune pathways for the most common retinal degenerative diseases. *EMBO Mol Med.* 2018;10.

110. Yerramothu P, Vijay AK, Willcox MDP. Inflammasomes, the eye and anti-inflammasome therapy. *Eye (Lond).* 2018;32:491-505.

111. Power M, Das S, Schütze K, Marigo V, Ekström P, Paquet-Durand F. Cellular mechanisms of hereditary photoreceptor degeneration - focus on cGMP. *Prog Retin Eye Res.* 2020;74:1-25.

**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

**How to cite this article:** Olivares-González L, Velasco S, Millán JM, Rodrigo R. Intravitreal administration of adalimumab delays retinal degeneration in *rd10* mice. *The FASEB Journal.* 2020;34:13839–13861. [https://doi.org/10.1096/fj.20200044RR](https://doi.org/10.1096/fj.20200044RR)