



Research article

Anti-inflammatory properties of antiangiogenic fucoidan in retinal pigment epithelium cells

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ABSTRACT

Age-related macular degeneration (AMD) is a multifactorial disease in which angiogenesis, oxidative stress and inflammation are important contributing factors. In this study, we investigated the anti-inflammatory effects of a fucoidan from the brown algae *Fucus vesiculosus* (FV) in primary porcine RPE cells. Inflammation was induced by lipopolysaccharide (LPS), polyinosinic: polycytidylic acid (Poly I:C), Pam2CSK4 (Pam), or tumor necrosis factor alpha (TNF- α). Cell viability was tested with thiazolyl blue tetrazolium bromide (MTT) test, barrier function by measuring transepithelial electric resistance (TEER), interleukin 6 (IL-6) and interleukin 8 (IL-8) secretion in ELISA, retinal pigment epithelium-specific 65 kDa protein (RPE65) and protectin (CD59) expression in Western blot, gene expression with quantitative polymerase chain reaction (qPCR) (*IL6*, *IL8*, *MERTK*, *PIK3CA*), and phagocytotic activity in a microscopic assay. FV fucoidan did not influence RPE cell viability. FV fucoidan reduced the Poly I:C proinflammatory cytokine secretion of IL-6 and IL-8. In addition, it decreased the expression of IL-6 and IL-8 in RT-PCR. LPS and TNF- α reduced the expression of CD59 in Western blot, this reduction was lost under FV fucoidan treatment. Also, LPS and TNF- α reduced the expression of visual cycle protein RPE65, this reduction was again lost under FV fucoidan treatment. Furthermore, the significant reduction of barrier function after Poly I:C stimulation is ameliorated by FV fucoidan. Concerning phagocytosis, however, the inflammation-induced reduction was not improved by FV fucoidan. FV and proinflammatory milieu did not relevantly influence phagocytosis relevant gene expression either. In conclusion, we show that fucoidan from FV can reduce proinflammatory stimulation in RPE induced by toll-like receptor 3 (TLR-3) activation and is of high interest as a potential compound for early AMD treatment.

1. Introduction

Age-related macular degeneration (AMD) is the main cause for blindness and severe vision loss in the industrialized world and its prevalence is on the rise [1]. Indeed, the global number of patients is currently estimated to be 196 million and rise to 288 million by 2040 [2]. Current treatment options are limited. The disease can be divided in early forms, which present with little symptoms for the patients, late atrophic forms, in which patches of retinal pigment epithelium (RPE) and photoreceptors degenerate and vision is slowly

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deteriorating, and a late exudative form, in which vessels grow from the choroid beneath and into the retina, which leads to a rapid loss of vision [3]. Only the late exudative form of this disease is currently treatable by the application of inhibitors of the angiogenic cytokine vascular endothelial growth factor (VEGF) [4]. While the advent of anti-VEGF therapies has revolutionized AMD treatment, unfortunately, the treatment is not curative and needs to be repeated regularly, often for many years. In addition, while a subset of patients will improve in visual acuity, the long-term outcome remains poor [5]. Better treatment options are clearly warranted.

AMD is a multifactorial disease, which is not completely understood. However, it is well accepted that oxidative stress, angiogenesis and inflammation play important parts in disease development [6–8]. On the cellular level, the development of AMD takes place at the interface between the photoreceptors, RPE and the choroid, with the RPE playing the pivotal role in AMD development, including a decline in function and degeneration of the cells [9,10]. The RPE has a plethora of functions in order to support the photoreceptors and to maintain visual function like phagocytosis of shed photoreceptor outer segments and renewal of the visual pigment [11]. It keeps up the fenestration of the choroidal endothelium by secreting VEGF [12], it protects the photoreceptors from oxidative stress [13] and it downregulates the immune response in the retina [14]. However, in age, oxidative stress resistance of the RPE decreases [15] and several noxious factors increase VEGF secretion in the RPE [16–19]. Indeed, molecular pathways in the RPE that contribute to AMD development are an increase in VEGF secretion, a decrease in expression of complement inhibitory protein, a dysfunction of mitochondria, a deregulated autophagy and cell loss due to different cell death mechanisms [10,20,21].

Furthermore, the RPE carries toll-like receptors (TLR) (pattern recognition receptors), which allows them to detect pathogen and danger-associated molecular patterns [22–24]. While the expression of different TLR are reported also for other cell types in the retina including glial cells [25], the expression of TLR in the RPE may be highly important in retinal inflammation and disease development, as these cells make up the outer blood-retinal-barrier and act as sentinels to detect not only retinal but also systemic danger signals, with the ability to react in a polarized manner [14,23,24,26,27]. Stimulation of RPE cells by TLR agonists or proinflammatory agents can induce a proinflammatory cytokine secretion and impact RPE function [23,28,29]. Indeed, proinflammatory stimuli activation of RPE cells has been implicated as an important factor in AMD development [7,24]. On our study, we used three different pro-inflammatory stimuli, the TLR-3 agonist polyinosinic:polycytidylic acid (Poly I:C, PIC) [30], the TLR-4 agonist lipopolysaccharide (LPS), and the cytokine TNF α . Besides their role as proinflammatory mediators, each of these has been implicated in AMD development. TLR-3 is highly expressed in the RPE and its activation induces cytokines that are implicated in AMD development as well as cell death in the retina, which contribute not only to tissue degeneration but to chronic/para-inflammation [16,23,28,31,32]. Furthermore, associations of specific polymorphisms of TLR-3 with the protection against development of geographic atrophy in AMD have been described, which seems to be dependent on the genetic background of the patient [33,34]. Systemic LPS has been implicated in AMD development. It has been shown that a high-fat and high-sugar diet causes increased permeability in the gut, resulting in a systemic increase in LPS (metabolic endotoxemia) which leads to chronic inflammation in the retina, increasing the risk of developing AMD, causing features of dry AMD in mice, and exacerbating choroidal neovascularization [27,35–37]. LPS induces a strong proinflammatory cytokine response in RPE as well as in retinal microglia [28,38]. Furthermore, long term exposure to LPS may interfere with RPE function and barrier properties [26,28]. In addition, certain TLR-4 variants have been associated with a greater susceptibility for AMD, though again this seems to be dependent on the genetic background of the carrier [24,39,40]. TNF α is implicated in the development of choroidal neovascularization (CNV), the hallmark of exudative AMD, as it is expressed in macrophages of AMD patients and increases choroidal sprouting in laser-induced CNV as well as VEGF expression [41,42]. Furthermore, certain TNF α polymorphism are more frequent in exudative AMD patients [43].

Fucoidans are sulphated polysaccharides derived from brown algae which exhibit bioactivities which makes them highly interesting for the development of new therapeutics for AMD [44–46]. It is important to consider, however, that fucoidans are a heterogenic group of polysaccharides with effects varying considerably depending on the species and the molecular properties of the fucoidan, which is also strongly influenced by environmental factors [47–49]. We have previously shown that this fucoidan reduces the secretion of VEGF. Moreover, it reduces the expression of VEGF even in the presence of another VEGF antagonist (bevacizumab) and reduces angiogenesis in an Matrigel-endothelial outgrowth angiogenesis model [50]. In addition, FV fucoidan showed protection against oxidative stress in a different ocular cell model [51]. In the current study, we investigated the effects of FV fucoidan on proinflammatory activation of RPE cells. Furthermore, as described above the RPE has many functions in the retina [11] which can be altered by inflammation. It is involved in the visual cycle and we have previously shown that pro-inflammatory stimulation can reduce the expression of the visual cycle protein RPE65 [28]. In addition, the RPE constitutes the blood-brain-barrier, and we previously showed that pro-inflammatory stimuli can interfere with RPE barrier functions [28]. Also, an important function of the RPE is the phagocytosis of photoreceptor outer segments and malfunction of this phagocytosis has been implicated in the development of AMD [52,53]. Therefore, we also investigated the effect of fucoidan on the pro-inflammatorily induced alterations of this functions in the RPE.

2. Methods

2.1. Cell culture

The RPE is strongly involved in the pathology of AMD and RPE cell cultures are widely used to investigate AMD pathology and model effects of potential therapeutics in AMD [54,55]. For this study, primary porcine RPE cells were used. Primary porcine RPE cells are a well described model for adult RPE cells, which (re-)differentiate in culture into a single monolayer of cobblestone morphology [54,56]. Porcine RPE cells were prepared from eyes of freshly slaughtered pigs. The eyes are waste materials and byproducts of food production. The usage of the eyes for experimental purposes was conducted in agreement with the animal welfare officer of Kiel

University. According to the German animal welfare act (TierSchG), it is not considered to be animal research, but an alternative to the use of animals in research. Procedure was conducted as previously described [57]. In brief, surrounding tissue of pig eye was removed, cornea, iris, ciliary body, retina and vitreous discarded and the RPE isolated with trypsin/ethylenediaminetetraacetic acid (EDTA) (Pan-Biotech, Aidenbach, Germany; #P10-023100). The primary cells were cultivated in HyClone Dulbecco's Modified Eagle's Medium (DMEM, GE Healthcare, München, Germany; #SH30022.01) with 1% non-essential amino acids (Pan-Biotech; #P08-32100), 2.5% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Pan-Biotech; #P05-01100), 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA; #P4333) and 10% fetal bovine Gibco® serum (Thermo Fisher Scientific, Waltham, MA, USA; #10270106). Twelve eyes were collected and all the cells were seeded in 12-well plates (Sarstedt, Nümbrecht, Germany; #83.3921) with or without cover slips as well as 24-well plates (Sarstedt; #83.3922) and cultivated for two weeks until reaching confluence. If coverslips were used, they were coated with collagen I (Pan Biotech; #P06-20030) according to the manufacturer's instructions. For transepithelial electrical resistance, primary porcine RPE cells were seeded on 12-well plates with transwell inserts (Sarstedt, Nümbrecht, Germany; #83.3931.040).

2.2. Stimulation

Cells were stimulated with 1 µg/mL lipopolysaccharide (LPS, Merck, Darmstadt, Germany; #L4524), 10 µg/mL polyinosinic: polycytidylic acid (Poly I:C, Tocris Bioscience, Bristol, UK; #4287/10), 50 ng/mL tumor necrosis factor alpha (TNF-α, R&D System, Minneapolis, USA; #210-TA/CF), or 10 ng/mL Pam2CSK4 (Pam, Tocris Biosciences; #4637) respectively, for various time periods to induce inflammation. For fucoidan application, cells were treated with 50 µg/mL fucoidan (FV fucoidan) from *Fucus vesiculosus* (Sigma Aldrich; Cat-No: F5631, Lot #SLBT5471). As different production batches may differ in their fucoidan content, all experiments were conducted with the same production batch. The concentrations have been chosen based on our previous studies [28,38,58,59].

2.3. Cell viability

The cell viability was investigated with thiazolyl blue tetrazolium bromide (MTT) assay [60]. Cells were washed with phosphate-buffered saline (PBS, Pan Biotech; #P04-37500) and 0.5 mg/mL MTT (Sigma-Aldrich; #M2128) was added for 2 h. The MTT was removed and formazan crystals were solved in dimethyl sulfoxide (Carl Roth, Karlsruhe, Germany; #A994.1). The absorbance was measured at 550 nm with plate reader Elx800 (BioTek, Bad Friedrichshall, Germany).

2.4. Transepithelial electrical resistance

Primary porcine RPE cells were prepared and seeded on a 12-well plate with *trans*-well inserts (refer Section 2.1). They were cultured for two weeks until reaching confluence. Before treatment the transepithelial electrical resistance (TEER) was measured with the Epithelial Volt/Ohm (TEER) Meter 3 (WPI, Sarasota, FL, USA). Measuring was performed by putting the electrodes into the well with the longer electrode in the outer well and the short one within the transwell [61]. After measuring, electrodes were washed with 70% ethanol and Aqua bidest. One transwell without cells was used as blank and the device subtracted it from all measured TEER data. A minimum of 150 ohm*cm² was set as a threshold for experimentation [62]. We measured the cells at time point zero before stimulation with FV fucoidan and inflammatory agents LPS, Poly I:C, TNF-α and Pam and then measured TEER again after one, three, seven, and 28 days.

2.5. Enzyme-linked immunosorbent assay

Secreted cytokines were measured with enzyme-linked immunosorbent assay (ELISA) from R&D Systems (Minneapolis, MN, USA) like described by the instructor's manual (porcine IL-6 DuoSet #DY686, porcine IL-8 DuoSet #DY535). These cytokines were chosen as a readout for pro-inflammatory activation as we previously have shown a robust and reproducible induction of their secretion in the RPE by the stimulants used [28]. We collected supernatants of treated RPE cells for 24 h after stimulating them with FV fucoidan, LPS, Poly I:C, TNF-α and Pam, respectively, for one, three, seven and 28 days.

2.6. Real-time polymerase chain reaction

RPE cells, were treated with 50 µg/mL FV fucoidan and/or 10 µg/mL Poly I:C for 72 h and RNA was isolated with NucleoSpin RNA Kit (Macherey-Nagel, Düren, Germany; #740955.50) as described in the manufacturer's instruction. For phagocytosis relevant genes *MERTK* and *PIK3CA* cells were also stimulated with 1 µg/mL LPS, 50 ng/mL TNF-α or 10 ng/mL Pam. Genomic DNA was digested with integrated DNase. Final RNA elution volume was set to 20 µl. The purity and concentration of the RNA was measured with the NanoDrop™ One system (Thermo Fisher Scientific). The cDNA was transcribed with High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific; # 4368814) accruing to the manual. A quantitative real-time polymerase chain reaction was conducted with TaqMan™ gene expression assays (dye label 5(6)-carboxyfluorescein-minor groove binder (FAM-MGB)) and TaqMan™ Fast Advanced Master Mix (both Thermo Fisher Scientific; #4331182 and #4444556) like described by the instructions of the master mix with primers for *GAPDH* (Glyceraldehyde-3-Phosphate Dehydrogenase, Ss03375629_u1), *IL6* (interleukin 6, Ss03384604_u1), *CXCL8* (interleukin 8, Ss03392437_m1), *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha, Ss00907960_m1) and *MERTK* (MER proto-oncogene, tyrosine kinase, Ss06909066_m1). ΔΔCT values and relative quantification were calculated in

cloud software Thermo Fisher Connect with the RQ module (relative quantification). Values of GAPDH were used for normalization and the *t*-test of the cloud software for calculation of significances.

2.7. Western blotting

Retinal pigment epithelium-specific 65 kDa protein (RPE65) and protectin (CD59) protein expression was detected via Western blot procedure like previously described [28]. Primary RPE cells were treated with FV fucoidan, and LPS, PIC, TNF or Pam, respectively, for one and three days. Cells were washed and cell lysates were prepared by lysis for 45 min with NP-40 buffer (1% Nonidet P40 Substitute (Sigma-Aldrich, #11332473001), 150 mM NaCl (Carl Roth AG, Karlsruhe, Germany; #3957.1) and 50 mM Tris (Sigma-Aldrich, #T1503) containing phosphatase and proteinase inhibitors (both Sigma-Aldrich; #P2850, #P5726, #P8340). Protein concentration was determined with BioRad protein assay (BioRad, Munich, Germany; #500-0006) with BSA as standard. Protein separation was performed with SDS-PAGE with 12% acrylamide gel. Western blot was conducted in a wet tank. Blotted membranes were blocked with 4% skim milk (Carl Roth AG; #T145.2) and primary antibodies were applied over night at 4 °C (mouse anti-RPE65 Abcam, 65 kDa, 1:6000, Berlin, Germany, #ab13826; rabbit anti-CD59, 18 kDa, 1:3000, Proteintech Group, Inc., Rosemont, IL, USA rabbit anti- β -actin, 37 kDa, 1:1000, Cell Signaling Technologies, Denver, CO, USA, #4967). Horseradish peroxidase conjugates were added (anti-mouse-HRP or anti-rabbit-HRP, Cell Signaling Technologies; #7076, #7074) for 1 h. Amersham ECL Western Blotting Detection Reagent (GE Healthcare, Chica-go, IL, USA; #RPN2106) was used for generating chemiluminescent signal. This was measured with

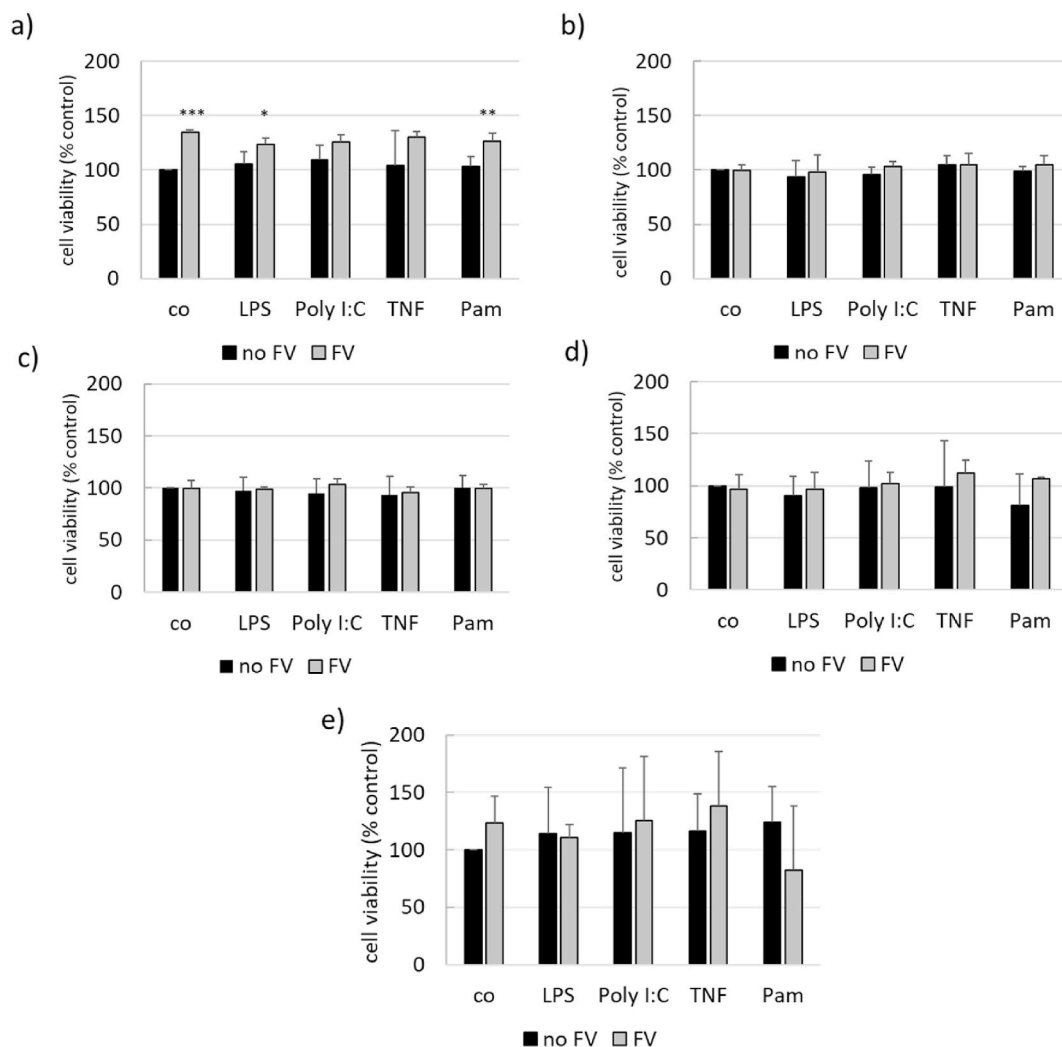


Fig. 1. Cell viability. Primary porcine retinal pigment epithelium (RPE) cells were treated with 50 µg/mL *Fucus vesiculosus* fucoidan from Sigma-Aldrich (FV) and/or 1 µg/mL lipopolysaccharide (LPS), 10 µg/mL polyinosinic:polycytidylic acid (Poly I:C), 50 ng/mL tumor necrosis factor alpha (TNF) and 10 ng/mL Pam2CSK4 (Pam) for 1 (a), 3 (b), 7 (c) and 28 (d) days in 24-well plates and for 28 days in 12-trans well plates (e). After that cell viability was measured with tetrazolium bromide (MTT) assay. Values are depicted in % compared to untreated control (co). One-sample *t*-test was conducted. $n \geq 3$.

MF-ChemiBIS 1.6 (Biostep, Jahnsdorf, Germany). Band volumes were converted into quantitative values by using TotalLab TL100 (Biostep) and β -actin band volumes were used for normalization.

2.8. Phagocytosis assay

Confluent RPE cells seeded on collagen I coated cover slip were treated with 50 $\mu\text{g}/\text{mL}$ FV fucoidan and inflammatory agents LPS, Poly I:C, TNF α and Pam, respectively, for one, three and seven days and an established phagocytosis assay was conducted [63]. In brief, fluorescent labeled latex beads (Sigma-Aldrich; #L1030) and photoreceptor outer segments (POS) from porcine retinae were mixed and given to the cells 4 h after treating them with the respective stimuli. After fixating and washing steps, the cells were mounted with Fluoromount-GTM and DAPI (Thermo Fisher Scientific; #00-4959-52). Fluorescence microscope Axiovert Imager M.2 and AxioVision Software (both from Zeiss Microscopy GmbH, Germany) were used for image capture. Cell nuclei, stained with DAPI, were counted manually. For counting of fluorescent beads, a self-designed macro from Fiji was used (<https://imagej.net/software/fiji/downloads>). Uptake of beads was set in relation to cell nuclei number for evaluation.

2.9. Statistical analysis

All experiments were independently repeated at least three times. Diagrams, mean values and standard deviation were created with Microsoft Excel (Excel 2010, Microsoft, Redmond, WA, USA). Statistical analysis was performed with GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA, USA, 2021). Test for normality was conducted with Shapiro-Wilk test. Normally distributed relative data was analyzed with one-sample *t*-test, not normally distributed data with Wilcoxon signed-rank test. Absolute data which was normally distributed was first analyzed with ANOVA (analysis of variance) followed by consecutive student's *t*-test or, when not normally distributed, with Kruskal-Wallis test followed by Mann-Whitney *U* test. Data was considered as significant if *p* values were ≤ 0.5 .

3. Results

3.1. Testing cell viability

We have previously shown that FV fucoidan is not toxic on RPE cells for up to seven days [50]. However, pro-inflammatory insults may reduce RPE cell viability [28]. Here, we have investigated the effect of fucoidan under pro-inflammatory stimulation. We treated primary porcine RPE with 50 $\mu\text{g}/\text{mL}$ FV fucoidan and/or 1 $\mu\text{g}/\text{mL}$ LPS, 10 $\mu\text{g}/\text{mL}$ Poly I:C, 50 ng/mL TNF and 10 ng/mL Pam, respectively for one, three, seven and 28 days in 24-well (Fig. 1a–d) and for 28 days in 12-transwell (Fig. 1e) plates. Cell viability was investigated with MTT assay. MTT data was normally distributed (Shapiro-Wilk test) and one-sample *t*-test was conducted. No significant toxic effects could be detected. Of interest, after one day cell viability signal increased significantly under FV fucoidan treatment compared to no treatment, treatment with LPS alone, and treatment with Pam alone, significance investigated with student's *t*-test, indicating a metabolic activation of the cells by FV fucoidan.

3.2. Testing barrier function

We have previously shown that pro-inflammatory stimuli can affect the barrier function of RPE cells [26]. To investigate the influence of fucoidan on the barrier function under inflammation, we cultivated primary porcine RPE cells in 12-transwell plates. We stimulated the cells with 50 $\mu\text{g}/\text{mL}$ FV fucoidan and/or 1 $\mu\text{g}/\text{mL}$ LPS, 10 $\mu\text{g}/\text{mL}$ Poly I:C, 50 ng/mL TNF α and 10 ng/mL Pam,

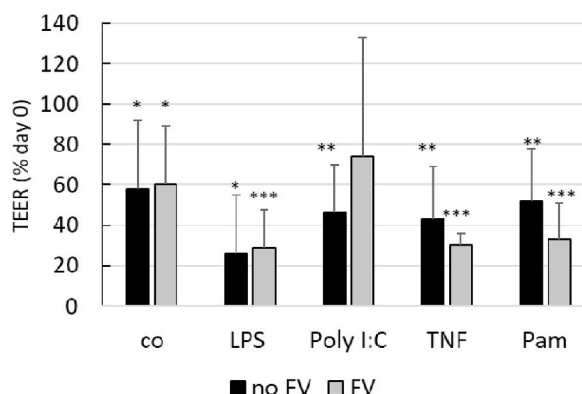


Fig. 2. Barrier function. Primary porcine RPE cells were treated with 50 $\mu\text{g}/\text{mL}$ FV and/or 1 $\mu\text{g}/\text{mL}$ LPS, 10 $\mu\text{g}/\text{mL}$ Poly I:C (PIC), 50 ng/mL TNF and 10 ng/mL Pam for 28 days in 12-transwell plates. Transepithelial electrical resistance (TEER) was measured in $\Omega\cdot\text{cm}^2$ before stimulation as well as after 28 days. TEER was set in relation to the day 0 values and are depicted in %. Data was normally distributed and one-sample *t*-test was conducted. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 (compared to day 0); *n* \geq 5.

respectively, for up to 28 days after each of the well reached at least $150 \Omega \cdot \text{cm}^2$. We measured TEER before stimulating as well as after one, three, seven and 28 days. We depicted the day 28 TEER values in % relative to the day 0 values (Fig. 2). Data was normally distributed (Shapiro-Wilk-test) and one-sample *t*-test was conducted. All TEER of each sample decreased slowly over the each of the measurements up until day 28 (data not shown) and they reached the lowest cell barrier at day 28. Only co-stimulation with FV fucoidan in combination with Poly I:C was not significant decreased which could indicate a possible protective effect of FV fucoidan against Poly I:C induced decrease of barrier integrity. For other time points and pro-inflammatory agents, we could not detect a protective effect on TEER if stimulated simultaneously with FV fucoidan (data not shown).

3.3. Testing cytokine secretion

We have previously shown that pro-inflammatory stimuli induce profound secretion of IL-6 and IL-8 in primary RPE cells [28]. Here, we tested the effect of FV fucoidan on the secretion of IL-6 (Fig. 3) and IL-8 (Fig. 4) in inflammatory activated primary porcine RPE cells. Cells were stimulated with 50 $\mu\text{g}/\text{mL}$ FV and/or 1 $\mu\text{g}/\text{mL}$ LPS, 10 $\mu\text{g}/\text{mL}$ PIC, 50 ng/mL TNF and 10 ng/mL Pam, respectively, for up to 28 days. Supernatants were taken after one, three, seven and 28 days, collected for 24 h each. These supernatants were analyzed with corresponding ELISA (IL-6: day 1 Fig. 3a, day 3 Fig. 3b, day 7 Fig. 3c, day 28 Fig. 3d; IL-8: day 1 Fig. 4a, day 3 Fig. 4b, day 7 Fig. 4c, day 28 Fig. 4d). For IL-6, data of day 1 were normally distributed (Shapiro-Wilk test), so ANOVA and consecutive student's *t*-test was conducted to assess significances. Data of day 3, 7 and 28 were not normally distributed, so a Kruskal-Wallis with consecutive Mann Whitney *U* test was conducted. All inflammatory stimuli induced IL-6. No effect of FV fucoidan could be seen on day 1. On day 3, 7, and 28, FV fucoidan significantly reduced IL-6 in cells treated with Poly I:C and on day 28 TNF induced IL-6 was also lowered. Regarding IL-8 secretion, all inflammatory agents significantly increased its secretion at all tested time points. Data of day 1, 7 and 28 days were normally distributed (Shapiro-Wilk test), so ANOVA and consecutive student's *t*-test was conducted to assess significances. Data of day 3 were not normally distributed, so a Kruskal-Wallis with consecutive Mann Whitney *U* test was conducted. Under Poly I:C stimulation, FV fucoidan significantly reduced IL-8 secretion at day 28. FV fucoidan did not affect pro-inflammatory cytokine secretion of any other tested stimulus.

3.4. Testing gene expression

We also investigated the gene expression of inflammation relevant interleukin 6 (gene *IL6*) and interleukin 8 (gene *CXCL8*) as well as RPE phagocytosis relevant *MERTK* and *PIK3CA* under proinflammatory treatment. Cells were stimulated with 50 $\mu\text{g}/\text{mL}$ FV fucoidan for 30 min and then additionally with 1 $\mu\text{g}/\text{mL}$ LPS, 10 $\mu\text{g}/\text{mL}$ Poly I:C, 50 ng/mL TNF- α or 10 ng/mL Pam for three days, including appropriate controls. RNA-isolation was performed after, then cDNA was generated and real-time PCR (qPCR) conducted. The data

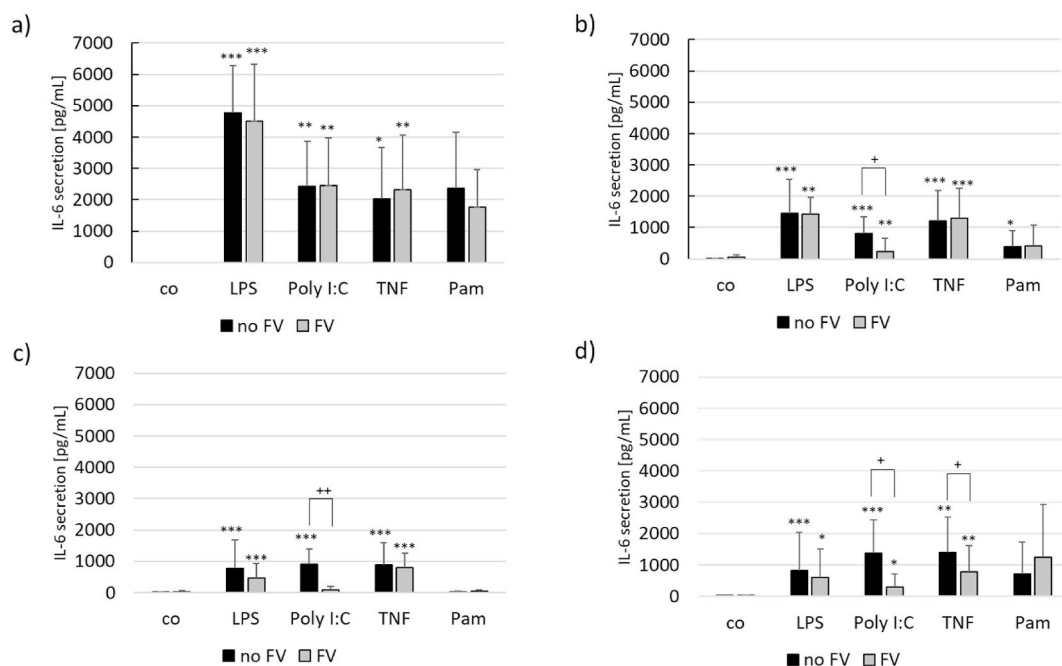


Fig. 3. Interleukin 6 secretion. Primary porcine RPE cells were treated with 50 $\mu\text{g}/\text{mL}$ FV and/or 1 $\mu\text{g}/\text{mL}$ LPS, 10 $\mu\text{g}/\text{mL}$ PIC, 50 ng/mL TNF and 10 ng/mL Pam for 28 days on 24-well plates. Supernatants were taken after one (a), three (b), seven (c) and 28 days (d) and analyzed for interleukin 6 (IL-6) with ELISA. Values are depicted as pg/mL IL-6. ANOVA and student's *t*-test or Kruskal-Wallis and Mann-Whitney *U* test were conducted. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 (compared to control), + *p* < 0.05, ++ *p* < 0.01 (compared to stress control); *n* ≥ 6.

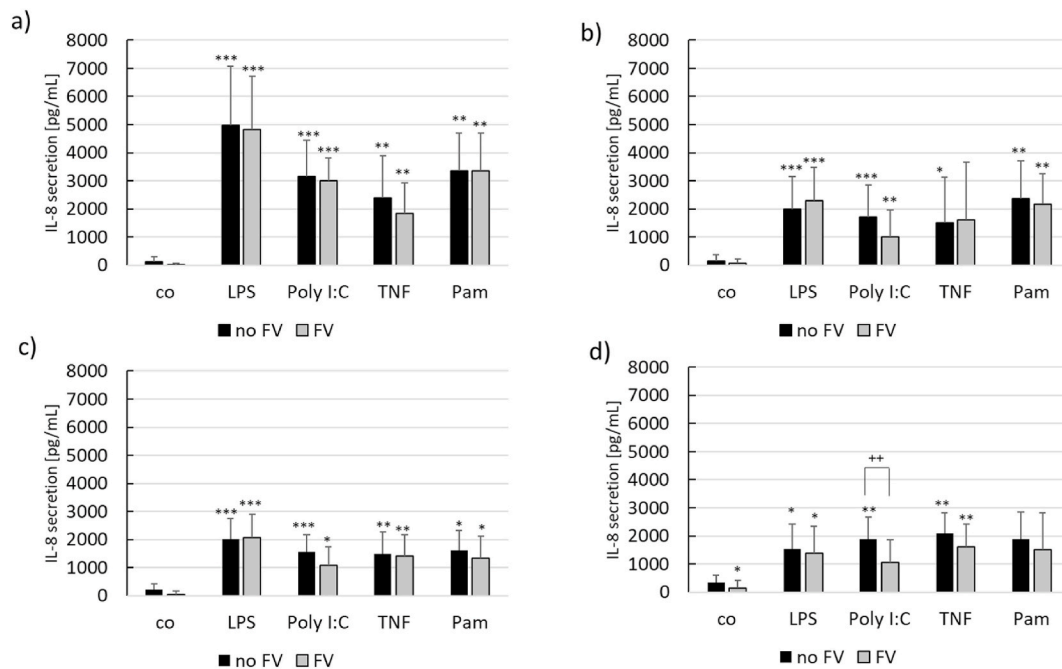


Fig. 4. Interleukin 8 secretion. Primary porcine RPE cells were treated with 50 µg/mL FV and/or 1 µg/mL LPS, 10 µg/mL PIC, 50 ng/mL TNF and 10 ng/mL Pam for 28 days on 24-well plates. Supernatants were taken after one (a), three (b), seven (c) and 28 days (d) and analyzed for interleukin 8 (IL-8) with ELISA. Values are depicted as pg/mL IL-8. ANOVA and student's *t*-test or Kruskal-Wallis and Mann-Whitney *U* test were conducted. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 (compared to control), ++ *p* < 0.01 (compared to stress control); *n* ≥ 6.

was evaluated with Thermo Fisher Connect and the integrated *t*-test was used for calculating significances. *GAPDH* was used as endogenous control for normalization. Relative quantification values and *p*-values are seen in Table 1 (*IL6*, *IL8*) and Table 2 (*MERTK*, *PIK3CA*). The Poly I:C stress control was set to 1.000 and other samples were calculated relative to it. Of note, Poly I:C did not significantly increase the expression of inflammatory genes. After three days FV displayed a significant reduction of *IL6* and *CXCL8* with 0.259 (*p* = 0.008) and 0.032 (*p* = 0.033) respectively. Concerning phagocytosis, no relevant gene expression bigger than 2-fold changes were achieved. Only Pam control increased expression of *PIK3CA* significantly to RQ of 1.598 (*p* = 0.049). TNF tended to increase *MERTK* gene expression but reached no significance.

3.5. Testing expression of CD59

We investigated the influence on protein expression of CD59, a complement regulating protein which is expressed in RPE cells and downregulated in late AMD [64]. Cell lysates were prepared after stimulating RPE cells for 1 and 3 days with FV fucoidan and/or LPS, Poly I:C, TNF and Pam, respectively. Exemplary blots are given in Fig. 5. Volumes of the Western blot bands were evaluated with TotalLab and set in relation to the untreated control. Data was normally distributed (Shapiro-Wilk test) and one-sample *t*-test was conducted. We could show that the CD59 was significantly reduced after one day by Poly I:C with 0.60 ± 0.19 [a.u.] (*p* = 0.0352) and

Table 1

Interleukin 6 and 8 gene expression. Gene expression of *IL6*, *CXCL8*, *MERTK* and *PIK3CA* after Poly I:C and/or FV fucoidan stimulation for three days. *GAPDH* data was used for normalization and values were set in relation to Poly I:C control.

Group Name	Gene	Rq	P-value
Control	<i>IL6</i>	1.649	0.661
Poly I:C	<i>IL6</i>	1.000	1.000
FV	<i>IL6</i>	0.259	0.008
FV + Poly I:C	<i>IL6</i>	0.422	0.229
Control	<i>CXCL8</i>	0.434	0.162
Poly I:C	<i>CXCL8</i>	1.000	1.000
FV	<i>CXCL8</i>	0.032	0.033
FV + Poly I:C	<i>CXCL8</i>	0.247	0.056

FV = Fucoidan from *Fucus vesiculosus* (Sigma-Aldrich), Poly I:C = polyinosinic:polycytidylic acid, *GAPDH* = gene for glyceraldehyd-3-phosphat-dehydrogenase, *IL6* = gene for interleukin 6, *CXCL8* = gene for interleukin 8, Rq = relative quantification quotient. *n* = 3 (number of independent experiments).

Table 2

Phagocytosis gene expression. Gene expression of *MERTK* and *PIK3CA* after LPS, Poly I:C, TNF, Pam and/or FV fucoidan stimulation for three days. *GAPDH* data was used for normalization and values were set in relation to control.

Group Name	Gene	Rq	P-value
Control	<i>MERTK</i>	1.000	1.000
FV	<i>MERTK</i>	0.849	0.750
LPS	<i>MERTK</i>	0.637	0.567
FV + LPS	<i>MERTK</i>	0.612	0.366
Poly I:C	<i>MERTK</i>	0.984	0.972
FV + Poly I:C	<i>MERTK</i>	0.583	0.302
TNF	<i>MERTK</i>	1.848	0.333
F2 + TNF	<i>MERTK</i>	0.678	0.484
Pam	<i>MERTK</i>	0.643	0.427
F2 + Pam	<i>MERTK</i>	0.670	0.594
Control	<i>PIK3CA</i>	1.000	1.000
FV	<i>PIK3CA</i>	1.213	0.568
LPS	<i>PIK3CA</i>	1.260	0.512
FV + LPS	<i>PIK3CA</i>	1.255	0.465
Poly I:C	<i>PIK3CA</i>	0.920	0.861
FV + Poly I:C	<i>PIK3CA</i>	1.109	0.663
TNF	<i>PIK3CA</i>	1.414	0.454
F2 + TNF	<i>PIK3CA</i>	1.082	0.704
Pam	<i>PIK3CA</i>	1.598	0.049
F2+Pam	<i>PIK3CA</i>	1.078	0.824

FV = Fucoidan from *Fucus vesiculosus* (Sigma-Aldrich), LPS = lipopolysaccharide, Poly I:C = polyinosinic:polycytidylic acid, TNF = tumor necrosis factor alpha, Pam = Pam2CSK4, *GAPDH* = gene for glyceraldehyde-3-phosphat-dehydrogenase, *MERTK* = gene for proto-oncogene tyrosine-protein kinase MER, *PIK3CA* = gene for phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha, Rq = relative quantification quotient. n = 3 (number of independent experiments).

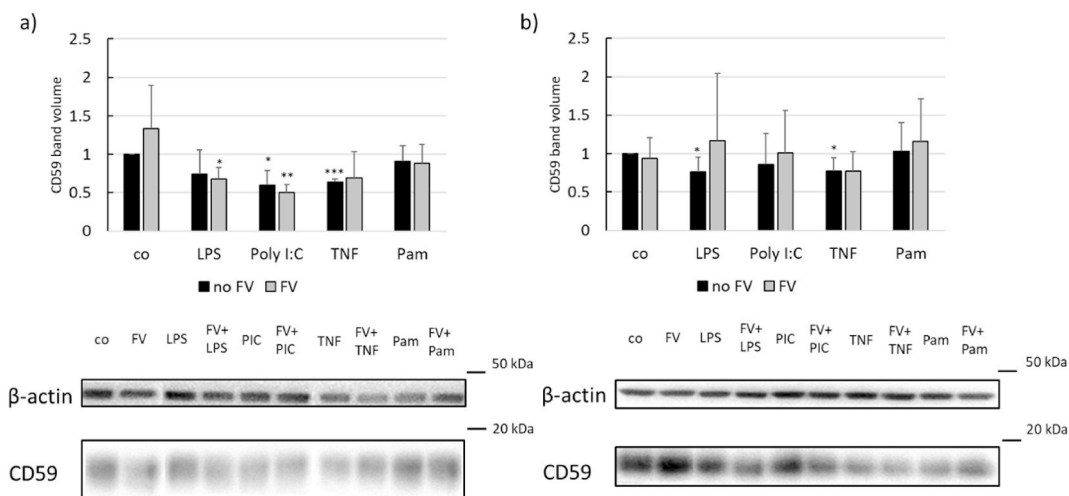


Fig. 5. Protectin protein expression. CD59 (protectin) expression in RPE cells was investigated after stimulating for one (a) and three (b) days with FV and/or LPS, PIC, TNF and Pam. Cell lysates were applied in Western blot. Band volumes were evaluated with TotalLab, normalized to β -actin and set in relation to the control (set to 1.00). Exemplary blots are depicted for one day (a) and three days (b) of stimulation. Data was normally distributed and one-sample *t*-test was applied. Original blots are depicted in supplement file “CD59 original blot”. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. n \geq 4.

TNF with 0.64 ± 0.04 [a.u.] (*p* = 0.0005) (Fig. 5a). Simultaneously stimulation with FV fucoidan and Poly I:C or TNF did not have significant differences to the stress controls. After three days TNF reduced CD59 to 0.77 ± 0.18 [a.u.] (*p* = 0.0348) and LPS reduced CD59 to 0.76 ± 0.19 [a.u.] (*p* = 0.0401) (Fig. 5b). Of note, the significance of this reduction was lost when cells were pretreated with FV fucoidan, indicating a protective effect on CD59 expression.

3.6. Testing expression of RPE65

RPE65 is a signature molecule of the RPE and a vital enzyme for the visual cycle [65,66]. RPE65 expression was investigated after

three days of stimulation. The expression of RPE65 was reduced expression by LPS to 0.57 ± 0.22 [a.u.] ($p = 0.0186$) and TNF to 0.39 ± 0.43 [a.u.] ($p = 0.0463$) (Fig. 6). The significance of the decrease was lost when the cells were preincubated with FV fucoidan, again indicating a protective effect on RPE65 gene expression by FV fucoidan.

3.7. Testing phagocytosis

Phagocytosis of shed photoreceptor outer segments is a major function of RPE cells [67]. We have previously shown that FV fucoidan does not interfere with phagocytotic function in RPE cells [50]. Here, we investigated the phagocytic ability of primary porcine RPE cells after stimulating them with FV fucoidan and/or LPS, Poly I:C, TNF and Pam, respectively for one (Fig. 7a), three (Fig. 7b) and seven (Fig. 7c) days. We counted them with DAPI stained cell nuclei which represents the cell number and used a self-programmed macro in Fiji to count the fluorescence beads which were opsonized with POS (photoreceptor outer segments). The bead number was normalized to the cell nuclei number. Exemplary photos are depicted in Fig. 7d. Data was distributed normally (Shapiro-Wilk test) and ANOVA with consecutive *t*-test was used to determine significances. After one day combined stimulation of FV fucoidan and the inflammatory agents LPS, Poly I:C and Pam reduced beads significantly in contrast to the agents alone (3–5 beads/cell compared to 9 beads/cell of the control). After three days, just Pam had an effect and reduced phagocytosis to 3 ± 1 beads/cell compared to control with 5 ± 1 beads/cell ($p = 0.004$). After seven days LPS, TNF, FV + LPS, FV + Poly I:C, FV + TNF and FV + Pam significantly reduced phagocytosis to 2–6 beads/cell compared to control with 8 ± 3 beads/cell. Taken together, we could show that inflammatory activation of RPE cells can affect phagocytotic activity which is not ameliorated by FV fucoidan.

4. Discussion

The pathogenesis of AMD includes four major (and interconnected) pathways: oxidative stress, inflammation, lipid accumulation and, in the exudative form of the disease, angiogenesis [7,8,68]. Current treatment options only target angiogenesis and are only administered after vision loss has already occurred [4]. Bioactive compounds that target several of these pathogenic pathways may offer a prospect to interfere earlier in disease development and preserve the vision loss of the affected patients [46].

Fucoidan is a sulphated polysaccharide derived from brown algae with interesting bioactivities [69] which is currently under investigation as a potential new treatment option for age-related macular degeneration [45,46]. Fucoidan from *Fucus vesiculosus* provided by Sigma Aldrich has shown to be anti-angiogenic and anti-oxidative in previous studies of our laboratory [50,51,58]. In this study, we have investigated the anti-inflammatory effect of FV fucoidan in primary porcine RPE cells.

Of note, primary porcine RPE cells are excellent models of adult human RPE cells [54,56]. Indeed, the porcine eye and the porcine retina are much closer to the human situation than e.g. the eye and retina of the mouse, considering animal behavior (e.g. diurnal vs. nocturnal activity) gross anatomy (e.g. size of eye ball and lens), cellular differences (e.g. number and distribution of cones) and molecular differences (e.g. proteins included in tight junction formation) [54,62,70]. Therefore, the porcine eye is an excellent model for the human eye, exceeding the mouse as model organism by far.

We have previously established the pro-inflammatory reaction of porcine RPE cells against different TLR agonists and the pro-inflammatory cytokine TNF α [28,38]. The concentrations and time lines for stimulation used in this study as well as the cytokines investigated (IL-6, IL-8) are based on the findings of these prior studies. Also, we decided on 50 μ g/mL fucoidan as the concentration of

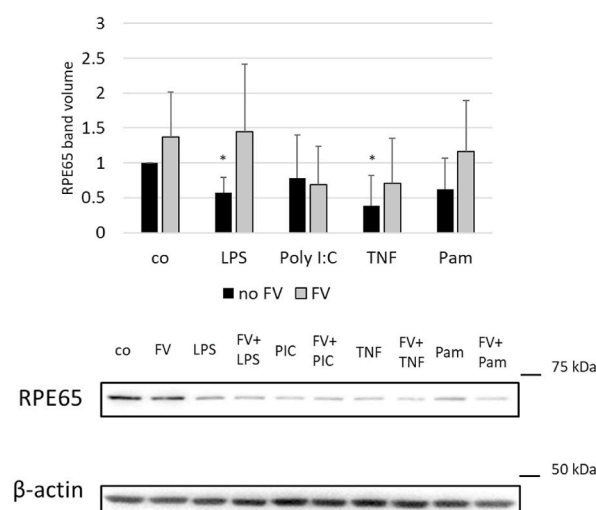


Fig. 6. Retinal pigment epithelium-specific 65 kDa protein expression. Retinal pigment epithelium-specific 65 kDa protein (RPE65) expression in RPE cells was investigated after stimulating for three days with FV and/or LPS, PIC, TNF and Pam. Cell lysates were applied in Western blot. Band volumes were evaluated with TotalLab, normalized to β -actin and set in relation to the control (set to 1.00). Example blot is depicted. Data was normally distributed and one-sample *t*-test was applied. Original blot is depicted in supplement file “RPE65 original blot”. * $p < 0.05$. $n = 5$.

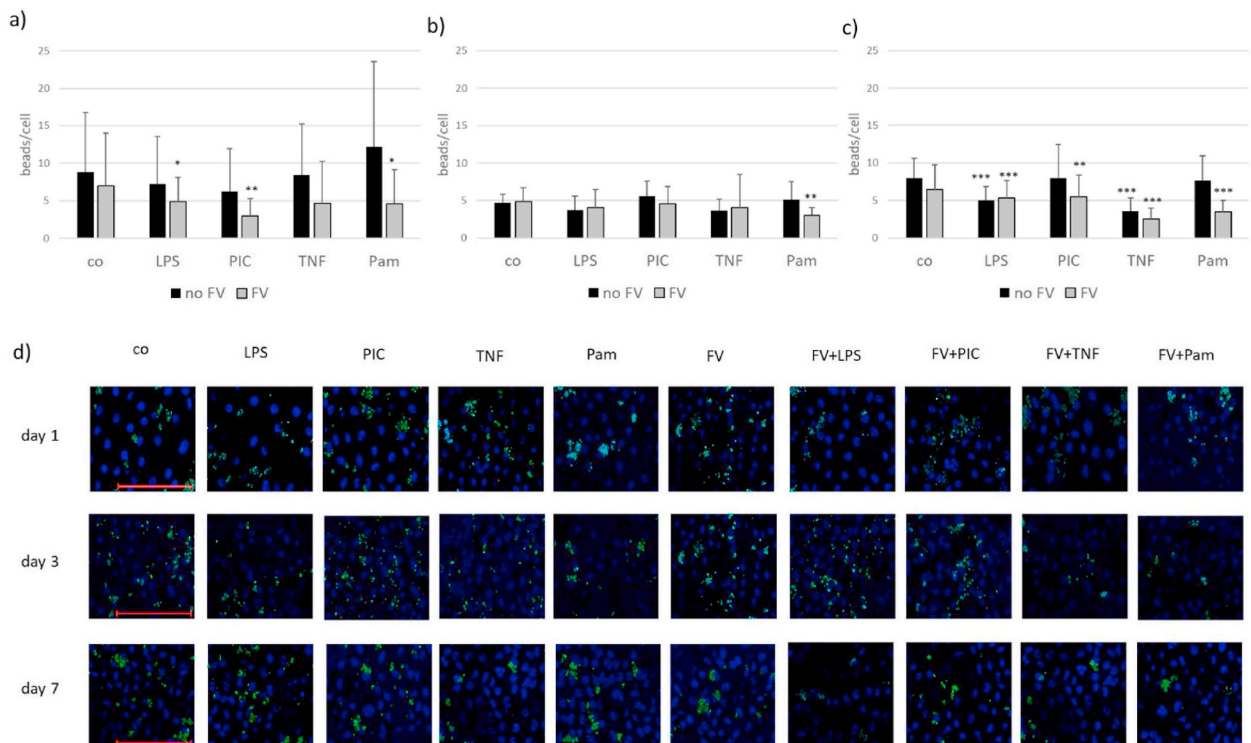


Fig. 7. Phagocytosis. Primary porcine RPE cells were treated with 50 $\mu\text{g/mL}$ FV and/or 1 $\mu\text{g/mL}$ LPS, 10 $\mu\text{g/mL}$ PIC, 50 ng/mL TNF and 10 ng/mL Pam for one (a), three (b) and seven (c) days. After that with photoreceptor outer segments phagocytized beads were given to the cells for 4 h and then fixated on cover slips. Fluorescence microscopy was applied and cell nuclei and beads were counted with Fiji. Exemplary photos are depicted (d). Cell nuclei as cell number was used for normalization of the bead data. Data was normally distributed and ANOVA and student's t-test applied. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 15$.

choice, as we have found this concentration of fucoidan to be highly effective in VEGF inhibition in primary RPE cells [58,59].

FV fucoidan with or without inflammatory stimuli did not reduce cell viability of our primary RPE, which corresponds to our previous findings [50,71]. As we have previously only investigated short term toxicity, this study confirms the safety of fucoidan in long term treatment even under pro-inflammatory stimulation.

Concerning FV fucoidan's influence on inflammation, our data showed a highly stimulus dependent effect, with cytokine release of IL-6 and, to a lesser extent IL-8, reduced only after induction by the TLR-3 agonist Poly I:C, but not by other tested stimuli. This indicates a specific effect of fucoidan, which is not generally interfering with a pro-inflammatory activation but targeting specifically TLR-3 induced pathways. This is of particular interest, as fucoidans have been shown to interact with TLR-2 and TLR-4 before and to reduce LPS induced proinflammatory activation. Conversely, in our study cytokines induced by agonists of TLR-2 (Pam) and TLR-4 (LPS) were not influenced by FV fucoidan. It should be noted, however, that the fucoidans tested in the cited studies were from different species (e.g. *Laminaria japonica*/*Saccharina japonica*, *Laminaria cichorioides*, or *Fucus distichus* subsp. *evanescens*) and furthermore tested in a different cell type (e.g. HEK293 cell; RAW264.7 macrophages) [72,73]. The dependence of fucoidan bioactivities on species and test system is well known [51,69]. In addition, the anti-inflammatory effect against LPS induced inflammation has been described to be dependent on molecular weight, with low molecular weight fucoidan showing the best effect [74]. Conversely, VEGF-inhibition and antiangiogenic effect is stronger with high molecular weight fucoidan [45,59,75,76], and we used FV fucoidan that has previously been shown to inhibit VEGF [50,58]. Most importantly, however, the anti-inflammatory effect of fucoidan against LPS induced inflammation is usually studied in macrophages [73,77–79], which differ profoundly from RPE cells [11]. Indeed, the role of the RPE in inflammation is complex. While being able to be pro-inflammatorily activated, the activation is far less than e.g. in retina microglia [38]. In addition, pro-inflammatorily activated RPE display a regulating effect on monocytic or microglia cells [38, 80]. Therefore, it is conceivable that the effect of fucoidan on RPE cells differ from those on macrophages. Indeed, the effect of fucoidan on TLR-3 induced inflammation has received little attention so far. To the best of our knowledge, an effect of fucoidan on TLR-3 mediated inflammation has only been shown for a fucoidan-rich extract from *Ascophyllum nodosum* in bronchial epithelial cells [81]. It is of interest that the effect of inflammation, including a reduction of IL-6, was similarly detected in another epithelial cell. This might be an indication of an effect specific for epithelial cells, however, further research is warranted to substantiate such a general indication.

As experienced before, the action of fucoidan proves to be highly dependent on the cells and tissues involved, as shown for its anti-angiogenic and anti-VEGF activity [50,51]. However, the specificity we see for TLR-3 may prove of high interest for its potential use in

AMD. TLR-3 is activated by dsRNA from viruses but also by degenerating cells, which are abundant in AMD development and suspected to be responsible for the low-grade chronic inflammation which drives AMD development. Furthermore, the activation of TLR-3 is discussed as an important contributor to AMD development [24]. Even though the data on genetics polymorphisms TLR-3 in association with AMD development is conflicting, with their effect being dependent on different cohorts and ethnicity [33,34,82–85], TLR-3 activation leads to several consequences in the RPE cell that are reminiscent of cellular AMD pathology. TLR-3 activation in the RPE has been shown to result in a reduction of RPE cell viability [16,31], and RPE degeneration is a major feature of AMD development [9]. TLR-3 activation can increase VEGF secretion in RPE cells [16], which is a hallmark of exudative AMD [86]. TLR-3 activation reduces the barrier function in RPE cells and can impede transcellular transportation [26,87], both important functions of the RPE and impaired in AMD [88]. We could show in this study, fucoidan can ameliorate the loss of barrier function in long-term treatment. Taken together, the ability of fucoidan to interfere with TLR-3 induced pro-inflammatory changes of the RPE may be highly relevant for the prevention of AMD development. As these experiments have been conducted in vitro only, further research including in vivo data will be needed to validate these findings.

In addition to its cytokine reducing effects, we found some modest effects of fucoidan on protein expression of CD59 and RPE65 which were independent of TLR-3, indicating a possible protecting effect of fucoidan on RPE function. However, the effects were mainly a loss of significance of the reduction and not universally seen for all stimuli. Furthermore, no protection of phagocytic activity and gene expression by fucoidan could be detected. Previous studies conducted in peripheral blood mononucleated cells as well as macrophages described a regulating or enhancing effect of fucoidan on phagocytosis [89,90]. The differences may be explained by the different phagocytotic roles of and the different utilization of receptors by RPE cells and macrophages [52]. Data of this study suggest that genes *MERTK* and *PIK3CA* are not relevantly influenced by fucoidan nor by proinflammatory activation.

Nevertheless, these data give at least some indication of some protection of function which should be investigated in further detail in further studies. After all, even a modest effect can help to maintain RPE function in a challenged retina.

In our study, we have shown that a fucoidan that was previously shown to be antiangiogenic and antioxidative is also anti-inflammatory, with a specificity towards TLR-3 mediated effects. These are exciting data, as they indicate a compound which could target three major pathways of AMD pathogenesis. Further studies are clearly warranted to further investigate and develop fucoidan for AMD treatment. A major step will be the translation of this in-vitro data into in-vivo models. Further research needs to prove the efficacy of fucoidan in animal models to pave the way for testing in human patients. But other further investigations are also of high interest. Fucoidan has been shown to interfere with lipid accumulation [91] – so there is a possibility that fucoidan could target the fourth major pathogenic pathway as well. This would be an exciting study to conduct. And finally, it would be of high scientific interest to decipher how exactly fucoidan interacts with the RPE cells and why it seems to specifically target TLR-3 mediated pathways, especially since it has been previously shown that it can interact with TLR-4 [92]. Indeed, even though the exact mode of action of fucoidan is not known, other studies not focusing on the retina showed that fucoidan can interact with several receptors (e.g. VEGFR-2, NRP-1) but also cytokines (e.g. TGF- β , VEGF) [93–95]. Depending on the study and molecular weight of the fucoidan, at least for VEGF it has been shown that it can either enhance receptor-ligand interaction or preventing it, preferably by inducing internalization of the receptor [94–96]. In addition, fucoidan can be taken up directly by clathrin-mediated endocytosis and might interfere with cellular signaling that way [97,98]. Understanding the mode of action and its dependence on fucoidan chemical and structural properties can give us indications how to enhance the efficacy of fucoidan.

In conclusion, we could show that fucoidan is not only anti-angiogenic and anti-oxidative, but also anti-inflammatory in our RPE cells and is of high interest for further development for AMD treatment and prevention.

Author contribution statement

Philipp Dörschmann: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Charlotte Seeba, Tabea Thalenhorst: Performed the experiments.

Johann Roider: Contributed reagents, materials, analysis tools or data.

Alexa Klettner: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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