

1 Title page

2 **Oxidative stress in retinal pigment epithelial cells increased endogenous complement-dependent**
3 **inflammatory and angiogenic responses - independent from exogenous complement sources**

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13 *Keywords:* oxidative stress, retinal pigment epithelial cells, complement system, inflammasome,
14 *foxp3*, olaparib

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17 **HIGHLIGHTS**

- 18 • Oxidative stress accumulates complement proteins and receptors in RPE cells
- 19 • Oxidative stress activates the RPE inflammasome without external complement proteins
- 20 • Oxidative stress increases *foxp3* expression and IL-8/VEGF secretion in RPE cells
- 21 • Olaparib enhances pro-inflammatory response of RPE

22

23 **ABSTRACT**

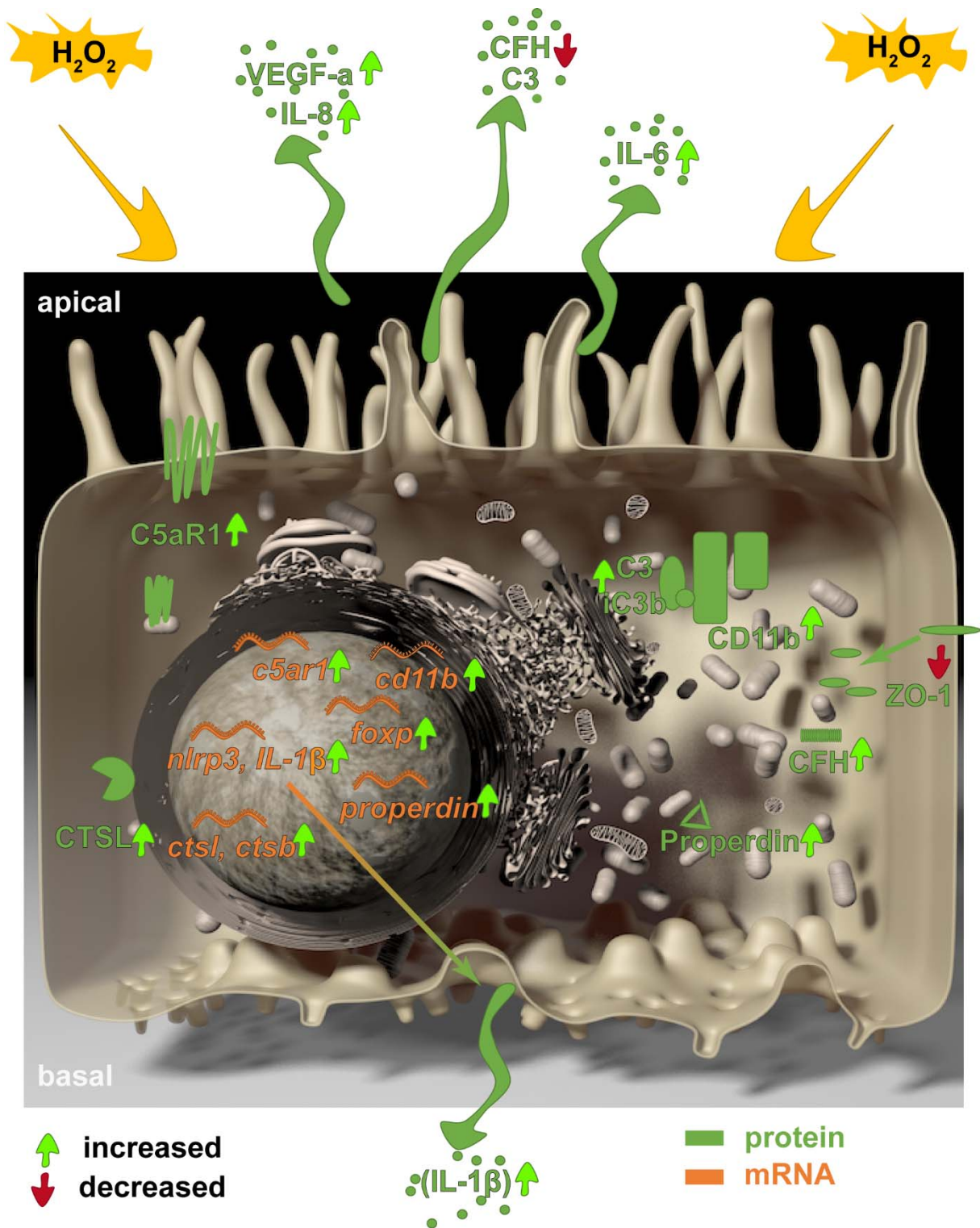
24 Oxidative stress-induced damage of the retinal pigment epithelium (RPE) together with chronic
25 inflammation has been suggested as major contributors to retinal diseases. Here, we examine the
26 effects of oxidative stress and endogenous complement components on the RPE and its pro-
27 inflammatory and –angiogenic responses.

28 The RPE cell line, ARPE-19, treated with H₂O₂ reduced cell-cell contacts, increased marker for
29 epithelial–mesenchymal transition but showed less cell death. Stressed ARPE-19 cells increased the
30 expression of complement receptors CR3 and C5aR1. CR3 was co-localized with cell-derived
31 complement protein C3, which was observed in its activated form in ARPE-19 cells. C3 as well as its
32 regulators CFH and properdin accumulated in ARPE-19 cells after oxidative stress independent from
33 external complement sources. This cell-associated complement accumulation promoted *nlrp3* and
34 *foxp3* expression and subsequent increased secretion of pro-inflammatory and pro-angiogenic factors.
35 The complement-associated ARPE-19 reaction to oxidative stress, independent from external
36 complement source, was increased by the PARP-inhibitor olaparib.

37 Our results indicated that RPE cell-derived complement proteins and receptors are involved in RPE cell
38 homeostasis following oxidative stress and should be considered as targets for treatment
39 developments for retinal degeneration.

40

41 **GRAPHICAL ABSTRACT**



42

43 We show a functional link between oxidative stress, complement receptors, endogenous complement
 44 proteins, pro-angiogenic and -inflammatory responses in ARPE-19 cells. These effects are independent
 45 from extracellularly added complement proteins or receptor ligands. We suggest an oxidative stress-
 46 associated autocrine mechanism of complement receptor regulation in ARPE-19 cells in connection
 47 with upregulated intracellular proteases.

48

49 INTRODUCTION

50 One of the most oxidative environments in the body is the retinal pigment epithelium (RPE) [1], which
51 is in close contact with the photoreceptors and maintains visual function [2]. Low levels of reactive
52 oxygen species are required to maintain physiological functions [3], but the combination of exposure
53 to visible light, elevated metabolic activity, accumulation of oxidized lipoproteins and decreased
54 antioxidant functions during aging make the retinal tissue vulnerable to oxidative stress [4,5]. Oxidative
55 damage to the RPE was therefore identified as a contributing factor to different retinal degenerative
56 diseases such as age-related macular degeneration or Stargardt disease [6–8].

57 In line with this, chronic oxidative stress can involve chronic inflammation subsequently leading to
58 cellular damage in the RPE/retina [6,9]. Based on genetic polymorphisms in genes of the complement
59 system, systemic complement activation and local complement deposition in degenerative retinal
60 tissue a contribution of the complement system to oxidative stress-related retinal degeneration was
61 hypothesized [7,10,11]. The complement system is composed of over 40 proteins, which bridge the
62 innate and adaptive immune defence [12]. The main functions are (I) removal of damaged cells, (II)
63 protection against invading pathogens and (III) attraction of immune cells.

64 Beside the traditional view, evidence is accumulating that complement is also involved in physiological
65 processes such response to oxidative stress and cellular survival programmes [6]. The complement
66 system comprises several soluble and membrane-bound proteins and receptors, which can be
67 produced by a number of cells, including non-immune cells and extrahepatic tissue, and contribute to
68 the autocrine cell physiology [13]. The role of endogenous complement-dependent regulation of
69 cellular homeostasis has been extensively studied in the recent years in T-cells [14]. T-cells, B-cells and
70 human airway epithelial cells contain intracellular stores of C3, which is endogenously cleaved into its
71 active forms C3a and C3b by intracellular proteases [15–17]. Activated C3 was correlated with the
72 activation of the NLRP3 inflammasome in T-cells [15], which lead to chronic pro-inflammation. An
73 antagonising complement modulation was described for regulatory T-cells, where C3aR and C5aR1
74 activation resulted in the activation of the forkhead box P3 (FOXP3) transcription factor [15,22]. The
75 FOXP3 transcription factor acts in multimodal fashion and stimulates the release of anti-inflammatory
76 cytokines and pro-angiogenic factors [22–24].

77 Oxidative stress and inflammasome activation were previously correlated to external complement
78 activity in RPE cells [6,25]. FOXP3 activation in RPE cells also depended on extracellularly added
79 complement components [26]. However, RPE-derived complement has not been discussed as source
80 for NLRP3 or FOXP3 modulation. Complement components can be produced by RPE cells [27] and their
81 expression is changed under oxidative stress [28–32]. Further activated forms of C3 (C3a), independent
82 from extracellular complement sources, were also secreted by RPE cells, suggesting a similar function
83 of the complement system in RPE cells compared to T-cells [18–21].

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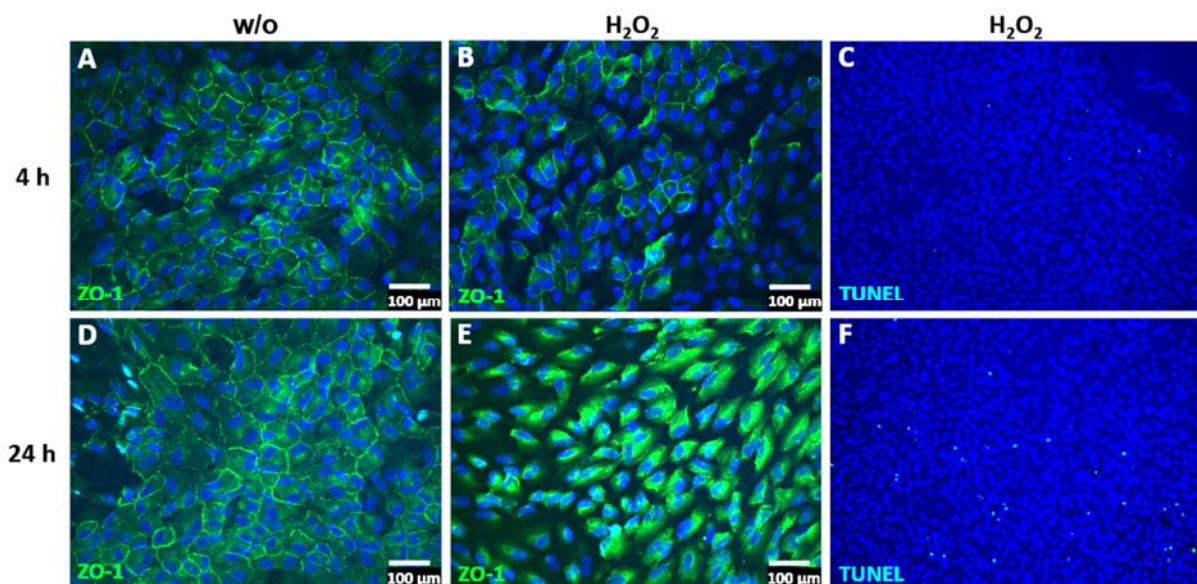
85 In this study, we report that H₂O₂ stimulated the accumulation of complement protein C3, CFH and
86 properdin in RPE cells and increased the expression of complement receptors C5aR1 and CR3. This was
87 accompanied with increased NLRP3 inflammasome activation and FOXP3-associated release of pro-
88 angiogenic factors. Our results indicate a cell homeostatic function of cell-derived complement
89 components, independent from external complement receptor ligands.

90

91 RESULTS

92 Stressed, *in vivo*-like cultivation of ARPE-19 cells

93 We investigated cellular stress response and cell-specific complement expression in a cell line of
94 human RPE cells, the ARPE-19 cell line. Aged ARPE-19 cells of passage 39 were cultivated under *in vivo*-
95 like, unstressed conditions. This was visualized by staining of *zonula occludens 1* (ZO-1), an important
96 protein for cell-cell-contact, showing formation of stable tight junctions and mainly mononuclear,
97 polarized cell growth on transwell filters (**Fig. 1A, D**). H₂O₂ treatment resulted in cellular stress
98 indicated by reduced cell-cell contacts after 4 h (**Fig. 1B**) and a time-dependent translocation of ZO-1
99 from the cell membrane to the cytoplasm after 24 h (**Fig. 1E**). Evidence of induced cellular stress by
100 H₂O₂ were also observed by increased mRNA expression of vimentin (*vim*) and α smooth muscle actin
101 (α -*sma*), typical mesenchymal marker indicating epithelial–mesenchymal transition (**Sup. Fig. 1**) [33–
102 35]. However, the majority of the ARPE-19 cells did not undergo apoptosis under these non-lethal
103 oxidative stress conditions, shown by a low number of TUNEL-positive cells (**Fig. 1C, F**).

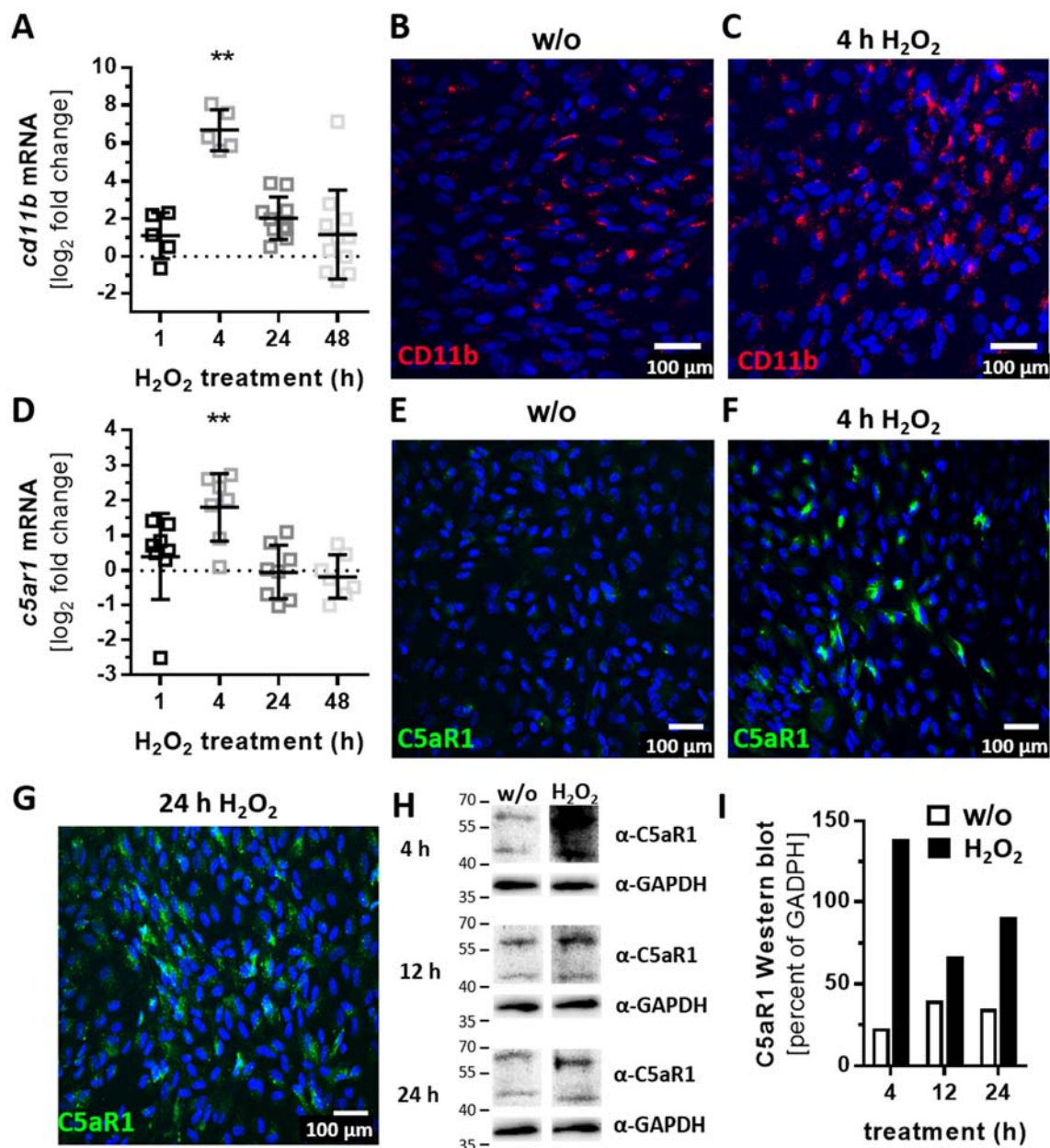


104
105 **Fig. 1 ARPE-19 cells reduced tight junctions and circumvent apoptosis under oxidative stress.**
106 (A, D) ARPE-19 cells untreated (w/o) and stressed with H₂O₂ for (B, C) 4 h or (E, F) 24 h
107 translocated time-dependently the *zonula occludens* protein 1 (ZO-1, green) from the (A, D)
108 cell membrane to the (B, E) cytoplasm. (C, F) ARPE-19 cells treated with oxidative stress
109 showed a minimal TUNEL-positive (light blue) apoptotic reaction after (F) 24 h.
110

111 ARPE-19 cells increase complement receptor expression under oxidative stress

112 ARPE-19 cells express cellular receptors, sense the cellular environment and can react to complement
113 activation products. Complement receptor 3 (CR3), is a heterodimer integrin consisting of two non-
114 covalently linked subunits CD11b and CD18 on leukocytes/ microglia and is activated by C3 cleavage
115 products (iC3b, C3d, C3dg). CD11b has been detected with low expression on mRNA and protein level

116 in ARPE-19 cells (**Fig. 2A, B**). Oxidative stress increased the *cd11b* mRNA expression after 4 h, which
 117 was also shown on protein level with immunostaining (**Fig. 2A, C**).
 118 Activation of complement protein C5 is detected by complement receptor C5aR1, which is expressed
 119 by ARPE-19 cells (**Fig. 2D**). H₂O₂ treatment increased *c5ar1* expression comparable to *cd11b* expression
 120 (**Fig. 2D – F**). C5aR1 protein accumulation was observed after 4 h at the cell nuclei (**Fig. 2F**), which was
 121 more distributed in/on the cell after 24 h (**Fig. 2G**). Increased C5aR1 protein level was also confirmed
 122 in Western blots (**Fig. 2H, I**).
 123 Transcription levels of complement receptor *c3aR* was not significantly changed in H₂O₂-treated ARPE-
 124 19 cells (**Sup. Fig. 2A**).



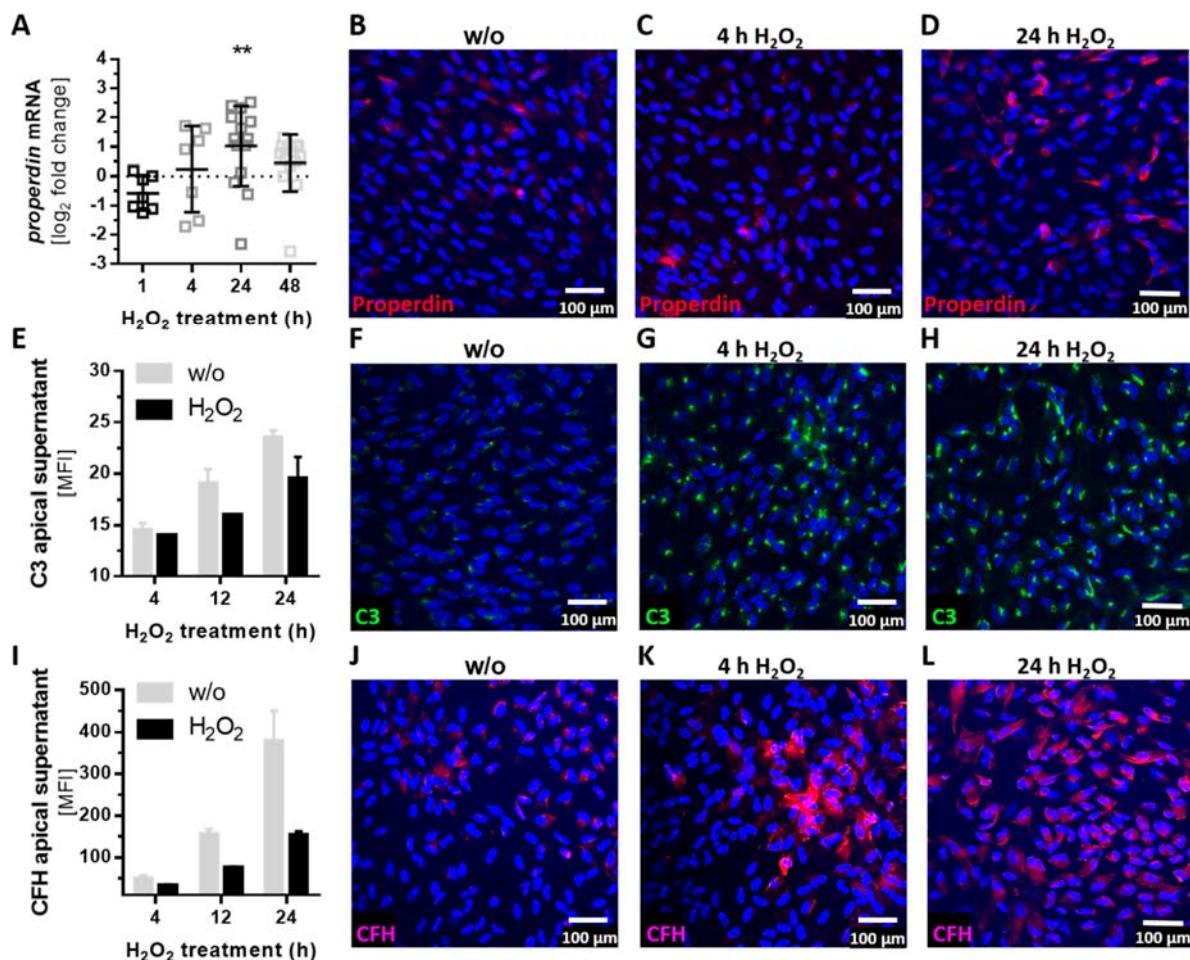
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Fig. 2 Oxidative stress increased expression of complement receptor subunit CD11b and C5aR1 in ARPE-19 cells.

128 (A, D) *Cd11b* and *c5aR1* mRNA expression was increased 4 h following H₂O₂ treatment. This
 129 effect was confirmed on protein level by immunohistochemistry using (B, C) anti-CD11b (red)
 130 and (E – G) anti-C5aR1 (green) antibodies. (H) Western Blots of ARPE-19 cell lysates detected
 131 C5aR1 between 40 – 60 kDa after 4 – 24 h H₂O₂ treatment. (I) Quantitatively, C5aR1
 132 expression was increased in H₂O₂ treated cells in Western blots. (A, D) Mean with standard
 133 deviation is shown, ** p < 0.01 unpaired, two-tailed, parametric t-test, dotted line depicts
 134 untreated control, (B, E, H, I) w/o untreated control.
 135

136 Complement proteins accumulated in ARPE-19 cells under oxidative stress

137 Complement proteins, which can modulate the activity of complement receptors at the RPE, are locally
 138 produced in the retina [27,36] and by RPE cells (Fig. 3, Sup. Fig. 2B – K). The mRNA expression and
 139 protein levels of the stabilizing complement regulator properdin were increased after 24 h of H₂O₂
 140 treatment (Fig. 3A – D), but apical properdin secretion was not detected (Sup. Fig. 3A). This indicated
 141 a properdin storage in the stressed ARPE-19 cells (Fig. 3B – D).



142
 143 **Fig. 3 Oxidative stress induced complement component accumulation in ARPE-19 cells.**
 144 (A) The *properdin* mRNA level was increased 24 h following H₂O₂ treatment. This effect was
 145 confirmed on protein level by immunohistochemistry using an (B – D) anti-properdin (red)
 146 antibody. (E) C3 and (I) CFH protein concentration in the apical supernatant of ARPE-19 cells
 147 decreases following H₂O₂ treatment. Immunohistochemistry using (F – H) anti-C3 (green) and
 148 (J – L) anti-CFH (purple) antibodies showed an increase of cell-associated (G, H) C3 and (K, L)
 149 CFH after oxidative stress treatment. (A, E, I) Mean with standard deviation is shown, (A) **

150 $p \leq 0.01$ unpaired, two-tailed, parametric t-test, dotted line depicts untreated control, (**E, B, F,**
151 **I, J**) w/o untreated control.

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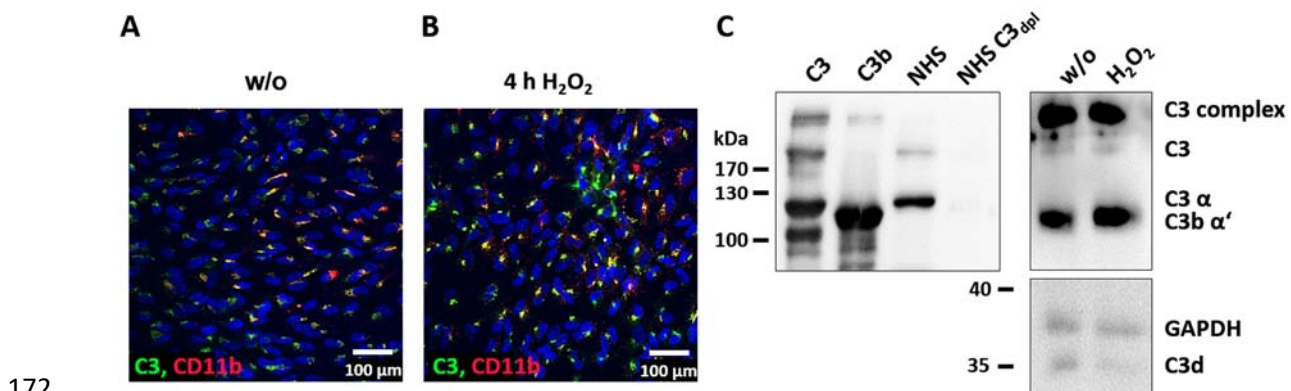
153 Transcription levels of additionally tested complement components (*c3*, *c4a*, *c4b*, *cfb*, *cfb*, *cfb*, *c5*), soluble
154 (*cfh*, *cfi*) and membrane-bound complement regulators (*cd46*, *cd59*) did not change under oxidative
155 stress conditions (**Sup. Fig. 2B – K**).

156 However, we observed a change in cellular accumulation and modulated secretion of complement
157 components on protein level by oxidative stress (**Fig. 3E – L**). Central complement component *c3* was
158 not regulated on mRNA expression by oxidative stress (**Sup. Fig. 2B**), but we detected an increase of
159 cellular C3 in immunostainings and a decrease of C3 secretion into the apical supernatant of ARPE-19
160 cells (**Fig. 3E – H**). Secretion of C3 was more observable in younger compared to older ARPE-19 cells
161 treated with H_2O_2 (**Sup. Fig. 3B**). A similar effect of cellular complement component accumulation and
162 reduced secretion was detectable for complement regulator CFH (**Fig. 3I – L, Sup. Fig. 3C**). Thus, the
163 mRNA expression was not changed under oxidative stress (**Sup. Fig. 2H**).

164

165 **Autocrine complement receptor activation following oxidative stress is correlated with the release** 166 **of pro-inflammatory and pro-angiogenic factors**

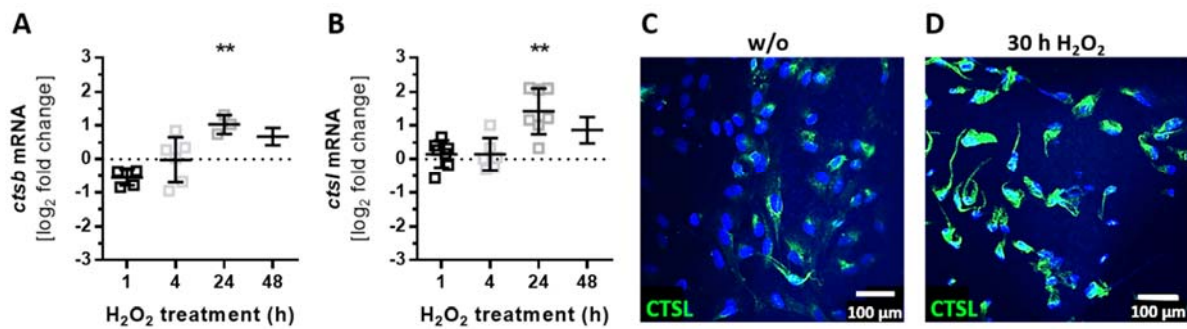
167 Intracellular complement proteins and cellular complement receptors were previously associated with
168 an autocrine regulation of cell differentiation and cell physiology in T-cells as well as lung epithelial
169 cells [17,37]. In line with this we found a co-localization of CD11b and C3 in ARPE-19 cells (**Fig. 4A, B**)
170 and activated C3 fragments ($C3b \alpha'$, $C3d$) in the ARPE-19 cells (**Fig. 4C**), without adding any external
171 complement source.



172 **Fig. 4 C3 and complement receptor CR3 are co-localized in ARPE-19.**
173 **(A)** Unstressed (w/o) and **(B)** H_2O_2 treated ARPE-19 cells were stained with anti-C3 (green) and
174 anti-CD11b (red) antibodies. Overlapping staining signals (yellow) suggested a co-localization
175 of C3 and CD11b. **(C)** C3 and activation products ($C3b \alpha'$ and $C3d$), were detected in untreated
176 and H_2O_2 treated ARPE-19 cells, together with native C3, C3b, human serum (NHS) and C3
177 depleted human serum (NHS $C3_{dpl}$), using Western Blot under reducing conditions.
178

179

180 Intracellular cleavage of complement proteins into active fragments, independent from the systemic
181 complement cascade, can be mediated by intracellular proteases, as cathepsin B (CTSB) or cathepsin L
182 (CTSL) [14,15]. Both proteases were expressed by ARPE-19 cells and they were upregulated following
183 oxidative stress (**Fig. 5**). The expression of *ctsb* and *ctsl* mRNA was increased after 24 h of H₂O₂
184 treatment (**Fig. 5A, B**). We confirmed the higher concentration of CTSL in ARPE-19 cells under stress
185 conditions also on protein level (**Fig. 5C, D**).



186
187 **Fig. 5 Expression of intracellular proteases is increased by oxidative stress in ARPE-19.**
188 (A) *Ctsb* and (B) *ctsl* mRNA expression increased 24 h following H₂O₂ treatment. This effect was
189 confirmed on protein level in immunostainings using an (C, D) anti-CTSL (green) antibody. (A,
190 B) Mean with standard deviation is shown, ** $p \leq 0.01$ unpaired, two-tailed, parametric t-test,
191 dotted line depicts untreated control, (C) w/o untreated control.
192

193 Activation of complement receptors on the one hand can induce inflammasome activation and on the
194 other hand can regulate the mTOR-pathway involving the FOXP3 transcription factor in T- and RPE cells
195 [25,26,38], the well-coordinated interplay of complement receptor signalling controls the pro- and
196 anti-inflammatory cytokine release [25,39]. After detection of cell-derived C3 co-localized with CD11b,
197 its activation products C3b and C3d (**Fig. 4**) as well as H₂O₂ dependent regulation of complement
198 receptors (**Fig. 2**) and cellular complement protein accumulation (**Fig. 3**), we supposed also an
199 autocrine, complement-dependent role of the NLRP3 inflammasome and FOXP3 in ARPE-19 cells
200 treated with H₂O₂ to induce oxidative stress. This regulation would be independent of blood-derived
201 complement components and involves release of cytokines and growth factors in stressed ARPE-19
202 cells (**Fig. 6**).

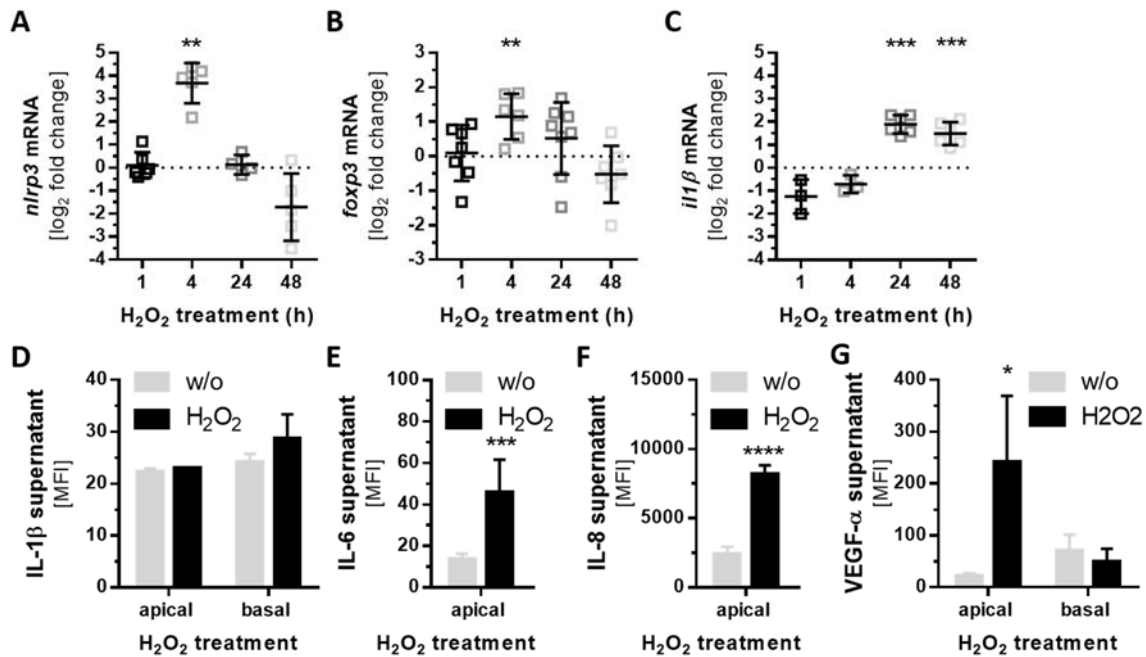


Fig. 6 Increased *nlrp3* and *foxp3* mRNA expression correlates with pro-inflammatory and pro-angiogenic factor secretion.

(A) *Nlrp3*, (B) *foxp3* and (C) *il1b* mRNA levels increased either (A, B) 4 h or (C) 24 h and 48 h following H₂O₂ treatment. Pro-inflammatory cytokine release of (D) IL-1β (after 4 h) and (E) IL-6 (after 48 h) was detected in stressed ARPE-19 cells. This was correlated with an enhanced secretion of pro-angiogenic factors (F) IL-8 (after 48 h) and (G) VEGF-α (after 4 h) H₂O₂ treated cells. MFI mean fluorescence intensity. Mean with standard deviation is shown, * p_≤ 0.05, ** p_≤ 0.01, *** p_≤ 0.001, **** p_≤ 0.0001 unpaired, two-tailed, parametric t-test, (A, B, C) dotted line depicts untreated control, (D – G) w/o untreated control.

Indeed, we detected an increased expression of *nlrp3* and *foxp3* mRNA after 4 h of H₂O₂ treatment (Fig. 6A, B). A subsequent enhanced expression of *il1b* mRNA after 24 h and 48 h indicated the activation of the NLRP3-inflammasome in stressed ARPE-19 cells (Fig. 6C), thus the mRNA expression of *il18* was not changed (Sup. Fig. 2L). Consequently, we found higher pro-inflammatory cytokine levels in H₂O₂ treated ARPE-19 cell supernatant compared to untreated control (Fig. 6D – E). IL-1β was slightly increased shortly after treatment (4 h), while IL-6 was significantly elevated in supernatant of stressed RPE cells (Fig. 6D).

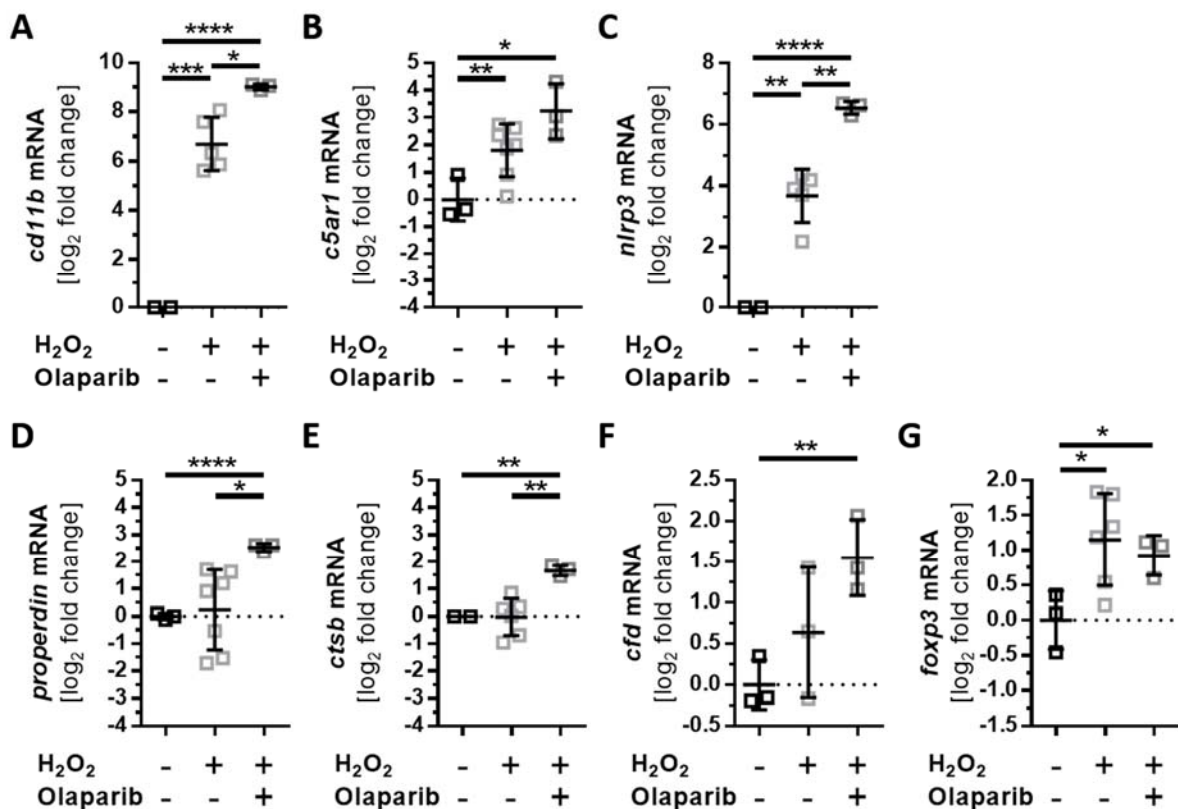
Increased *foxp3* expression is an attribute of anti-inflammatory regulatory T-cells, which secrete mainly TGF-β and IL-10. We did not detect a change in *tgfb* expression (Sup. Fig. 2M) or IL-10 secretion (data not shown) by H₂O₂ treated ARPE-19 cells. Therefore, we assumed a pro-angiogenic function of *foxp3* in the cells as previously reported [23,24]. In line with this, we observed an increase of IL-8 (after 48 h) and VEGF-α (after 4 h) secretion in stressed ARPE-19 cells (Fig. 6F, G). This correlation between complement components, *foxp3* expression and pro-angiogenic reaction in RPE cells needs to be further investigated.

228 [As a side note: IL-17, IFN γ , IL-18, IL-2 and TNF- α were not detected in the apical and basal supernatant
 229 of 4 h, 24 h and 48 h untreated and H₂O₂ treated ARPE-19 cells (data not shown).]

230

231 **Olaparib boosted the pro-inflammatory response of ARPE-19 cells to oxidative stimuli**

232 Oxidative stress-induced cellular reactions were previously ameliorated by an approved anti-cancer
 233 drug olaparib, which is an inhibitor of the poly(ADP-ribose) polymerase (PARP) [40–42]. We
 234 investigated the effect of olaparib on H₂O₂-dependent mRNA expression changes of complement
 235 receptors, components and inflammation-related transcripts (**Fig. 7, Sup. Fig. 4**). Oxidative stress
 236 increased expression of *cd11b*, *c5ar1* and *nlrp3* after 4 h of H₂O₂ treatment, this was further enhanced
 237 by olaparib-treatment (**Fig. 7A, B, C**). An increase of *properdin* and *ctsb* transcripts was observed after
 238 24 h following oxidative stress alone (**Fig. 3A, 5A**). A combination of H₂O₂ and olaparib accelerated this
 239 reaction with a significant increase of *properdin* and *ctsb* mRNA expression already after 4 h (**Fig. 7D,**
 240 **E**). The expression of *cfb* (**Sup. Fig. 2F**) was not modulated under oxidative stress, however H₂O₂ and
 241 olaparib together increased the *cfb* transcript level (**Fig. 7F**). Olaparib did not interfere with
 242 transcription of *foxp3* (**Fig. 7G**) and other transcripts (*c3*, *c4a*, *c5*, *cfb*, *cfh*, *cfi*, *c3ar*, *ctsl*) (**Sup. Fig. 4**) in
 243 ARPE-19 cells treated with H₂O₂.



244

245 **Fig. 7 Olaparib enhanced oxidative stress dependent expression changes in ARPE-19 cells.**

246 ARPE-19 cells were treated for 4 h with H₂O₂ and the effect of simultaneously added olaparib
247 on transcription was investigated. **(A)** *Cd11b*, **(C)** *nlrp3*, **(D)** *properdin* and **(E)** *ctsb* transcripts
248 were significantly increased in olaparib-treated, stressed cells compared to stressed cells
249 alone. **(B)** *C5aR1*, **(F)** *cfp* and **(G)** *foxp3* mRNA expression was not significantly changed in
250 stressed ARPE-19 cells following olaparib addition. Mean with standard deviation is shown, *
251 p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001 unpaired, two-tailed, parametric t-test,
252 dotted line depicted untreated control.
253

254 DISCUSSION

255 The RPE is exposed to high-energy light and it conducts phagocytosis of oxidized photoreceptor outer
256 segments, both is accompanied by a rapid release of reactive oxygen species [6,43,44]. Reactive oxygen
257 species, including H₂O₂, are on the one hand major cellular stressors [6,45] and, on the other hand,
258 cellular survival factors [3,46]. Antioxidants are decreased in light-exposed retinae, allowing the intra-
259 ocular accumulation of H₂O₂ [47]. We used H₂O₂ treatment to mimic physiological oxidative stress in
260 serum-free cultivated RPE cells to investigate the endogenous complement response in RPE cells
261 independent from external complement sources [48,49].

262 Oxidative stress increased the concentration of complement regulators CFH, properdin and of the
263 central complement protein C3 in RPE cells time-dependently without access to any extracellular
264 complement source. Previous studies reported mostly a reduced expression of *cfh* mRNA in RPE cells
265 exposed to oxidative stress [29–32], but these studies did not include further CFH protein analysis. Our
266 reported CFH protein accumulation after H₂O₂ treatment in polarized, monolayer ARPE-19 cells, using
267 immunohistochemistry, is in contrast to reduced CFH protein detection results in Western Blots of
268 non-*in vivo*-like cultivated ARPE-19 cells following H₂O₂ treatment [31].

269 However, it is known that intracellular CFH can enhance the cleavage of endogenously expressed C3
270 by a cathepsin L (CTSL)-mediated mechanism [50]. The lysosomal protease CTSL and the central
271 complement protein C3 concentrations were both enhanced under oxidative stress conditions in ARPE-
272 19 cells. Previous studies of RPE cell-derived complement components only focused on *c3* mRNA
273 expression, which was not changed under low H₂O₂ concentrations [51]. We went a step further and
274 showed that C3 was retained in the RPE cells and less secreted following oxidative stress. This RPE cell
275 dependent local accumulation of C3 was also shown for ARPE-19 cells treated with cigarette smoke
276 [28]. If C3 is activated in the blood, this is inhibited by CFH and promoted by complement regulator
277 properdin. We showed for the first time, that oxidative stress increased *properdin* mRNA expression
278 in ARPE-19 cells. This resulted in a higher properdin protein concentration in these cells, which could
279 promote cellular C3 cleavage. In summary, our data described a local production of complement
280 proteins in RPE cells and an enhanced cellular storage of complement proteins in the cells after H₂O₂
281 treatment. This cellular accumulation suggested an autocrine, cellular function of complement
282 proteins in RPE cells following oxidative stress.

283 Our studies revealed a co-localization of accumulated, endogenous C3 with complement receptor 3
284 (CR3, CD11b/CD18) in ARPE-19 cells exposed to oxidative stress and an increase of CR3 after 4 h. CR3
285 expression had been associated with inflammasome activation as a reaction to complement
286 components or/and oxidative stress in white blood and RPE cells [52,53]. In agreement with this, the
287 addition of H₂O₂ to ARPE-19 cells increased a time-dependent expression of *nlrp3* and *il-1β* mRNA and
288 subsequently enhanced the secretion of pro-inflammatory cytokines IL-1β and IL-6, which indicated an

289 enhanced inflammasome activity. Inflammasome activation depends on reactive oxygen species and
290 has been associated with lipid peroxidation end products and phototoxicity in RPE cells [54,55].
291 Involvement of the complement components in this oxidative stress response of RPE cells had been
292 only described in relation to extracellular added anaphylatoxins so far [25], but endogenous
293 complement of RPE cells hasn't been suggested as potential priming factors for the inflammasome. On
294 the one hand, we detected activated C3 cleavage products in ARPE-19 cells and previous studies
295 showed that activated C3a can be intracellularly generated in RPE cells independent from the systemic
296 canonical complement system [18–21]. On the other hand, C3 receptors are expressed and regulated
297 under oxidative stress in ARPE-19 cells indicating a role of endogenous complement components in
298 stressed ARPE-19 cells. Cellular C3 is cleaved by lysosomal CTSL [15,50] and NLRP3-inflammasome
299 activation depended on this CTSL activity [55]. Previously, CTSL inhibition reduced inflammasome
300 activity in ARPE-19 cells exposed to oxidative stress [56], showing the interaction of cell-specific
301 complement component cleavage and inflammasome activity. It is already known, that endogenous
302 C3-driven complement activation was required for the IL-1 β and IL-6, as well as inflammasome
303 activation in immune cells [57]. Our data suggest now, that this could be also a autocrine mechanism
304 in RPE cells.

305 Additionally to C3, C5 has been identified as a key player in cell homeostasis [25]. The *c5aR1* receptor
306 is expressed in RPE cells [58,59] and was increased during oxidative stress. *C5* mRNA expression was
307 not changed, as the expression of *c3* mRNA. However, the biologically highly active C5a fragment, a
308 ligand for C5aR1 has a half-life of approximately 1 min [60,61], due to rapid receptor binding. This rapid
309 signalling might have interfered with our detection scheduled. C5aR1 stimulation is associated with IL-
310 8 and VEGF-a secretion in ARPE-19 cells [58,59]. Increased secretion of these pro-angiogenic factors
311 was also observed following the H₂O₂ stimuli, but the signalling pathway is not exactly known so far. In
312 regulatory T-cells the transcription factor FOXP3 promotes the expression of IL-8 [23] and in bladder
313 cancer cells a knock-down of *foxp3* resulted in a reduced expression of *vegf* [24]. *Foxp3* mRNA was
314 expressed in ARPE-19 cells and increased under oxidative stress conditions. Previous studies showed,
315 that extracellular C5a can activate FOXP3 in ARPE-19 cells, which was associated with increased IL-8
316 secretion [26]. We showed that this can be also due to endogenous activation of C5aR1 following
317 oxidative stress in RPE cells.

318 These changes in expression and cellular complement protein accumulation following oxidative stress
319 were time-dependent (**Sup. Fig. 5**). The first changes of complement receptor (CR3, C5aR1) and
320 component (CFH, C3) levels in the RPE cells occurred after 4 h and were accompanied with changes in
321 *nlrp3* and *foxp3* mRNA expression. Downstream alterations in properdin expression, intracellular
322 proteases and an increase of epithelial–mesenchymal transition marker as well as loss of tight-

323 junctions were described. This indicates that complement receptor signalling could be involved in early
324 response of RPE cells to H₂O₂ treatment.

325 Oxidative stress-related cell damage of ARPE-19 cells and retinal degeneration in mouse models for
326 RPE degeneration as well as hereditary retinal degeneration were successfully ameliorated using
327 olaparib in previous studies [40–42]. Olaparib is a clinically developed poly-ADP-ribose-polymerase
328 inhibitor developed for cancer treatment by blocking the DNA-repair mechanism. ARPE-19 cells were
329 resistant to H₂O₂ induced mitochondrial dysfunction and to energy failure, when olaparib was added
330 [40]. We ask the question if olaparib can also normalize complement-associated pro-inflammatory
331 expression profiles in H₂O₂-treated cells. Surprisingly, olaparib accelerated the effect of oxidative stress
332 in RPE cells and enhanced the expression of complement receptors, complement components and the
333 *nlrp3* mRNA. This shows that endogenous complement-related, pro-inflammatory response of ARPE-
334 19 cells could be correlated with defective DNA repair mechanisms.

335

336 **CONCLUSION**

337 Oxidative stress and activation of the complement system cause retinal degeneration, but the
338 mechanism behind this is still a matter of investigation. We showed for the first time, that oxidative
339 stress can increase endogenous RPE cell complement components and receptors and that the process
340 was associated with release of pro-inflammatory and pro-angiogenic factors. Our data offer a stepping
341 stone for numerous further investigations regarding the function of a cell-associated complement
342 system in the RPE. Many questions were raised during this project: How are the complement
343 components activated? What is (are) the signalling pathway(s) of the complement receptors
344 independent from external complement sources? How are inflammasome regulation and FOXP3
345 activity modulated by endogenous complement components in RPE cells? Can endogenous
346 complement factors be targeted to affect cell-associated signalling pathways? These new perspectives
347 will hopefully help to decipher the function of intracellular complement components in retinal health
348 and disease and offer new strategies for treatment of retinal degeneration.

349 **METHODS**

350 **Cell culture and treatment**

351 Human ARPE-19 cells (passage 39; American Type Culture Collection) were cultivated for 6 days in cell
352 culture flasks with DMEM/F12 (Sigma-Aldrich) and 10% fetal calf serum (FCS; PanBiotech) and 1%
353 penicillin/ streptomycin (37°C, 5% CO₂). Cells were trypsinized (0.05% trypsin/ 0.02% EDTA) and seeded
354 in a concentration of 1.6×10^5 cells/cm² (passage 39) on mouse laminin (5 µg/cm², Sigma-Aldrich)
355 coated 0.4 µm pore polyester membrane inserts (Corning). Cells were cultivated for 4 weeks with
356 apical and basal media exchanges (first day medium with 10% FCS, remaining time medium with 5%
357 FCS were used). Before treatment FCS concentration was reduced within 3 days from 5% to 0%. ARPE-
358 19 cells were treated either with 0.5 mM H₂O₂ for 1, 4, 24 and 48 h, or 0.5 mM H₂O₂ and 0.01 mM
359 Olaparib (Biomol, Hamburg, DE) for 4 h.

360 **Immunohistochemistry and TUNEL assay**

361 PBS (Sigma-Aldrich) washed, paraformaldehyde (4%, 20 min; Merck) fixated ARPE-19 cells were
362 permeabilized (PBS/ 0.2% Tween20 (PBS-T), 45 min) and unspecific bindings were blocked (3% BSA
363 (Carl Roth)/PBS-T, 1 h). Antigens were detected using primary antibody (**Sup. Table 1**, overnight,
364 3% BSA/PBS-T) and fluorescence-conjugated anti-species antibody (**Sup. Table 1**, 45 min,
365 3% BSA/PBS). HOECHST 33342 (1:1000) stained DNA. Cells were covered with fluorescenting mounting
366 medium (Dako, Agilent). Images were taken with a confocal microscope (Zeiss).

367 The TUNEL assay was performed with DeadEnd™ Fluorometric TUNEL System (Promega) on
368 paraformaldehyde fixated, washed and permeabilized (0.2% Triton X-100 in PBS) cells. Images were
369 taken with confocal microscope a by Zeiss.

370 **RT-qPCR**

371 mRNA was isolated using the NucleoSpin® RNA/Protein kit (Macherey-Nagel). Purified mRNA was
372 transcribed into cDNA with the QuantiTect® Reverse Transcription Kit (Qiagen). Transcripts of
373 complement components, receptors and inflammation-associated markers were analyzed using the
374 Rotor-Gene SYBR® Green PCR Kit either with QuantiTect Primer Assays (**Sup. Table 2**), or in-house
375 designed primer pairs (Metabion) described in **Sup. Table 3** in the Rotor Gene Q 2plex cyclers (Qiagen).

376 **Western Blot**

377 Proteins were purified using RIPA buffer (Sigma-Aldrich) with protease and phosphatase inhibitors
378 (1:100, Sigma-Aldrich). Samples were dissolved in reducing Laemmli sample buffer and denatured
379 (95 °C, 10 min). Samples were separated in a 12% SDS-PAGE and transferred on to an activated
380 polyvinylidene difluoride membrane using a wet blotting system. Membranes were blocked (1 h, 5%

381 BSA/PBS-T) and incubated with the primary antibody (**Sup. Table 1**, overnight, 5% BSA/PBS-T).
382 Peroxidase-conjugated anti-species antibodies were used for detection (**Sup. Table 1**, 1 h, PBS-T).
383 WesternSure PREMIUM Chemiluminescent Substrate (LI-COR) visualized the antigen in the Alpha
384 Innotech Fluor Chem FC2 Imaging System.

385 **Multiplex-Immunoassays**

386 Cytokine concentration of basal and apical supernatants of treated and untreated ARPE-19 cells were
387 determined according to the protocol of a custom ProcartaPlex® multiplex immunoassay kit
388 (ThermoFisher). Complement components in the cellular supernatant were quantified using the
389 MILLIPLEX MAP Human Complement Panel (Merck). The read out of the multiplex assay was
390 performed in a Magpix instrument (Luminex).

391 **Statistics**

392 Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software Inc.).

393

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- 584

585 **DATA AVAILABILITY**

586 Original data supporting the findings of this study are available from the corresponding author upon
587 reasonable request.

588

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592

593 **AUTHOR CONTRIBUTIONS**

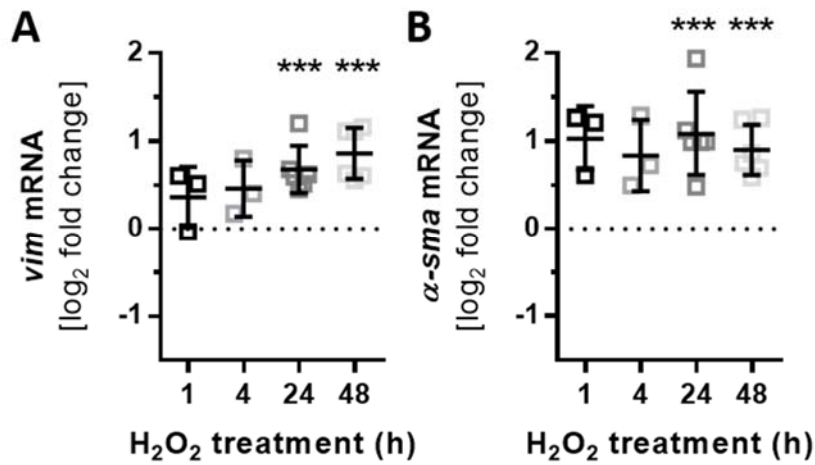
594 TT, NS, VE and DP designed research; TT, NS, MR, KK, VE and DP performed research; TT, NS, MR, KK,
595 VE and DP analysed and interpreted the data; TT, NS, VE and DP wrote the manuscript. All authors
596 provided input to edit the manuscript.

597

598 **COMPETING INTERESTS**

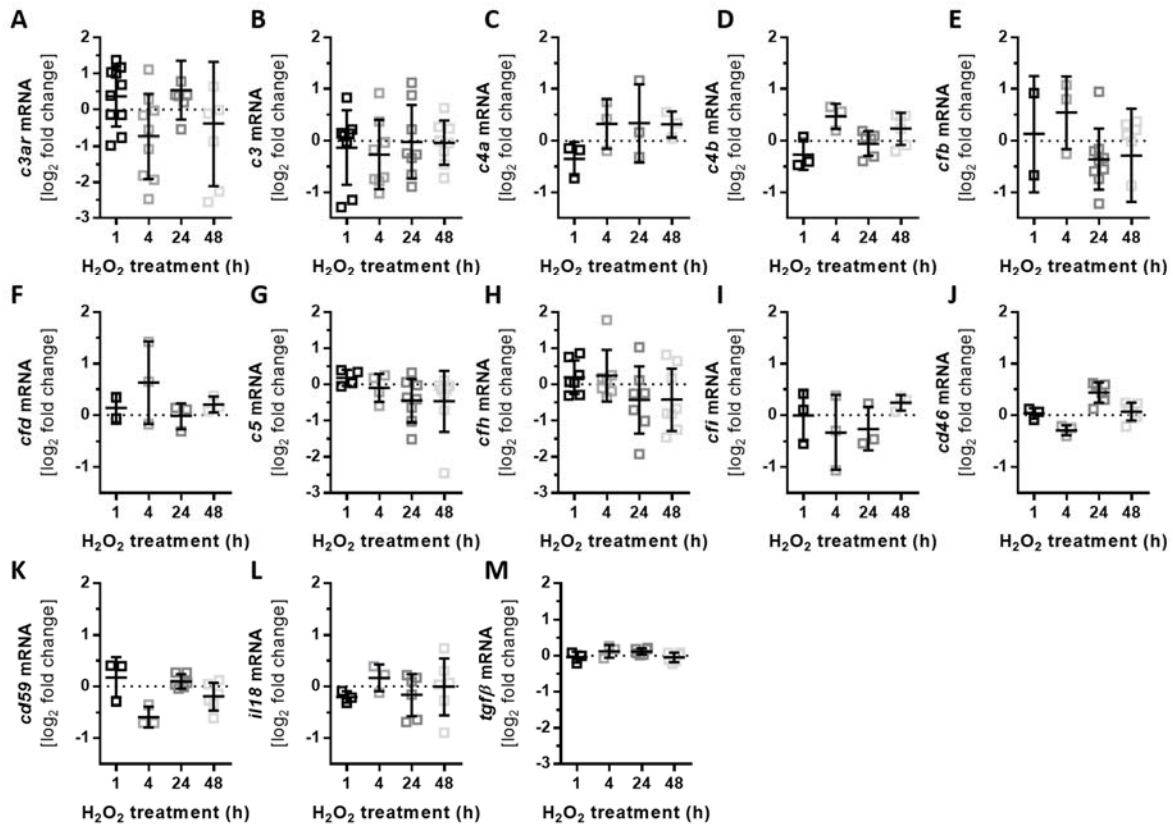
599 The authors declare no competing interests.

600 SUPPLEMENT



601
602 **Sup. Fig. 1** H₂O₂ treatment increased expression of epithelial-mesenchymal transition markers
603 in ARPE-19 cells.
604 ARPE-19 cells were treated either for 1, 4, 24 or 48 h with H₂O₂. (A) *vim* and (B) *α-sma*
605 transcription was significantly increased after 24 h compared to the untreated control.
606 Mean with standard deviation is shown, *** p < 0.001, unpaired, two-tailed,
607 parametric t-test, dotted line depicts untreated control.

608

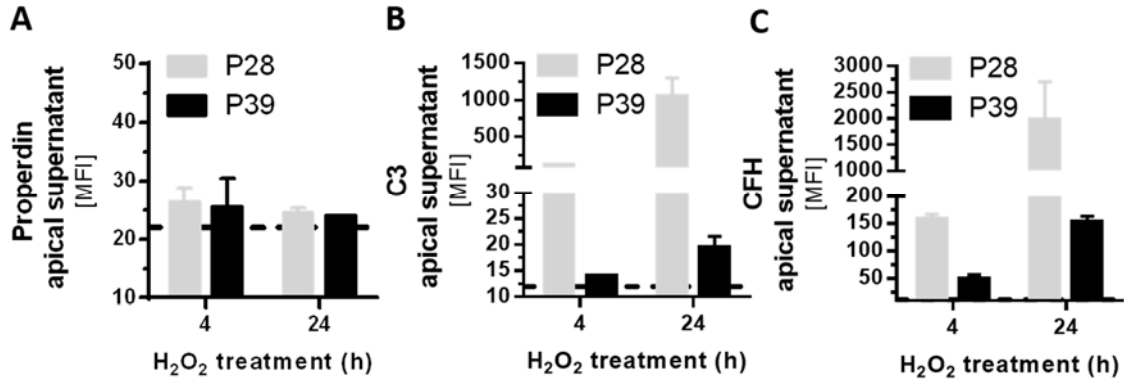


609

610 **Sup. Fig. 2** H₂O₂ treatment did not influence the transcription levels of several genes in ARPE-
611 19 cells.

612 ARPE-19 cells were treated either for 1, 4, 24 or 48 h with H₂O₂. mRNA levels were not
613 significantly changed for: (A) *c3ar*, (B) *c3*, (C) *c4a*, (D) *c4b*, (E) *cfb*, (F) *cfd*, (G) *c5*, (H)
614 *cfh*, (I) *cfi*, (J) *cd46*, (K) *cd59*, (L) *il18* and (M) *tgfb*. Mean with standard deviation,
615 dotted line untreated control.

616

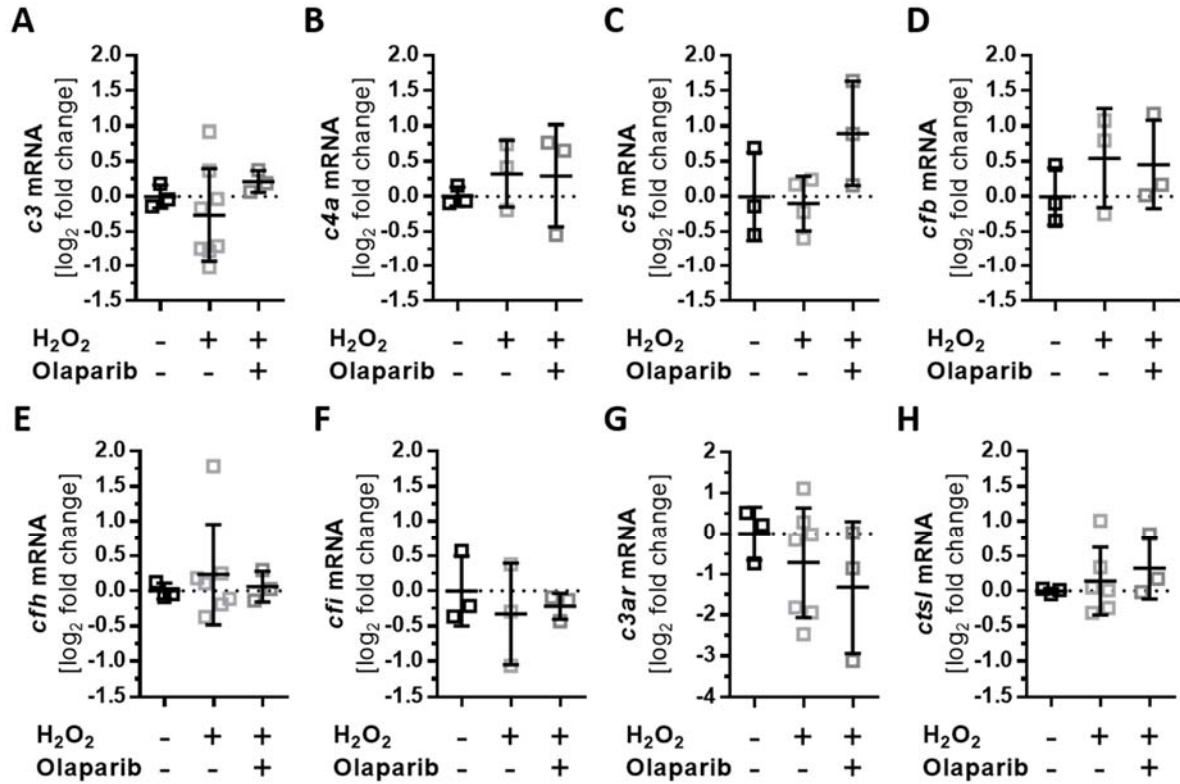


617

618 **Sup. Fig. 3** **Increased complement component secretion in lower passage number of ARPE-19**
619 **cells compared to higher passage number.**

620 ARPE-19 cells with passages 28 or 39 (latter used in the rest of this study) were treated
621 either for 4 or 24 h with H₂O₂. The protein concentration of **(A)** properdin, **(B)** C3 and
622 **(C)** CFH was determined in the apical supernatant using a multiplex immunoassay. **(A)**
623 Properdin was not secreted by ARPE-19 cells of varied passages. ARPE-19 cells with
624 lower passage number secreted more **(B)** C3 and **(C)** CFH than ARPE-19 cell with higher
625 passage number into the apical supernatant.

626

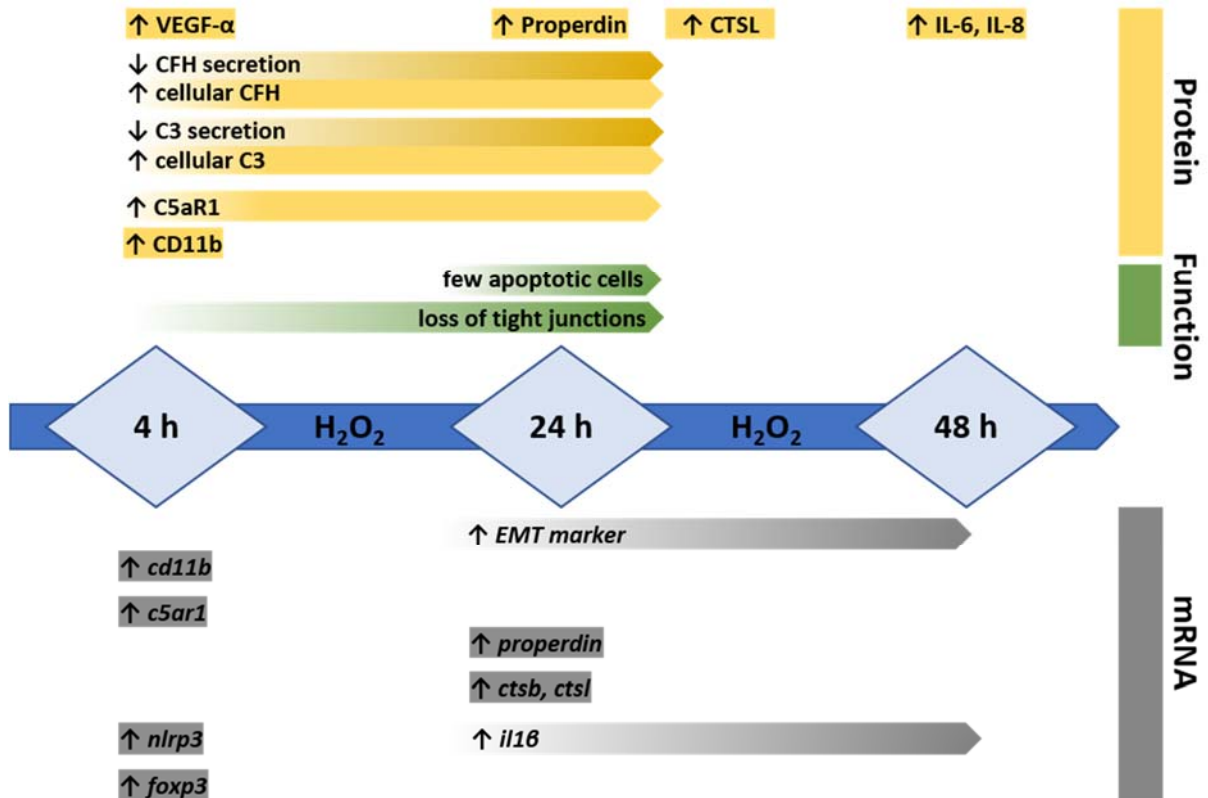


627

628 **Sup. Fig. 4** **Stable expression of complement components and related genes after Olaparib and**
 629 **oxidative stress treatment in ARPE-19 cells.**

630 ARPE-19 cells were treated for 4 h with H₂O₂ and the effect of simultaneously added
 631 olaparib on transcription was investigated. (A) *c3*, (B) *c4a*, (C) *c5*, (D) *cfb*, (E) *cfh*, (F)
 632 *cfi*, (G) *c3aR* and (H) *ctsl* did not significantly change in stressed ARPE-19 cells following
 633 olaparib addition.

634



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636

Sup. Fig. 5 Time-dependent changes of H₂O₂ treatment in ARPE-19 cells.

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ARPE-19 cells were treated for 4, 24 and 48 h with H₂O₂. Changes in mRNA expression (grey), function (green) and on protein level (yellow), which are described in this manuscript, are summarized in this scheme.

638

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641 **Sup. Table 1: Primary and secondary antibodies**

Primary antibody	Species	Company	Catalogue number	Dilution
anti-ZO-1	rabbit	ThermoFisher	61-7300	IS 1: 300
anti-CD11b	goat	Biorbyt	orb19554	IS 1:500
anti-C5aR1	mouse	Hycult	HM2094	IS 1:100, WB 1:1000
Anti-GAPDH-HRP	rabbit	Cell signaling technology	3683	
anti-Propertdin	goat	Complement Technology	A239	IS 1: 250
anti-C3	goat	Bio Rad	AHP1752	IS 1:250
anti-C3	rabbit	Abcam	Ab181147	WB 1: 1000
anti-CFH	goat	Quidel	A312	IS 1:250
anti-CTSL	mouse	Abcam	ab6314	IS 1:500
Secondary antibody				
anti-mouse Ig-HRP	goat	Dianova	115-035-003	WB 1:5000
anti-rabbit Ig-HRP	goat	Dianova	111-035-003	WB 1:5000
anti-goat Ig-HRP	rabbit	Dianova	305-035-003	WB 1:5000
Anti-goat IgG Cy3	donkey	Dianova	705-165-147	IS 1:500
anti-Mouse IgG (H+L) Alexa Fluor 488	donkey	ThermoFisher	AB_2534069	IS 1:500

642 WB – Western blot, IS – Immunostaining

643

644 **Sup. Table 2: QuantiTec PrimerAssays**

mRNA transcript	name	Catalogue number
<i>gapdh</i>	Hs_GAPDH_1_SG	QT00079247
<i>c3</i>	Hs_C3_1_SG	QT00089698
<i>c3ar</i>	Hs_C3AR1_1_SG	QT00090398
<i>cd11b</i>	Hs_ITGAM_1_SG	QT00031500
<i>c4a</i>	Hs_C4A_1_SG	QT00237160
<i>c4b</i>	Hs_C4B_1_SG	QT00237167
<i>c5</i>	Hs_C5_1_SG	QT00088011
<i>c5ar1</i>	Hs_C5R1_1_SG	QT00997766
<i>cd46</i>	Hs_MCP_1_SG	QT00073689
<i>cd59</i>	Hs_CD59_1_SG	QT00035952
<i>cathepsin b</i>	Hs_CTSB_1_SG	QT00088641
<i>cathepsin l</i>	Hs_CTSL_1_SG	QT01664978
<i>complement factor b</i>	Hs_BF_1_SG	QT00012138
<i>complement factor d</i>	Hs_CFD_1_SG	QT00212191
<i>complement Factor h</i>	Hs_CFH_1_SG	QT00001624
<i>complement Factor i</i>	Hs_CFI_1_SG	QT00213794
<i>complement Factor p</i>	Hs_CFP_1_SG	QT00010514
<i>nlrp3</i>	Hs_NLRP3_1_SG	QT00029771
<i>forkhead-box-protein P3</i>	Hs_FOXP3_1_SG	QT00048286

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647 **Sup. Table 3: In-house designed RT-qPCR primers**

mRNA transcript	sequence
<i>il1β</i>	fw: CTCGCCAGTGAAATGATGGCT rv: GTCGGAGATTCGTAGCTGGAT
<i>il18</i>	fw: ACTGTAGAGATAATGCACCCCG rv: AGTTACAGCCATACCTCTAGGC
<i>tgfbβ</i>	fw: CATAGCTGACTTCAAGATGTGGT rv: CCTAGTGAGACTTTGAACCGT
<i>vim</i>	fw: TGTCCAATCGATGTGGATGTTTC rv: TTGTACCATTCTTCTGCCTCCTG
<i>α-sma</i>	fw: GCCTGGTGTGTGACAATGG rv: AAAACAGCCCTGGGAGCAT

648 fw - forward primer, rv - reverse primer

649