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1 **Overexpression of STARD3 attenuates oxidized LDL-induced oxidative stress and inflammation in**
2 **retinal pigment epithelial cells**

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27 **Abstract**

28 Age-related macular degeneration (AMD) is the most common cause of visual disorder in aged
29 people and may lead to complete blindness with ageing. The major clinical feature of AMD is the
30 presence of cholesterol enriched deposits underneath the retinal pigment epithelium (RPE) cells.
31 The deposits can induce oxidative stress and inflammation. It has been suggested that abnormal
32 cholesterol homeostasis contributes to the pathogenesis of AMD. However, the functional role of
33 defective cholesterol homeostasis in AMD remains elusive. STARD proteins are a family of proteins
34 that contain a steroidogenic acute regulatory protein-related lipid transfer domain. There are fifteen
35 STARD proteins in mammals and some, such as STARD3, are responsible for cholesterol trafficking.
36 Previously there was no study of STARD proteins in retinal cholesterol metabolism and trafficking.
37 Here we examined expression of the *Stard3* gene in mouse retinal and RPE cells at ages of 2 and 20
38 months. We found that expression of *Stard 3* gene transcripts in both mouse RPE and retina was
39 significantly decreased at age of 20 months when compared to that of age 2 months old. We created
40 a stable ARPE-19 cell line overexpressing STARD3 and found this resulted in increased cholesterol
41 efflux, reduced accumulation of intracellular oxidized LDL, increased antioxidant capacity and lower
42 levels of inflammatory cytokines. The data suggested that STARD3 is a potential target for AMD
43 through promoting the removal of intracellular cholesterol and slowing the disease progression.

44 **Key words** Age-related macular degeneration, STARD3, retinal pigment epithelial cells, cholesterol
45 efflux, oxidized low density lipoprotein

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53 **1. Introduction**

54 Age-related macular degeneration (AMD) is characterized by severe visual impairment of the central
55 retina and a leading cause of blindness among aged people worldwide. It is predicted that the
56 number of AMD patients will increase from 196 million by 2020 to 288 million by 2040 due to
57 population ageing (Wong et al., 2014). AMD is a chronic retinal disease and can progress to
58 neovascular (wet) AMD and geographic atrophy (dry AMD) at advanced stage. Wet AMD has new
59 vessels grown from the choroid into the sub-retinal space, leading to the leakage of fluid, lipid or
60 blood into the sub-retinal space. Clinically, wet AMD can present as haemorrhage, lipid exudates,
61 pigment epithelial detachment or sub-retinal fluid (Bolin and Kang, 2012). Dry AMD is characterised
62 as drusen formation (yellow-white spots) beneath the retinal pigment epithelium (RPE), Bruch's
63 membrane thickening and abnormal architecture, and lipofuscin accumulation in the RPE; abnormal
64 deposits are also formed in the subretinal space, called reticular pseudodrusen (Bolin and Kang,
65 2012). Analyses of AMD patient eye samples have shown both cholesterol and lipoprotein are
66 accumulated in drusen, reticular pseudodrusen and Bruch's membrane, suggesting there is possibly
67 a defect in cholesterol transport in photoreceptor/RPE/Bruch's membrane (Pikuleva and Curcio,
68 2014). It is supposed surplus cholesterol in peripheral tissue cells, e.g. the RPE, moves back to the
69 liver via reverse cholesterol transport (RCT) (Ikonen, 2006). The components of the RCT pathway
70 have been shown to express in human and mouse RPE and in a human RPE cell line, ARPE-19
71 (Duncan et al., 2009; Biswas et al., 2017; Storti et al., 2017; Zheng et al., 2012); while active
72 cholesterol efflux is shown to present in human and mouse primary RPE and in ARPE-19 cells (Biswas
73 et al., 2017; Ishida et al., 2006; Storti et al., 2017). Genome-wide association studies have
74 demonstrated that genes involved in the RCT are link to AMD development (Burgess and Smith,
75 2017; DeAngelis et al., 2017; Yuan et al., 2018). Recently Storti et al. (2019) reported mice with RPE-
76 specific knockout of the cholesterol efflux gene, *Abca1*, displayed AMD-like retinal phenotypes,
77 including lipid accumulation in the RPE, increased inflammation, RPE and photoreceptor
78 degeneration, and decreased visual function. These findings suggested the RCT pathway may play a

79 critical role in the pathogenesis of AMD. Therefore, promotion of the removal of RPE cholesterol
80 may be an effective therapeutic strategy for the treatment of early AMD patients.

81 The steroidogenic acute regulatory proteins (StAR) belong to a family of proteins in mammals
82 that are classified to fifteen various members, STARD1-STARD15 and are divided into six subfamilies.
83 All members consist of the StAR-related lipid transfer (START) domain and are believed to play major
84 roles in the transport and metabolism of lipids (Alpy and Tomasetto, 2005). The STARD1 subfamily
85 contains cholesterol-specific binding proteins: STARD1 and STARD3. STARD1 is a mitochondrial
86 protein and regulates steroidogenesis by delivering cholesterol from the mitochondrial outer
87 membrane to the mitochondrial inner membrane, where cholesterol is converted to pregnenolone
88 by cytochrome P450 enzymes (Clark and Stocco, 1995). STARD3 is located to the membrane of late
89 endosomes and is responsible for cholesterol transport from late endosomes to the endoplasmic
90 reticulum (ER) and mitochondria (Charman et al., 2010; Wilhelm et al., 2017). Depletion of STARD3
91 reduced cholesterol transported from late endosomes to mitochondria and decreased production of
92 CYP11A1-catalysed pregnenolone in Chinese hamster ovary cells (Charman et al., 2010). Knockdown
93 of STARD3 also affects actin-dependent late endosome dynamics and cholesterol transport (Hölttä-
94 Vuori et al., 2005). Overexpression of STARD3 in breast cancer cells resulted in decreased total free
95 cholesterol and alteration of cellular cholesterol distribution (Vassilev et al., 2015). Overexpression
96 of STARD3 also caused higher production of mitochondrial superoxide, decreased mitochondrial
97 membrane potential and ATP synthesis, and mitochondrial fragmentation in a human liver cancer
98 cell line (HepG2) (Balboa et al., 2017). In addition, overexpression of STARD3 also enhances
99 cholesterol efflux in human macrophages and rat hepatoma cells but not in rat insulinoma cells,
100 suggesting STARD3 plays different roles in different organs (Borthwick et al., 2010; Pinto and
101 Graham, 2016; Soffientini et al., 2014). STARD 3 is the only member of STARD family, which is
102 enriched in macula and which binds Lutein (Li et al., 2011). High levels of Lutein in sera or in ocular
103 tissues are associated with a reduced risk for AMD (Feng et al., 2019). However, the function of
104 STARD3 in RPE cells has been not investigated.

105 In the current study, we examined STARD expression in mouse retina and established a STARD3
106 overexpressing cell line. We found STARD3 expression in the retina was significantly decreased in
107 aged mice, overexpression of STARD3 in ARPE-19 cells enhanced cholesterol efflux, decreased
108 uptake and accumulation of oxidized low density lipoprotein (oxLDL) and suppressed oxLDL-induced
109 oxidative stress and inflammation.

110 **2 Materials and methods**

111 **2.1 Set-up of a STARD3-overexpressing stable cell line**

112 ARPE-19 cells were seeded at a density of 1×10^5 cells per well in a 24-well plate with 0.5ml
113 DMEM/F12 media and incubated overnight in an incubator at 37 °C with 5 % CO₂. 500 ng FLAG-
114 tagged STARD3 plasmid with pCMV6-Entry vector backbone (Cat. RC228357, OriGene Technologies,
115 Inc., Rockville, USA), or pCMV6-Entry vector (Cat. PS100001, OriGene Technologies, Inc., Rockville,
116 USA) were transfected into ARPE-19 cells using Lipofectamine-2000 (Cat. 11668- 027, Life technology)
117 following the manufacturer's protocol. After incubation at 37°C for 48 hours, the media were
118 removed. Media with G418 (1mg/ml) were added to kill non-resistant cells without integration of
119 transfected plasmids. Media with G418 were replaced every 3 days. After 21 days, cells were
120 colonised. These colonised cells were trypsinized and transferred into 6-well plates. Overexpression
121 of STARD3 in individual colonies was verified by Western blotting with anti-FLAG antibody.

122 **2.2 Treatment of oxidized low density lipoprotein (oxLDL) in ARPE-19 cells**

123 ARPE-19 cells were seeded in 12-well plates and treated with oxLDL (200µg/ml) or fluorescence
124 labelled oxLDL (Dil oxLDL, 10µg/ml) for 4 or 24 hours. Both oxLDL and Dil-oxLDL were purchased
125 from Alfa Aesar (Cat. J65591 and J64164, respectively). Dil oxLDL treated cells were washed with PBS
126 for three times, fixed with 4% Paraformaldehyde for 10 min at room temperature and then mounted
127 with DAPI. The intracellular Dil OxLDL was visualized under a confocal microscope and quantified
128 using the Image J software. oxLDL treated cells were collected and subject to biochemical analysis.

129 **2.3 Measurement of cholesterol efflux**

130 ARPE-19 cells were seeded on 12 well plates and labelled with [3H] cholesterol for 24 hours with 2%
131 BSA in serum free culture media. Efflux was initiated by the addition of serum-free DMEM/ F12
132 consisting human apoAI (10 g/ml), apoE (10 mg/ml), HDL (20 mg/ml) or human serum (1%, v/v) for
133 24 hours. The media were collected and centrifuged for 10 minutes at 12,000 rpm to remove
134 residual dead cells. 200µl of media were transferred into scintillation counter and radioactivities
135 were counted. The cholesterol efflux was then calculated according to our previous description
136 (Biswas et al., 2017).

137 **2.4 Measurement of reactive oxygen species (ROS)**

138 ROS production in control or STARD3 overexpressing cells exposed with or without oxLDL for 4 hours
139 and 24 hours was detected using DCFH-DA Assay Kit (Cat. D6883, Sigma, UK) following the
140 manufacture's guidance. The relative ROS level was represented as (fluorescence of treated cells/
141 fluorescence of untreated cells)×100%.

142 **2.5 Quantitative real-time polymerase chain reaction (qRT-PCR)**

143 All animal work was approved by the Animal Welfare and Ethical Review Body, University of
144 Strathclyde (Project licence P8C815DC9). Retinas and RPE/choroid were collected from four male
145 mice at each age point (2, 6, 12 and 20 months old). The samples of each age group were pooled
146 together and homogenized. RNA was extracted using Tri Reagent (Cat. T9424, Sigma, UK) according
147 to the manufacture's protocol. cDNA synthesis was performed using a High-Capacity cDNA Reverse
148 Transcription Kit (Cat. 436814, Applied Biosystems, UK). The quantification of targeted expression
149 was detected by qRT-PCR assay using a Platinum® SYBR® Green QPCR SuperMix-UDG w/ROX kit (Cat.
150 11744100, Invitrogen, UK). The sequences for primers used for qRT-PCR were listed in Table S1.

151 **2.6 Western blotting**

152 Cells and mouse tissues (retinas and RPE/choroid pooled from 4 male mice at age of 2 or 20 months)
153 were lysed using lysis buffer and concentration of extracted protein was quantified using a Bradford
154 assay. 50 µg of individual samples were separated by SDS-PAGE and transferred to nitrocellulose
155 membrane. The membrane was blocked with 5% non-fat dried milk in Tris-buffered saline, 0.1%

156 Tween 20 (TBST), incubated with primary and secondary antibodies. Signals of targeted proteins
157 were detected using the LICOR Odyssey FC Imaging System. The densities of targeting protein bands
158 were measured by the Odyssey Fc Imager with Image Studio™ Software
159 (<https://www.licor.com/bio/odyssey-fc/>). The intensity of target protein was normalized to the
160 intensity of GAPDH to determine relative protein intensity.

161 2.7 Immunostaining

162 Cells were seeded on sterilized round coverslips in a 12-well plate. These cells were fixed with 100%
163 methanol at -20°C for 5 minutes, washed 3 times with PBS and blocked with PBS containing 2%
164 bovine serum albumin (BSA) for 30 minutes at room temperature. Cells were then incubated with
165 anti-STARD3 antibody (Cat. ab3478, Abcam, 1:200 dilution) or anti-FLAG antibody (Cat. F1804, Sigma,
166 1:200 dilution) overnight at 4°C. Further, cells were washed with PBS for three times (5
167 minutes/each) and blocked with 2% sheep serum in 2% BSA/PBS for 30 minutes at room
168 temperature. In addition, cells were incubated with secondary antibody (Alexa Fluor® Plus 488 goat
169 anti-mouse IgG, Cat. A32723, 1:500 dilution or Alexa Fluor® Plus 488 goat anti-rabbit IgG, Cat.
170 A32731, 1:500 dilution, Thermo Fisher Scientific) for 45 minutes at room temperature in dark. Cells
171 were then washed 5 times with PBS and mounted using DAPI. Images were captured under confocal
172 microscope.

173 Cryosections of eyes from 3 months old male mice were blocked by 2% BSA/PBS for 30 minutes
174 at room temperature and incubated with anti-STARD3 antibody and anti-rhodopsin antibody (Cat.
175 Ab98887, 1:200 dilution, Abcam) overnight at 4°C, follow by the procedures described above.

176 2.8 Oil red O staining

177 Cells were seeded on cover slips in 12-well plates and exposed to oxLDL for 4 and 24 hours. Cells
178 were incubated with Propylene Glycol for 2 minutes then with Oil Red O solution (Cat. ab150678,
179 Abcam) for 6 minutes. Further cells were exposed to 85% Propylene Glycol for 1 minute and rinsed
180 with distilled water for 2 minutes twice. Finally, cells were incubated in haematoxylin for 2 minutes

181 and further rinsed with distilled water for three times. Intracellular stained lipids were imaged using
182 an EVOS M5000 microscope.

183 **2.9 Biochemical assays**

184 Measurement of superoxide dismutase (SOD) and catalase (CAT) activity and quantification of
185 glutathione (GSH) and malondialdehyde (MDA) were carried out using the OxiSelect Superoxide
186 Dismutase Activity Assay Kit (Cat. STA-340), the catalase (CAT) Activity Assay kit (Cat. STA-341), the
187 total glutathione (GSSG/GSH) assay kit (Cat. STA-312) and the total OxiSelect™ TBARS Assay Kit (Cat.
188 STA-312), respectively, purchased from Cell Biolabs UK, according to the manufacture's guidance.

189 **2.10 Enzyme-linked immunosorbent assay (ELISA)**

190 Media from control and STARD3 over-expressing cells treated with or without oxLDL (200µg/ml)
191 were collected. Secreted IL-1 and TNF-α in the media were detected using Human IL-1β Mini TMB
192 ELISA Development Kit (Cat. 900-TM95) and Human TNF-α Mini TMB ELISA Development Kit (Cat.
193 900-M25), respectively, according to the manufacture's protocols.

194 **2.11 Statistical analysis**

195 Data were analysed using unpaired t-test or one-way ANOVA followed by Bonferroni multiple
196 comparison test using GraphPad Prism version 7 software ([https://www.graphpad.com/scientific-](https://www.graphpad.com/scientific-software/prism/)
197 [software/prism/](https://www.graphpad.com/scientific-software/prism/)) and presented as mean ±SEM. A minimum of three independent experiments in
198 triplicate were performed for statistical analysis. P value less than 0.05 was considered significant.
199 *p<0.05, **p<0.01 and ***p<0.001.

200 **3 Results**

201 **3.1 Expression of STARD3 in mouse retinas**

202 Previous studies demonstrated STARD3 protein was expressed in human retinas and RPE/choroid
203 and mouse RPE/choroid but not mouse retina (Li et al., 2011). We also examined the expression of
204 STARD3 in mouse retinas using immunostaining with anti-rhodopsin and anti-STARD antibodies and
205 found that STARD3 was predominantly localized to outer and inner segments of photoreceptors,
206 overlapping with rhodopsin staining. STARD3 were also localized to outer and inner plexiform layers

207 and ganglion cells. Weak signals were also detected in RPE (Figure 1A). We also examined expression
208 of *Stard3* gene transcripts in mouse retina and RPE/choroid at age of 2, 6, 12 and 20 months by qRT-
209 PCR. *Stard3* mRNA levels in mouse retina and RPE/choroid was decreased with ageing, though there
210 was no significantly difference in mice at 6 month old compared to that of mice at age of two
211 months; there was significantly decreased expression in mouse retina and RPE/choroid at age of 12
212 and 20 months (Figure 1B). STARD3 protein level in the retina and RPE/choroid of mice at age of 2
213 and 20 months was also examined by Western blotting. We found that STARD3 protein was
214 markedly lower in aged mouse retinas (Figure 1C). However, STARD3 protein was undetectable in
215 the RPE by Western blotting (data not shown), possibly due to its expression at low level.

216 **3.2 Overexpression of STARD3 in ARPE-19 cells**

217 ARPE-19 cells were transfected with empty vector (pCMV6) and STARD3 plasmid (pCMV6-STARD3)
218 and stable cell lines were selected using G418 screening. Human STARD3 was fused with a FLAG tag
219 at the C terminal. To confirm overexpression of STARD3 protein, we used Western blotting to
220 examine STARD3 protein level using anti-STARD3 and anti-Flag tag antibodies. The anti-STARD3
221 antibody detected both native STARD3 and FLAG-tagged STARD3; the anti-FLAG antibody only
222 detected overexpressed STARD3. STARD3 was expressed in control cells; however, the stable cell
223 line transfected with pCMV6-STARD3 had higher STARD3 expression (Figure 2). Overexpression of
224 STARD3 protein was also detected by immunostaining using anti-STARD3 and anti-FLAG antibodies.
225 Stronger fluorescent signals were detected with anti-STARD3 antibody in the stable cell line which
226 contained native and overexpressed STARD3 when compared to control cells containing native
227 STARD3 protein only (Figure S1).

228 **3.3 Overexpression of STARD3 increased cholesterol efflux in ARPE-19 cells**

229 Overexpression of STARD3 in macrophages enhanced cholesterol efflux and upregulated ABCA1
230 expression (Borthwick et al., 2010). We also examined whether overexpression of STARD3 affected
231 cholesterol efflux in ARPE-19 cells. Under basal condition, there was no significant difference for
232 cholesterol efflux between STARD3-overexpressing and control cells. In the presence of HLD and

233 human serum (HS), there is significantly increased cholesterol efflux in STARD3-overexpressing cells
234 when compared to control cells ($p < 0.0001$). However, cholesterol efflux to ApoA1 was not
235 significantly changed in STARD3-overexpressing cells (Figure 3).

236 Our early work showed the mitochondrial translocator protein, TSPO, mediated cholesterol efflux
237 in RPE cells (Biswas et al., 2017). Though STARD3 is responsible for transporting cholesterol from
238 endosome to mitochondria, its relation with TSPO has not been investigated. We measured TSPO
239 and STARD3 expression by qRT-PCR and Western blotting and found that STARD3-overexpressing
240 cells had significantly increased TSPO expression at mRNA and protein level compared to that of
241 control cells (Figures 4A, S2A). In TSPO knockout cells, whose cholesterol efflux is defective (Biswas
242 et al., 2017), expression of STARD3 was notably lower than that of control cells (Figures 4B, S2B).

243 **3.4 Overexpression of STARD3 decreased the uptake and accumulation of cholesterol**

244 Since overexpression of STARD3 promoted cholesterol efflux, we examined whether STARD3
245 overexpression affects oxLDL uptake and accumulation. Both control and STARD3-overexpressing
246 cells were exposed to fluorescence labelled oxLDL (Dil oxLDL) for 4 hours (uptake) or 24 hours
247 (accumulation). Images were taken under confocal microscopy and fluorescence signals were
248 quantified using Image J software. Results showed uptake of oxLDL was significantly lower in
249 STARD3-overexpressing cells when compared to control cells; similarly, intracellular accumulation of
250 oxLDL was markedly reduced in STARD3-overexpressed cells when compared to control cells (Figure
251 5).

252 Oil Red O staining is a commonly used method to detect neutral lipid and lipid droplets. Control
253 and STARD3-overexpressed cells were fed with oxLDL for 4 and 24 hours then stained with oil Red
254 O. Intracellular lipid accumulation was significantly reduced in STARD3-overexpressed cells with
255 oxLDL treatment for 4 and 24 hours when compared to control cells (Figure 6).

256 **3.5 Overexpression of STARD3 reduced production of reactive oxygen species (ROS) and increased** 257 **antioxidant capacity**

258 OxLDL exposure is known to induce ROS production (Biswas et al., 2017). We also examined ROS
259 production in control and STARD3-overexpressed cells with or without exposure to oxLDL. ROS
260 production is significantly increased in control cells treated with oxLDL for 4 and 24 hours when
261 compared to control cells without treatment. However, the STARD3 overexpressed cells treated with
262 oxLDL for 4 and 24 hours had significantly lower levels of ROS when compared to control cells
263 receiving similar treatment (Figure 7A).

264 OxLDL can induce oxidative stress and reduce antioxidant capacity. Control and STARD3-
265 overexpressed cells were treated with oxLDL for 24 hours. Superoxide dismutase (SOD) activity was
266 significantly increased by 129% in STARD3-overexpressed cells when compared to control cells with
267 the same treatment; similarly, the activity of catalase (CAT) was significantly raised by 245% when
268 compared to control cells (Figure 7B).

269 Glutathione (GSH) is an important antioxidant, which protects cells from reactive oxygen species
270 (ROS) caused damage. GSH levels in STARD3-overexpressed cells were significantly increased 860%
271 when compared to control cells (Figure 7B). Malondialdehyde (MDA) is a biomarker for lipid
272 peroxidation. MDA was markedly decreased by 63% in STARD3-overexpressed cells when compared
273 to control cells (Figure 7B).

274 **3.6 Overexpression of STARD3 decreased secretion of inflammatory cytokines**

275 We also examined expression of inflammatory cytokines (IL-1 β and TNF- α) in control and STARD3-
276 overexpressing cells. Both control and STARD3-overexpressing cells without oxLDL treatment had
277 similar levels of IL-1 β at 4 and 24 hours. However, IL-1 β secretion was significantly reduced in
278 STARD3-overexpressing cells when compared to control cells incubated with oxLDL for 4 and 24
279 hours (Figure 8A). The level of TNF- α was not different between control and STARD3-overexpressing
280 cells without oxLDL feeding for 4 and 24 hours. TNF α was only significantly decreased in STARD3-
281 overexpressed cells when compared to control cells exposed to oxLDL for 24 hours (Figure 8B).

282 **4. Discussion**

283 STARD1 and STARD3 belong to the same subfamily of STARD proteins with 37% identity and 50%
284 similarity of amino acids in the START domain (Alpy and Tomasetto, 2005). STARD1 transfers
285 cholesterol from the endoplasmic reticulum (ER) to mitochondria and STARD3 transfers cholesterol
286 from late endosomes to mitochondria. STARD1 associates with voltage-dependent anion channel
287 (VDAC), TSPO, acyl coenzyme A-binding domain containing 3 (ACBD3) and the regulatory subunit
288 R1of PKA (PKA α) to form a multiprotein complex which is in charge of cholesterol trafficking (Liu et
289 al., 2006). Elustondo et al postulate that STARDs also forms a multiprotein complex with VDAC and
290 TSPO to mediate the transport of cholesterol from late endosomes to mitochondria (Elustondo et al.,
291 2017). STARD3 interacts with vesicle-associated membrane protein-associated protein (VAP) family
292 members (VAP-A, VAP-B and MOSPD3, motile sperm domain-containing protein2), facilitating the
293 movement of cholesterol from endosomes to ER, where STARD1 delivers cholesterol to
294 mitochondria (Alp et al., 2013; Mattia et al., 2020; Wilhelm et al., 2017). TSPO is responsible for
295 transporting cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial
296 membrane (IMM), where cholesterol is catalysed to oxysterols by CYP27A1 in non-steroidogenic
297 cells (Rone et al., 2009). Oxysterols activate the liver X receptor (LXR) pathway and promote
298 cholesterol efflux. Overexpression of STARD1 or STARD3 has been shown to enhance cholesterol
299 efflux in macrophages (Borthwick et al., 2010; Taylor et al., 2010). In the current study we also
300 demonstrated that overexpression of STARD3 resulted in a significant increase in cholesterol efflux
301 to HDL and human serum (HS) (Figure 3). TSPO controls the import of STARD1 to IMM and mediates
302 STARD1's activity (Rone et al., 2009). However, it is unclear whether TSPO cooperates with STARD3.
303 Here we found a possible functional relationship between TSPO and STARD3 in RPE cells, since
304 overexpression of STARD3 upregulated TSPO expression and loss of TSPO decreased STARD3
305 expression (Figure 4). Since overexpression of STARD1 or STARD3 upregulated cholesterol trafficking
306 gene expression and facilitated lipid biosynthesis in non-retinal cells (Borthwick et al., 2010; Taylor
307 et al., 2010), it is worth investigating whether overexpression of STARD3 has an impact on
308 expression of cholesterol trafficking and metabolism genes in RPE cells.

309 Defects in cholesterol efflux by loss of TSPO increased uptake and accumulation of oxLDL in RPE
310 cells (Biswas et al., 2017). Enhanced cholesterol efflux by TSPO ligands reduced oxLDL uptake and
311 accumulation in choroidal endothelial cells (Biswas et al., 2018). Here we also found overexpression
312 of STARD3 decreased uptake and accumulation of oxLDL in RPE cells and lowered intracellular
313 neutral lipids (Figure 5). These results are compatible with a previous study that demonstrated a
314 STARD3 (Δ START-STARD3) mutant leads to increased cholesterol accumulation in CHO cells and
315 limits late endosomal trafficking (Zhang et al., 2002). Another study reported that the level of sterol
316 ester storage was increased in Stard3 mutant mice fed a high fat diet (Kishida et al., 2004). OxLDL
317 has been localized to the drusen and is supposed to induce oxidative stress, contributing to the
318 progression of AMD (Yamada et al., 2008). Our previous work has shown that accumulation of oxLDL
319 induces ROS production and decreases intracellular oxLDL resulting in less ROS production and
320 elevated antioxidant capacity (Biswas et al., 2017, 2020). STARD3 overexpression significantly
321 reduced ROS and MDA production, and increased SOD and CAT activity and the level of GSH in RPE
322 cells exposed to oxLDL.

323 Raised ROS production by intracellular ox-LDL activates the NF- κ B pathway and elevates the
324 production of pro-inflammatory cytokines such as IL-1 β and TNF α and IL-1 β (Cominacini et al., 2000;
325 Rodríguez and Larrayoz, 2010). OxLDL also mediates activation of the NLRP3 inflammasome,
326 resulting in release of proinflammatory cytokines, IL-1 β and IL-18 (Baldrighi et al., 2017). Early work
327 demonstrated the oxLDL activated NLRP3 inflammasome in human primary fetal RPE and ARPE-19
328 cells (Gnanaguru et al., 2016). Our recent work showed oxLDL activated the NF- κ B pathway (Biswas
329 et al., 2020). OxLDL has been shown to induce secretion of pro-inflammatory cytokines in RPE cells
330 (AnandBabu et al., 2019; Biswas et al., 2017; 2020). Our data showed that IL-1 β and TNF- α levels
331 were significantly decreased in STARD3 overexpressed cells when compared to control cells exposed
332 to oxLDL. This is compatible with other studies which demonstrated oxidative stress increased
333 inflammatory gene expression, including IL-1 β , IL-8, TNF- α , VCAM-1 and INF- γ , and caused retinal
334 disorders (Semeraro et al., 2015).

335 Cholesterol plays an important structural and metabolic role, which is required for cellular
336 function. Cholesterol is also a key component to produce steroid hormones. Cellular level of
337 cholesterol is well controlled, excess cholesterol in the RPE is removed via the reverse cholesterol
338 transport to the liver for excretion or storage. Abnormal cholesterol homeostasis causes different
339 types of diseases including AMD and atherosclerosis (Pikuleva and Curcio, 2014). Cholesterol is
340 enriched in sub-RPE deposits of AMD patients and the Bruch's membrane also has enriched
341 cholesterol, suggesting defects in reverse cholesterol transport. When aging, the Bruch membrane
342 and the RPE cells become atrophied and degenerated, leading slowly to severe visual loss (Frankel
343 and Sowka, 2011). Components of drusen, including cholesterol, have been shown to induce
344 oxidative stress and inflammation. It will be a good therapeutic strategy to promote the removal of
345 intracellular cholesterol by trafficking back to the liver and reduce drusen formation, which will slow
346 disease progression in AMD patients. STARD3 overexpression enhanced cholesterol removal in RPE
347 cells suggests it may be a therapeutic target for AMD. Further studies in animal models to address
348 the functional roles of STARD3 are required.

349 In conclusion, overexpression of STARD3 enhanced cholesterol efflux, reduced intracellular
350 accumulation of cholesterol, suppressed ROS production, increased antioxidant capacity and
351 inhibited inflammation. Overexpression of STARD3 in RPE cells or development of ligands enhancing
352 STARD3 function may be an effective strategy to treat AMD patients.

353

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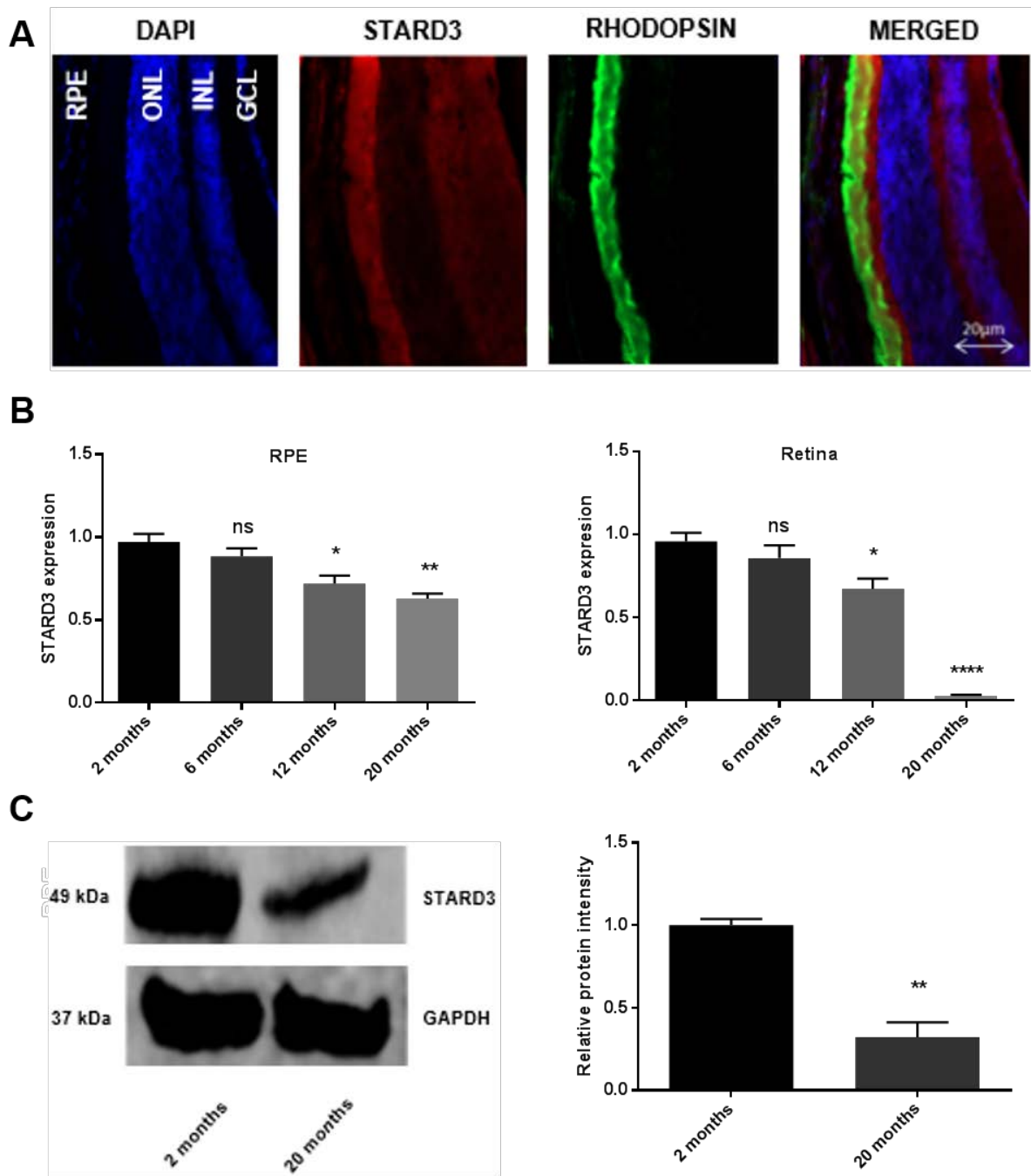
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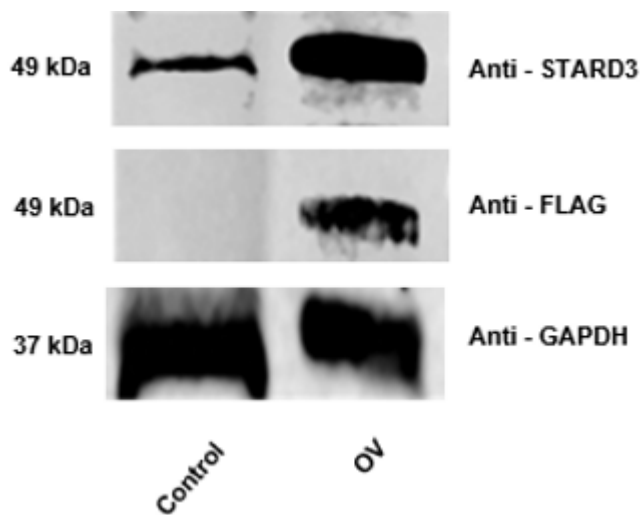
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490 **Figure 1** Stard3 expression in mouse retina and RPE. (A) Immunolocalization of STARD3 in mouse
 491 retina. Two-month old mouse eye sections were immune stained with an STARD3 antibody for
 492 STARD3 expression and DAPI for labelling nuclei. (B) Retinas and RPE/choroid were dissected from
 493 four male mice at age of 2, 6, 12 and 20 months. The samples at each age point were pooled
 494 together. RNAs were extracted and cDNAs were synthesized. mRNA levels of *Stard3* in the retina and

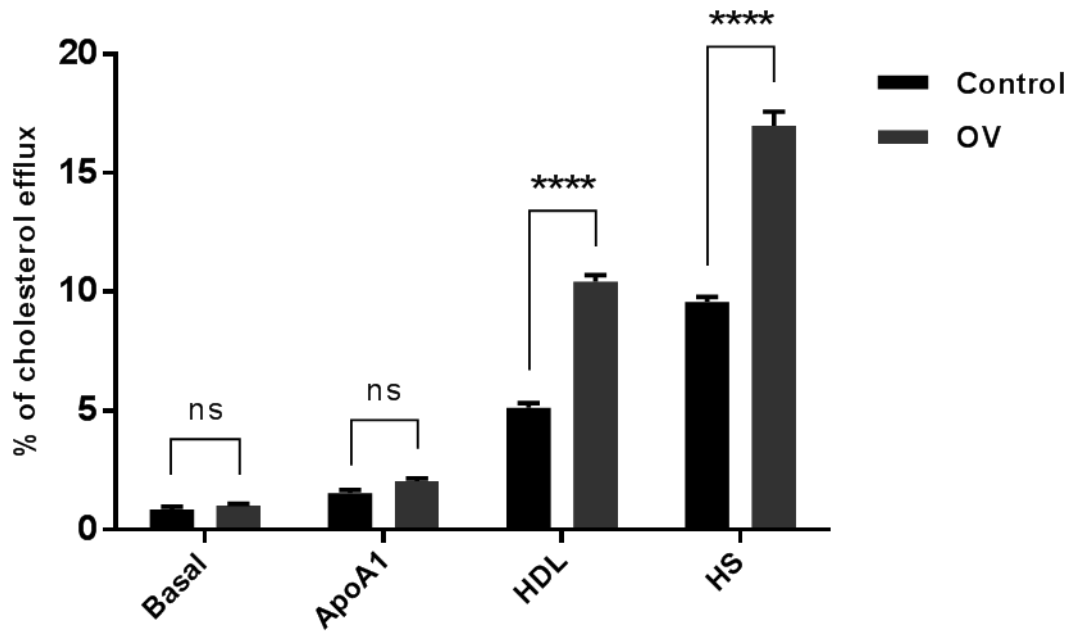
495 RPE/choroid were measured by qRT-PCR (n=4). Data was analysed by one-way ANOVA followed by
496 Bonferroni multiple comparison test. (C) Retinas were dissected from four male mice at age of 2 or
497 20 months and pooled together. Proteins were extracted from the retinal samples using Tissue
498 Extraction Buffer. STARD3 protein was detected by Western blotting and quantified by LI-COR Image
499 Studio Software. Data were analysed by unpaired t-test followed by Bonferoni post hoc test and
500 presented as mean \pm SEM. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer;
501 RPE, retinal pigment epithelial cells. ns, no significance; *P<0.05, **p<0.01, ****p<0.0001.
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504 **Figure 2** Overexpression of STARD3 protein was detected by Western blot with anti-STARD antibody
505 and anti-FLAG antibody respectively. OV, STARD3-overexpressing cells.

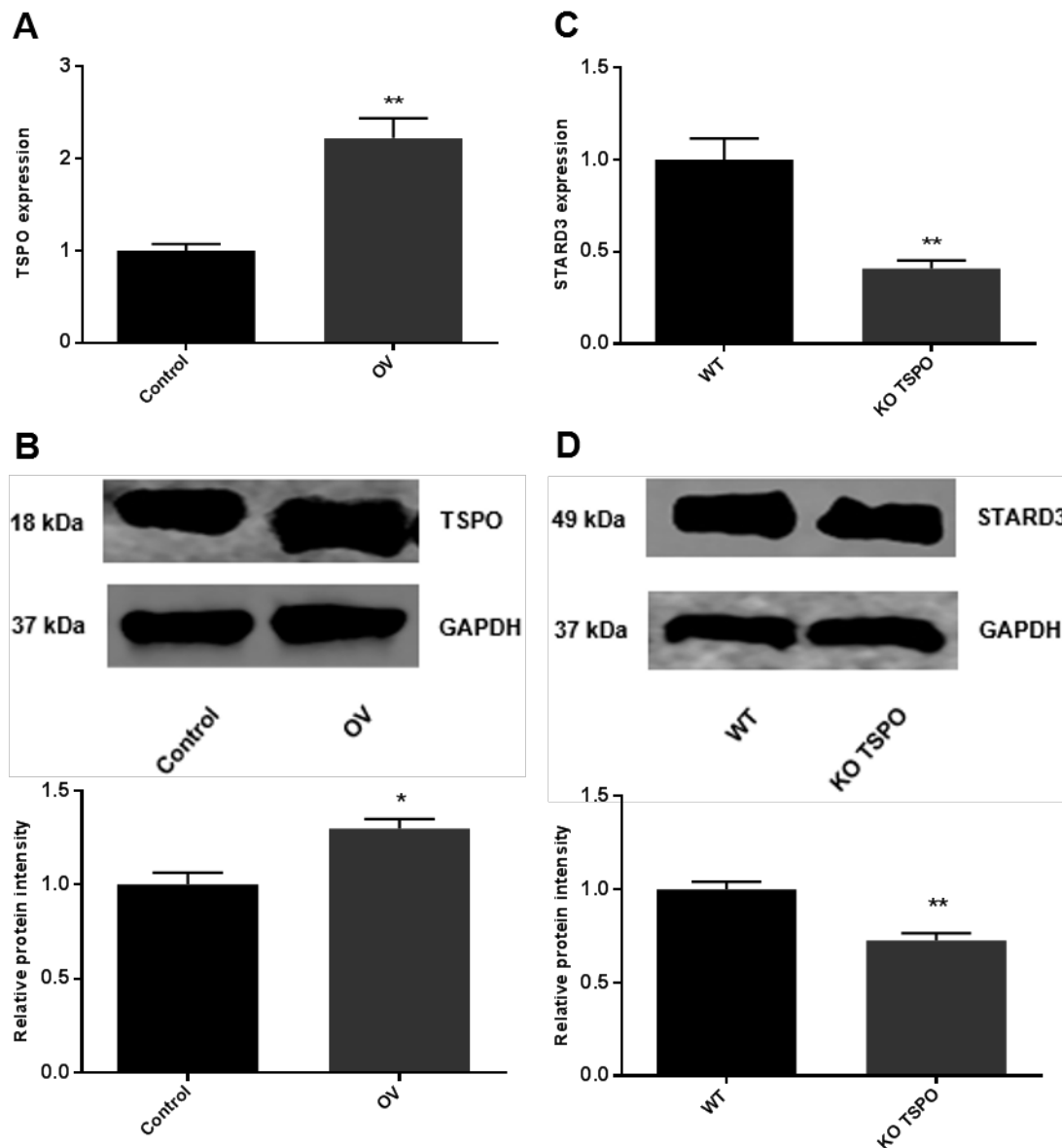
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508 **Figure 3** Comparison of cholesterol efflux to ApoA1, HDL and human serum (HS) between cells with
 509 overexpression of STARD3 (OV) and control cells (transfected with empty vector, pCMV6). Data were
 510 Data were analysed by unpaired t-test with Bonferoni post hoc test and presented as mean \pm SEM
 511 (n=5). Ns, no significance, ****P<0.0001.

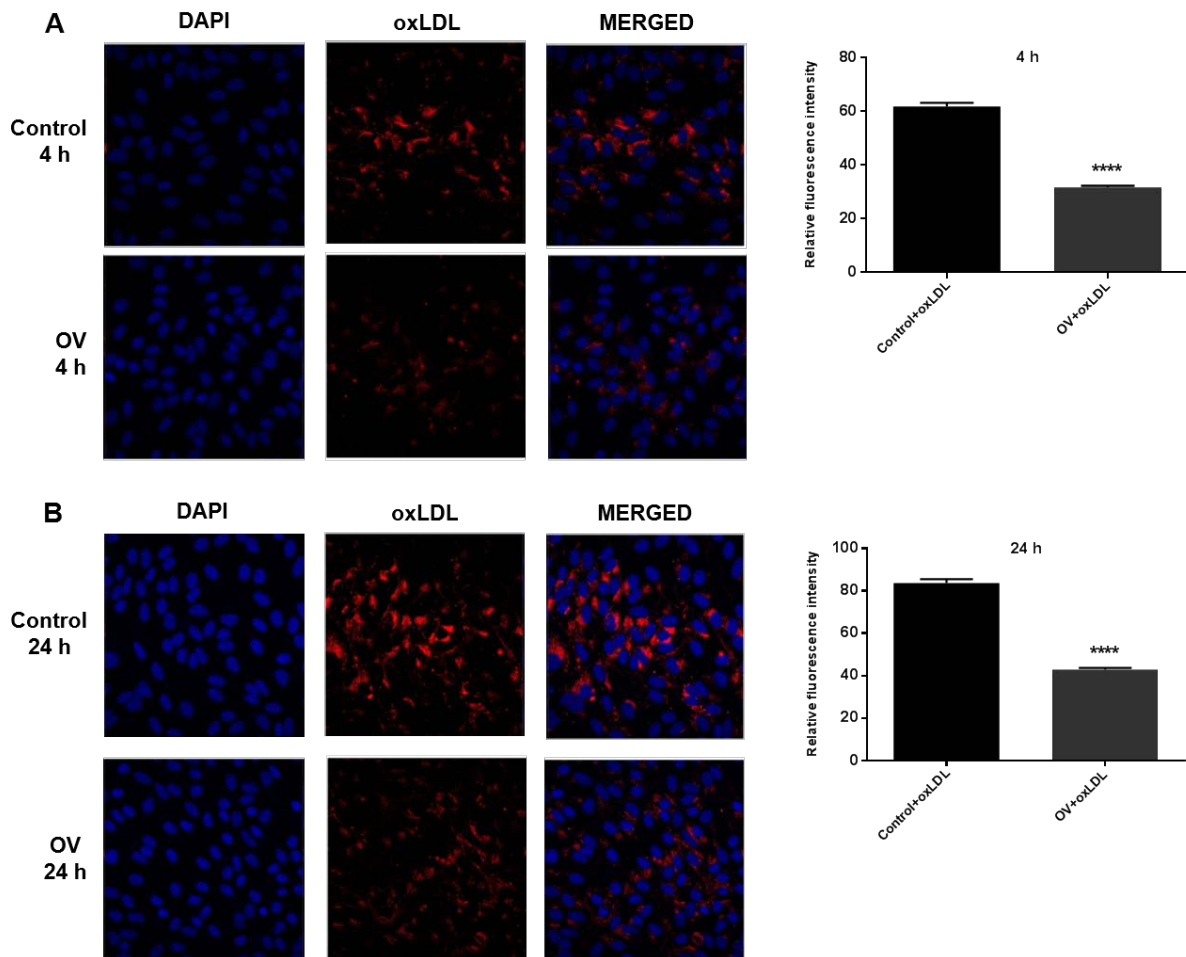
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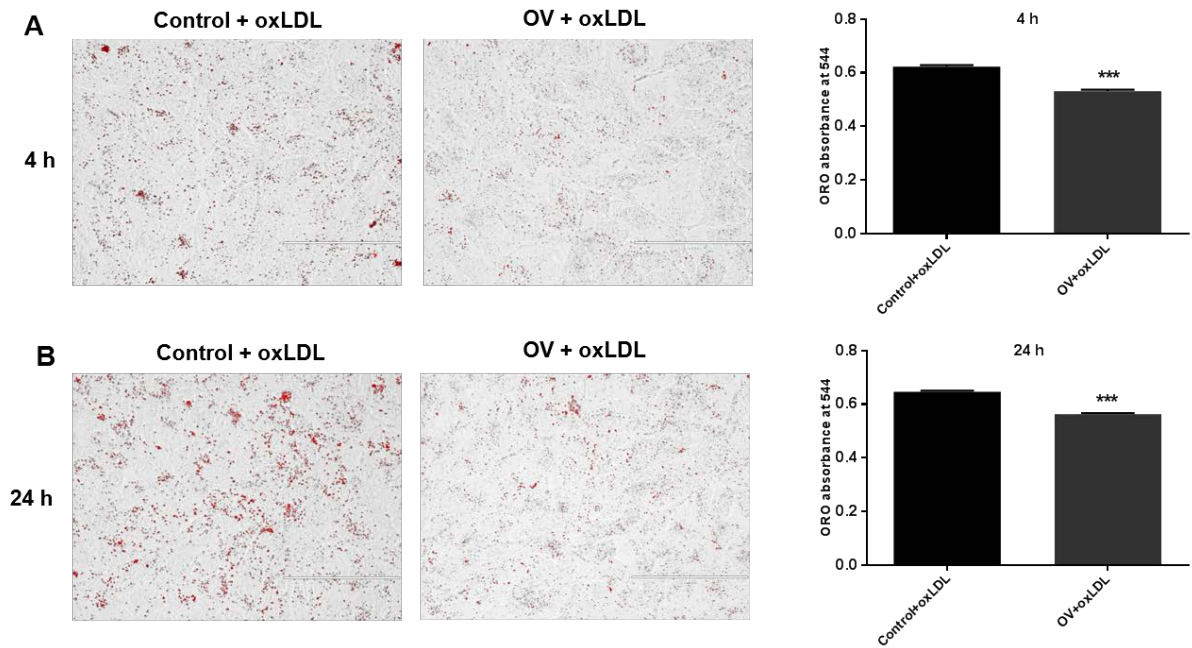
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514 **Figure 4** Overexpression of STARD3 upregulated TSPO expression. (A) TSPO mRNA in control and
 515 STARD3-overexpressing cells was detected by qRT-PCR. The data was analysed by unpaired t-test
 516 followed by Bonferroni post hoc test and presented as mean \pm SEM (n=4). (B) TSPO protein in control
 517 and STARD-overexpressing cells was detected by Western blotting and quantified using LI-COR
 518 Image Studio Software. The data was analysed by unpaired t-test followed by Bonferroni post hoc
 519 test and presented as mean \pm SEM (n=3). (C) STARD3 mRNA in wildtype and TSPO knockout cells.
 520 The data was analysed by unpaired t-test followed by Bonferroni post hoc test and presented as
 521 mean \pm SEM (n=4). (D) STARD3 protein in wildtype and TSPO knockout cells was examined by

522 Western blotting and quantified by LI-COR Image Studio Software. The data was analysed by
 523 unpaired t-test followed by Bonferoni post hoc test and presented as mean \pm SEM (n=3). OV:
 524 STARD3-overexpressing cells; KO TSPO, TSPO knockout cells. *p<0.05; **p<0.01.
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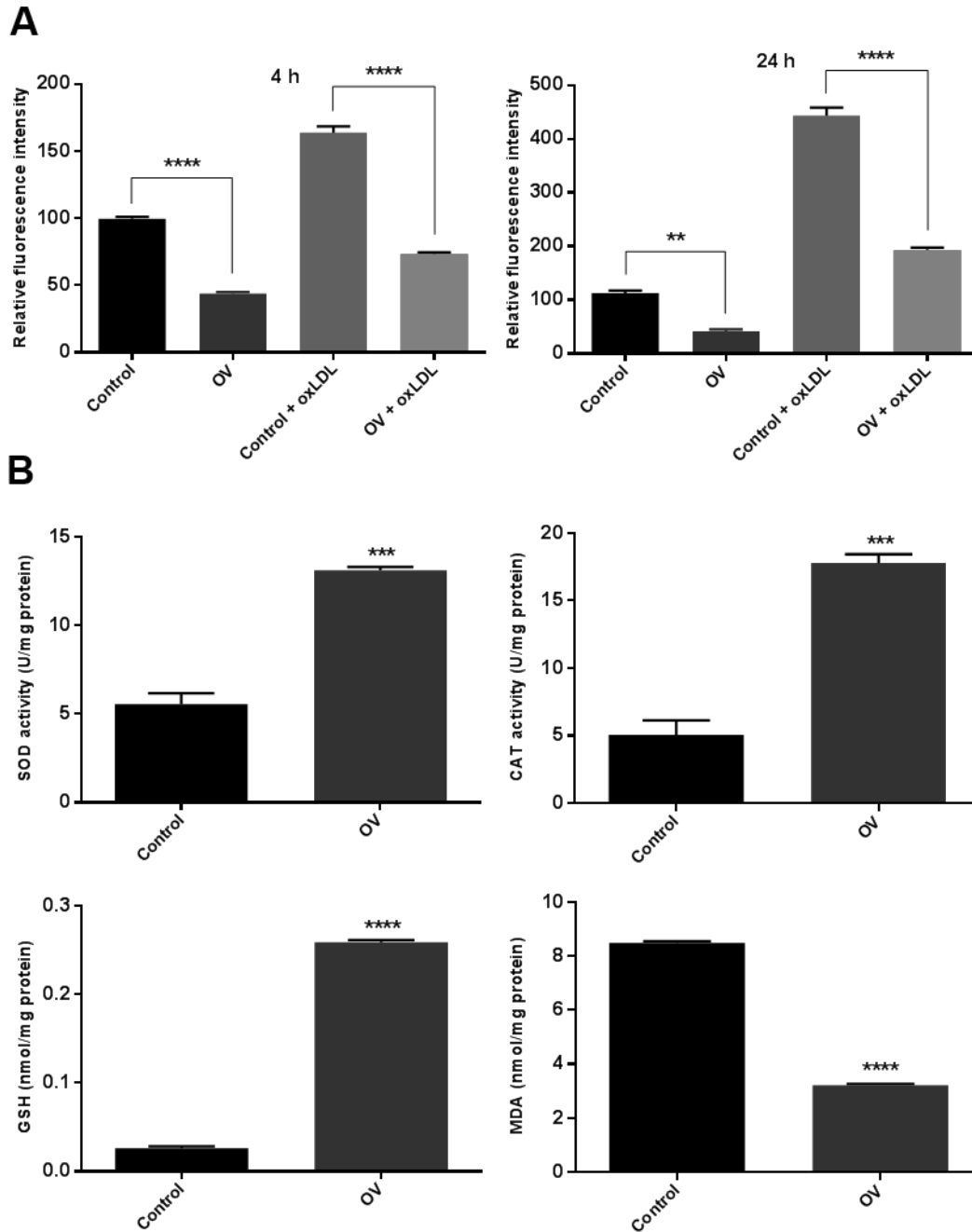
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 527 **Figure 5** Uptake (A) and accumulation (B) of oxLDL in control and STARD3-overexpressing cells
 528 exposed to Dil oxLDL for 4 hours and 24 hours, respectively. Images were taken under confocal
 529 microscopy and fluorescent signals of intracellular oxLDL were quantified using Image J software and
 530 calculated the corrected total cell fluorescence (CTCF = Integrated Density – (Area of selected cell X
 531 Mean fluorescence of background readings)). The data was analysed by unpaired t-test followed by
 532 Bonferoni test and presented as mean \pm SEM (n=5). OV, STARD3-verexpressing cells. Data were
 533 presented as mean \pm SEM. ****p<0.0001.
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536 **Figure 6** The oil Red O (ORO) staining showed intracellular lipid droplets in control and STARD3-
 537 overexpressing cells incubated with oxLDL for 4 hours (A) and 24 hours (B). Images were taken
 538 under light microscopy. After staining, lipid droplets were extracted with isopropanol and measured
 539 using spectrometry at the absorbance of 544 nm and the data was analysed by unpaired t-test
 540 followed by Bonferroni post hoc test and presented as mean \pm SEM (n=4). OV: STARD3
 541 overexpressing cells. Data were presented as mean \pm SEM. ***P<0.001.

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544 **Figure 7** (A) Production of reactive oxygen species (ROS) in control and STARD3-overexpressing cells

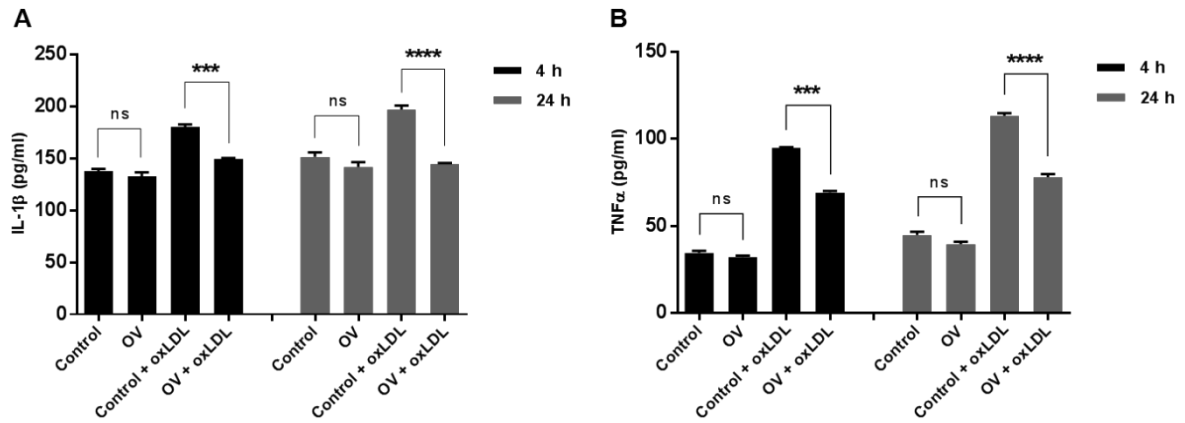
545 treated with/without oxLDL for 4 hours and 24 hours. (B) The effects of overexpressed STARD3 on

546 activities of superoxide dismutase (SOD) and catalase (CAT) and on production of glutathione (GSH)

547 and malonaldehyde (MDA) in control and STARD3-overexpressing cells (OV) fed with oxLDL for 24

548 hours. All the data were analysed by unpaired t-test followed by Bonferroni test and presented as

549 mean \pm SEM (n=5). **P<0.01; ***p<0.001; ****p<0.0001.



550

551 **Figure 8** Overexpressed STARD3 decreased production of proinflammatory cytokines in LDL-exposed

552 RPE cells. IL-1 β (A) and TNF α (B) in control and STARD3-overexpressing cells treated with/without

553 oxLDL for 4 hours and 24 hours were measured by ELISA. All the data were analysed by unpaired t-

554 test followed by Bonferroni post hoc test and presented as mean \pm SEM (n=5). OV: STARD3-

555 overexpressing cells. Ns, no significance; ****P

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