Transgenic Mice Over-Expressing RBP4 Have RBP4-Dependent and Light-Independent Retinal Degeneration

Mei Du,[1] Eric Phelps,[2] Michael J. Balangue,[2] Aaron Dockins,[2] Gennadiy Moiseyev,[1] Younghwa Shin,[1] Shelley Kane,[1] Laura Otalora,[1] Jian-Xing Ma,[1] Rafal Farjo,[2] and Krysten M. Farjo[1]

[1]Department of Physiology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, United States
[2]EyeCRO LLC, Oklahoma City, Oklahoma, United States

Correspondence: Krysten M. Farjo, University of Oklahoma Health Sciences Center, 940 Stanton L. Young Boulevard, BMSB 617, Oklahoma City, OK 73104, USA; krystenfarjo@gmail.com.

Submitted: April 24, 2017
Accepted: July 18, 2017

Citation: Du M, Phelps E, Balangue MJ, et al. Transgenic mice over-expressing RBP4 have RBP4-dependent and light-independent retinal degeneration. *Invest Ophthalmol Vis Sci*. 2017;58:4095–4103. DOI:10.1167/iovs.17-22107

Purpose. Transgenic mice overexpressing serum retinol-binding protein (*RBP4-Tg*) develop progressive retinal degeneration, characterized by microglia activation, yet the precise mechanisms underlying retinal degeneration are unclear. Previous studies showed *RBP4-Tg* mice have normal ocular retinoid levels, suggesting that degeneration is independent of the retinoid visual cycle or light exposure. The present study addresses whether retinal degeneration is light-dependent and RBP4-dependent by testing the effects of dark-rearing and pharmacological lowering of serum RBP4 levels, respectively.

Methods. *RBP4-Tg* mice reared on normal mouse chow in normal cyclic light conditions were directly compared to *RBP4-Tg* mice exposed to chow supplemented with the RBP4-lowering compound A1120 or dark-rearing conditions. Quantitative retinal histological analysis was conducted to assess retinal degeneration, and electroretinography (ERG) and optokinetic tracking (OKT) tests were performed to assess retinal and visual function. Ocular retinoids and bis-retinoid A2E were quantified.

Results. Dark-rearing *RBP4-Tg* mice effectively reduced ocular bis-retinoid A2E levels, but had no significant effect on retinal degeneration or dysfunction in *RBP4-Tg* mice, demonstrating that retinal degeneration is light-independent. A1120 treatment lowered serum RBP4 levels similar to wild-type mice, and prevented structural retinal degeneration. However, A1120 treatment did not prevent retinal dysfunction in *RBP4-Tg* mice. Moreover, *RBP4-Tg* mice on A1120 diet had significant worsening of OKT response and loss of cone photoreceptors compared to *RBP4-Tg* mice on normal chow. This may be related to the very significant reduction in retinyl ester levels in the retina of mice on A1120-supplemented diet.

Conclusions. Retinal degeneration in *RBP4-Tg* mice is RBP4-dependent and light-independent.

Keywords: serum retinol-binding protein, retinal degeneration, RBP4

Serum retinol-binding protein (RBP4) is the protein responsible for binding and transporting retinol (vitamin A) through the blood stream to target tissues.[1–4] RBP4 is especially important for delivering retinol to the retinal pigment epithelium,[5] where retinol is used in a biochemical pathway known as the "retinoid visual cycle" to generate the light-responsive visual chromophore.[6–9] RBP4-knockout mice (*RBP4-KO*) have reduced retinol uptake into the retina, resulting in reduced levels of visual chromophore and impaired vision, although vision can be restored to normal through administration of sufficient dietary vitamin A.[5] Thus, RBP4 supports optimal visual function, but is not completely essential for vision, since retinol can still get into the retina through RBP4-independent pathways.

The classical function of RBP4 in retinoid trafficking has been well characterized[1,5]; however, several clinical studies have found that elevated concentrations of RBP4 in serum is correlated with systemic disease, including obesity,[10,11] insulin resistance,[10–15] and type 2 diabetes.[11,15] Further studies have shown that high concentrations of RBP4 elicit retinol-independent proinflammatory activity, which contributes to adipose tissue inflammation and insulin resistance in mice.[16,17] We recently discovered that mice overexpressing RBP4 (*RBP4-Tg*) develop early-onset progressive retinal degeneration,[18] yet the underlying mechanisms are unclear.

Retinal degeneration in *RBP4-Tg* mice is characterized by microglia activation and neuroinflammatory expression of interleukin (IL)-18 in the retina.[18] Retinal degeneration in *RBP4-Tg* mice begins as early as 1 month of age and deteriorates rapidly so that by 6 months of age ERG responses are very significantly and severely reduced.[18] Importantly, *RBP4-Tg* mice aged up to 6 months have no signs of systemic disease, and are normoglycemic with normal body weight and insulin responses,[18] which indicates that retinal degeneration is caused by a specific effect on the retina rather than an indirect result of systemic disease. We found that *RBP4-Tg* mice retinae have normal steady-state retinoid profiles and only slightly elevated bis-retinoid A2E levels,[18] suggesting that retinal degeneration occurs through a mechanism that is unrelated to retinoid trafficking, and likely caused by the proinflammatory action of RBP4. The present study investigated whether the classical function of RBP4 in retinoid trafficking plays a role in retinal degeneration in *RBP4-Tg* mice by dark-rearing mice to inhibit the retinoid visual cycle. In addition, we tested whether pharmacological lowering of serum RBP4 levels can alleviate retinal degeneration in *RBP4-Tg* mice.

iovs.arvojournals.org | ISSN: 1552-5783

METHODS

Mice

RBP4-Tg mice, which overexpress human RBP4 under control of the mouse muscle creatine kinase (MCK) promoter on a C57BL/6J background, have been described previously.[19] *RBP4-KO* mice were generated and described previously.[5] Both *RBP4-Tg* and *RBP4-KO* mice were kindly provided by Loredana Quadro. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center, and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All studies were performed on age-, sex-, and strain-matched wild-type controls. Both sexes were used in all studies, and no sex-dependent differences in phenotype were observed. Importantly, *RBP4-Tg* mice were confirmed to be free of the retinal degeneration rd8 mutation that is known to be present in some C57Bl/6 colonies (data not shown).

Unless otherwise noted, mice were reared under normal cyclic light conditions (12-hour light/12-hour dark), with cage rack light intensities of 9.5 to 40 lux. Dark-reared mice were born and reared in a specially designed dark room, and only exposed to filtered dim red light to allow for staff to provide proper care. Unless otherwise noted, mice were fed a standard chow diet (Labdiet 5001) ad libitum. This diet contains a sufficient amount of vitamin A (15 IU g^{-1}). For studies involving A1120, A1120 was purchased from Tocris Bioscience, United Kingdom (catalog no. 3793). Standard mouse chow (Labdiet 5001) was supplemented with A1120 at 0.03% or 0.05% (0.3 or 0.5 g A1120 per Kg chow, prepared by Envigo, United Kingdom), and mice were fed ad libitum. A1120 diet administration began at weaning (P21), as testing showed no A1120-mediated lowering of serum RBP4 in pups when the maternal diet was supplemented with A1120 prior to weaning.

Mouse serum was collected by superficial temporal vein puncture from live mice that had fasted for 6 hours. ELISA kits for mouse RBP4 (catalog #MRB400: R&D Systems, Minneapolis, MN, USA) and human RBP4 (catalog# DRB400; R&D Systems) were used to quantify target molecules in serum.

Electroretinography (ERG)

ERG was performed as described previously.[18] Mice were weighed the day prior to ERG test and dark adapted overnight. Thirty minutes prior to test, one drop of 1% cyclogyl was delivered to each cornea for pupil dilation. Mice were anesthetized with an intraperitoneal injection of a cocktail of ketamine/xylazine at 85/14 mg/kg, respectively. Body temperature was maintained at 37°C using a heating pad. Pupils were dilated a second time with Phenylephrine Hydrochloride Ophthalmic Solution (10%) 5 minutes prior to performing the ERG procedure. Excess fluid was wicked from eyes, whiskers were trimmed, and Gonak (2.5%) was applied to each cornea. A stainless-steel needle electrode was gently hooked into the right cheek to serve as a reference. A stainless-steel needle electrode was placed just under the skin of the tail to serve as the ground. Platinum-wire electrodes were placed on the corneal surface of each eye, and a drop of saline was added after baseline was established. A seven-step protocol was run using an Espion E2 system with a Ganzfeld Colordome (Diagnosys LLC; Lowell, MA, USA). The first five steps were performed under scotopic conditions and consisted of five single flashes of 10-fold increasing flash intensity, starting at 0.004 (S)cd.s/m^2 light intensity. After light adaptation, two separate photopic readings were taken at light intensities of 3 (S)cd.s/m^2 and 10 (S)cd.s/m^2 using a 10-(S)cd/m^2 background light; for each photopic light intensity reading, 15 flashes were averaged.

For bleach-recovery ERG measurements, mice were dark-adapted overnight and then a prebleach ERG measurement was taken at flash intensity of 0.04 (S)cd.s/m^2. Then mice were exposed to a light at 500 cd/m^2 for 2 minutes to "bleach" ~85% to 90% of visual pigment. The postbleach ERG measurements [0.04 (S)cd.s/m^2] were taken immediately after the "bleach," and every 4 minutes thereafter for up to 40 minutes.

Quantitative Histological Analyses of Retinal Thickness and Immunohistochemistry

A flamed needle was used to scorch the superior side of the cornea to demarcate the vertical meridian, and eyes were enucleated with part of the optic nerve still attached. Eyes were fixed in 4% paraformaldehyde for 24-hours, subsequently transferred into PBS, then dehydrated and paraffin-embedded for sectioning along the superior-inferior retinal axis. Sections were deparaffinized, stained with hematoxylin and eosin, and images were scanned at 40× magnification on a Ventana Coreo Au Slide Scanner (Tissue Sciences Facility, University of Nebraska Medical Center). Scanned images underwent quantitative analysis using Ventana image viewer software (Ventana Medical Systems, Inc., Tucson, AZ, USA). Beginning at the optic nerve head and extending into the retinal periphery in 250-μm increments, thickness of the total retina was measured.

For immunohistochemistry, sections were deparaffinized, and antigen retrieval was performed using 10 mM sodium citrate pH 6.0. Then sections were permeabilized in PBS containing 0.03% Triton X-100 (PBST) for 15 minutes. Sections were coated in blocking solution (PBST with 5% bovine serum albumin) for 1 hour before incubation overnight at 4°C in primary antibodies diluted in blocking buffer. After sections were washed in PBST, they were incubated with appropriate fluorophore-conjugated secondary antibodies for 1 hour at room temperature. Then sections were washed in PBST, and 0.1% Sudan black in 70% ethanol was applied to the sections to reduce background autofluorescence. The sections were then rinsed, and Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) containing 4′,6-diamidino-2-phenylindole (DAPI) was applied as a coverslip was placed over the sections. Sections were imaged at 20× magnification using a Zeiss Observer Z1 microscope. The following EMD Millipore (Billerica, MA, USA) antibodies were used: cone arrestin (catalog no. AB15282), cone S-opsin (catalog no. ab5407), and cone M-opsin (catalog no. ab5405).

Quantification of Visual Cycle Retinoids and Bis-Retinoid A2E in Mouse Eyes

Visual cycle retinoids were quantified as described previously.[18] Mice were dark-adapted overnight for 16 hours, killed in the dark under dim red light, and eyes were enucleated and stored at −80°C protected from light. All further sample processing and HPLC was performed in the dark under dim red light. The whole eyes were homogenized by glass grinder in lysis buffer [10 mM NH2OH, 50% ethanol, 50% 2-(N-morpholino)ethanesulfonic acid, pH 6.5], and retinoids were extracted with hexane. Solvent was evaporated under argon gas, and dried retinoid samples were resuspended in 200 μL mobile phase (11.2% ethylacetate, 2.0% dioxane, 1.4% octanol, 85.4% hexane) and injected into the HPLC machine (515 HPLC pump, Waters Corp., Milford, MA, USA) at an isocratic flow rate of 1 mL/min for separation using a normal phase 5-μm column (Lichosphere SI-60; 4.6 mm inner diameter × 250 mm length; Alltech, Deerfield, IL, USA). Each retinoid isomer was quantified from the area of its corresponding peak, determined by using synthetic purified retinoid standards for calibration (empower software; Waters Corp.).

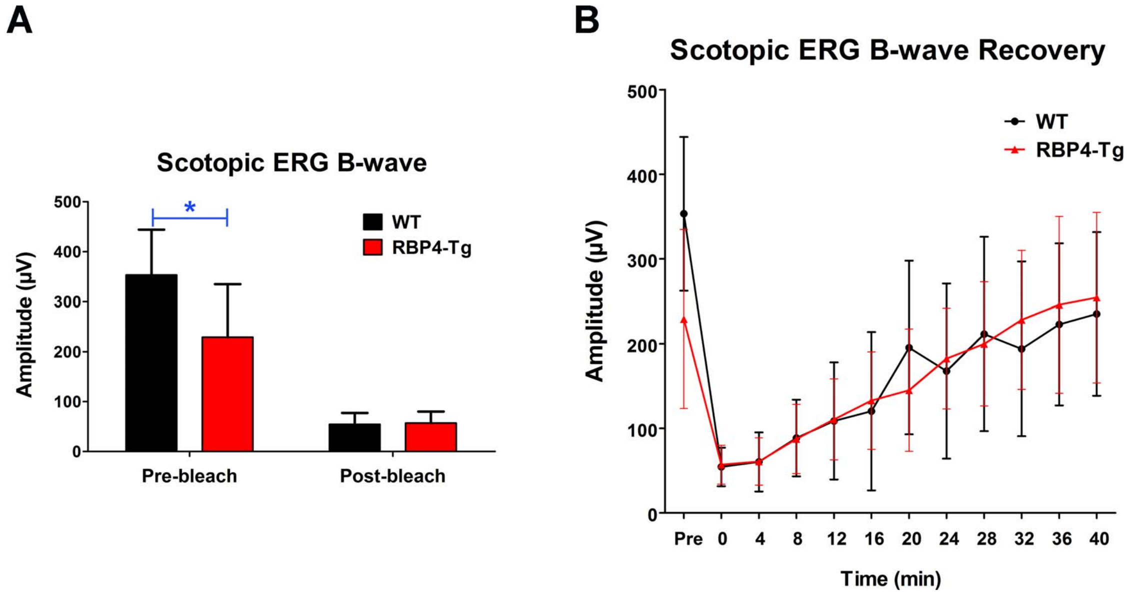

Figure 1. *RBP4-Tg* mice have normal ERG bleach-recovery kinetics. ERG bleach-recovery kinetics were measured in wild-type (WT) and *RBP4-Tg* mice at 1 month of age. (**A**) Scotopic ERG B-wave amplitudes (at flash light intensity of 0.04 cd.s/m^2) in fully dark-adapted mice pre-bleach and immediately following a 2-minute exposure to light at 500 cd/m^2 (post-bleach). Graph shows the mean ± standard deviation from at least six mice per group. **P* < 0.05 for *RBP4-Tg* compared to WT mice based on Student's *t*-test. (**B**) The scotopic ERG B-wave amplitude recovery was measured (at flash light intensity of 0.04 cd.s/m^2) every 4 minutes after the bleaching light exposure. The graph shows the mean ± standard deviation from at least six mice per group. Note there was no statistically significant difference between WT and *RBP4-Tg* ERG recovery kinetics.

Bis-retinoid A2E was quantified as described previously.[18] Mice were reared either under normal cyclic light or in a dark room under dim red light. All mice were fed standard chow ad libidum. At 9 months of age, mice were killed, eyes were enucleated, and the eyecup (RPE, sclera, and choroid) was dissected out. A total of four eyecups (from two mice of the same genotype and sex) were pooled to generate a single sample for HPLC measurement. Eyecups were stored at –80°C until analysis. Samples were homogenized in a glass grinder in PBS, and bis-retinoids were extracted three times using chloroform/methanol (2:1 vol/vol). The pooled organic phases were dried under a stream of argon gas and resuspended in 100 µL methanol. A2E was separated using a mobile phase gradient of methanol in water (85%–96% methanol + 0.1% trifluoroacetic acid) through a C18 reverse phase column (3.5 µM, 4.6 × 150 mm, Waters Corp.) at a flow rate of 1 mL/min. A Waters 2996 photodiode array detector was used to monitor absorbance of A2E at 430 nm. A2E levels were quantified from the area of the peak using purified A2E standard for calibration (Empower software; Waters Corp.).

Optokinetic Tracking (OKT) Analyses. As described previously,[18] visual function was measured using a virtual OKT system (OptoMotry, CerebralMechanics, Inc., Medicine Hat, Alberta, Canada) designed for rapid, quantifiable behavioral measurements of spatial vision in a virtual environment.[20] All OKT experiments were performed using an OptoMotry designed for rodent use (CerebralMechanics, Inc.). The animals were placed on a platform surrounded by four computer monitors forming a square inside an enclosed box. The monitors display continuous vertical sine wave gratings rotating across the monitors at 12°/s, which appear to the animal as a virtual three-dimensional rotating cylinder. The animal's ability to visualize the sine wave was monitored via a video camera positioned directly above the animal to display an image perpendicular to the animal's field of vision. The rotation of the virtual cylinder was constantly centered at the animal's viewing position to ensure a consistent viewing distance. Tracking movements were identified as slow, steady head movements in the direction of the rotating grating. For measurements of spatial frequency threshold, the mice were tested at a range of spatial frequencies from 0.034 to 0.664 cyc/deg. The OptoMotry device employs a proprietary algorithm to accept the input from the masked observer and automatically adjusts the testing stimuli based upon whether the animal exhibited the correct or incorrect tracking reflex. All measurements of contrast threshold were performed at a spatial frequency threshold of 0.064 cyc/deg. The contrast sensitivity was calculated as a reciprocal of the Michelson contrast from the screen's luminance (maximum − minimum)/(maximum + minimum).

Statistical Analysis

Data are presented as mean ± standard deviation. Statistical analyses were conducted using Prism 4 software (La Jolla, CA, USA) with 1-way ANOVA using Tukey's post hoc analysis to compare differences among three or more groups. A value of $P < 0.05$ was accepted as significant.

Results

RBP4-Tg Mice Have Normal ERG Bleach-Recovery Kinetics

Since we have previously shown that *RBP4-Tg* mice retinae have a slight elevation in toxic bis-retinoid A2E levels,[18] it could be that *RBP4-Tg* mice have an increased level or rate of retinoid visual cycle activity. In order to estimate visual chromophore regeneration kinetics, we performed ERG bleach-recovery experiments, in which mice were exposed to a prolonged light stimulus to effectively bleach >85% of light-responsive visual pigments, followed by measurement of retinal response to light stimuli every 4 minutes. As shown previously,[18] *RBP4-Tg* mice have a significantly reduced scotopic b-wave amplitude prebleach (Fig. 1A), and the bleaching protocol effectively reduced the postbleach ERG response by 85% to 90% so that wild-type and *RBP4-Tg* mice had similar scotopic b-wave amplitude responses postbleach (Fig. 1A). ERG measurements during the recovery phase show no statistical difference in scotopic b-wave recovery kinetics between wild-type and *RBP4-Tg* mice (Fig. 1B). These data establish that *RBP4-Tg* mice have normal visual pigment regeneration kinetics.

Retinal Degeneration Is Light-Independent in *RBP4-Tg* Mice

To determine whether retinoid visual cycle activity and its toxic by-products, such as bis-retinoid A2E, contribute to retinal degeneration in *RBP4-Tg* mice, the retinoid visual cycle was inhibited by dark-rearing mice. As expected, dark-rearing had no effect on serum RBP4 levels, and *RBP4-Tg* mice at 6 months of age maintained a ~8-fold increase in serum RBP4 compared to wild-type (Fig. 2A). As shown previously,[18] *RBP4-Tg* mice reared under normal cyclic light conditions have a slight but significant increase in bis-retinoid A2E levels in their eyecups by 9 months of age (Fig. 2B). As expected, dark-rearing effectively inhibited the retinoid visual cycle to prevent the elevation of A2E in *RBP4-Tg* eyecups (Fig. 2B). Despite the effectiveness of dark-rearing to inhibit A2E production, dark-rearing had no effect on ERG response or structural retinal degeneration in *RBP4-Tg* mice (Figs. 2C–F). Dark-reared *RBP4-Tg* mice had significantly reduced scotopic a- and b-wave and photopic b-wave responses compared to wild-type mice, and there was no statistically significant difference in ERG responses from *RBP4-Tg* mice reared in the dark versus normal cyclic light (Figs. 2C–E). Likewise, central retinal degeneration was very similar in *RBP4-Tg* mice reared in the dark versus normal cyclic light (Fig. 2F). These data demonstrate that retinal degeneration in *RBP4-Tg* mice is light-independent, and therefore unrelated to the retinoid visual cycle or the classical role of RBP4 in supplying retinol to the retinal pigment epithelium.

Pharmacological Lowering of Serum RBP4 Prevents Retinal Degeneration in *RBP4-Tg* Mice

To test whether pharmacological lowering of serum RBP4 can alleviate retinal degeneration in *RBP4-Tg* mice, mouse chow was supplemented with a nonretinoid RBP4 antagonist, A1120.[21–23] The A1120 compound binds to the retinol-binding pocket of RBP4, thereby blocking retinol-binding and promoting renal excretion of RBP4.[21–23] A1120 supplemented chow significantly and dose-dependently reduced serum RBP4 levels in *RBP4-Tg* mice (Fig. 3A). In fact, the highest dose of A1120 (0.05%) resulted in serum RBP4 levels that were not statistically different from wild-type level (Fig. 3A). We found that the lower dose (0.03%) of A1120, which resulted in a 70% decrease in serum RBP4 level, was sufficient to prevent structural retinal degeneration in *RBP4-Tg* mice (Fig. 3B). These data show that structural retinal degeneration in *RBP4-Tg* mice is RBP4-dependent. However, the A1120 treatment did not block or reduce retinal dysfunction in *RBP4-Tg* mice, since there was no statistically significant difference in ERG results between *RBP4-Tg* mice on standard diet versus A1120-supplemented diet (Figs. 3C–E).

A1120 Treatment Reduces Retinyl Ester Stores in the Retina, Impairs OKT, and Results in Loss of Cone Photoreceptors

In an effort to understand why A1120-mediated lowering of serum RBP4 levels prevents structural retinal degeneration but not retinal dysfunction, we measured retinal retinoid profiles. As shown previously,[18] *RBP4-Tg* mice had a significant increase in all-*trans*-retinal (Fig. 4A). As expected, the all-*trans*-retinal level was significantly reduced and similar to wild-type level in A1120-treated *RBP4-Tg* mice (Fig. 4A). The level of visual chromophore 11-*cis*-retinal was similar to wild-type in all groups of *RBP4-Tg* mice, regardless of A1120 treatment (Fig. 4A). However, *RBP4-Tg* mice treated with A1120 had a very statistically significant and severe reduction in retinyl esters stores in the retina, which was comparable to the retinyl ester level found in RBP4-knockout (*RBP4-KO*) mice (Fig. 4A). This finding suggests that the mice treated with A1120 have suboptimal retinol uptake into the retinal pigment epithelium.

Our previous study showed that the OKT response is reduced in *RBP4-Tg* mice,[18] and we observed similar results in the present study (Figs. 4B, 4C). Surprisingly, the OKT response was very significantly and severely reduced in A1120-treated *RBP4-Tg* mice (Figs. 4B, 4C). Since the OKT response relies on high acuity bright light vision, which is mediated by cone photoreceptors, we performed immunohistochemical staining to evaluate cone distribution in the retina. We found that A1120-treated *RBP4-Tg* mice had a clear decline in the abundance of cones, based on the staining pattern of M- and S-opsins, as well as cone arrestin (Figs. 4D, 4E).

Discussion

This study definitively shows that retinal degeneration is light-independent in *RBP4-Tg* mice, thereby confirming that the classical function of RBP4 in retinoid trafficking is unrelated to the mechanisms of retinal degeneration in *RBP4-Tg* mice. We have previously shown that *RBP4-Tg* mice have retinal microglia activation and significant induction of IL-18 expression in the retina,[18] indicating that neuroinflammation is a major feature of retinal degeneration in *RBP4-Tg* mice. In addition, numerous studies have shown that high concentrations of RBP4 exert proinflammatory effects in cells and tissues.[16–18,24,25] Recently, we and others have shown that the proinflammatory effects of RBP4 in adipose tissue macrophages[17] and human endothelial cells[24] are largely mediated through activation of toll-like receptor 4 (TLR4). TLR4 is involved in the pathogenesis of different types of retinal degeneration.[26–30] Retinal microglia, which are activated as early as 1 month of age in *RBP4-Tg* mice,[18] express TLR4.[26,27] Moreover, TLR4-mediated signaling has been shown to induce IL-18 mRNA and protein activation.[31] Therefore, we speculate that RBP4 may activate TLR4 signaling in retinal microglia to release IL-18, but this is yet to be determined.

The present study also demonstrates that pharmacological lowering of serum RBP4 levels prevents retinal degeneration in *RBP4-Tg* mice (Figs. 3A, 3B), confirming that it is an RBP4-dependent phenomenon. However, the A1120 compound used for lowering serum RBP4 levels severely reduces retinyl ester stores in the retina by over 80%, to a level that is similar to *RBP4-KO* mice (Fig. 4A). Similar to our result, a previous study showed that 12 days of A1120 treatment depleted retinyl ester stores in the mouse retina by almost 30%.[22] Previous studies have shown that A1120 does not directly interfere with the RPE visual cycle activity by using bovine RPE microsomes to test isomerohydrolase (RPE65) activity in the presence or absence of A1120.[22] Rather, A1120 indirectly inhibits the RPE visual cycle activity by binding to serum RBP4 and reducing RBP4-mediated uptake of retinol by the RPE.[22] Moreover, A1120 has less off-target effects compared to other RBP4-lowering compounds, such as Fenretinide [N-(4-hydroxyphe-

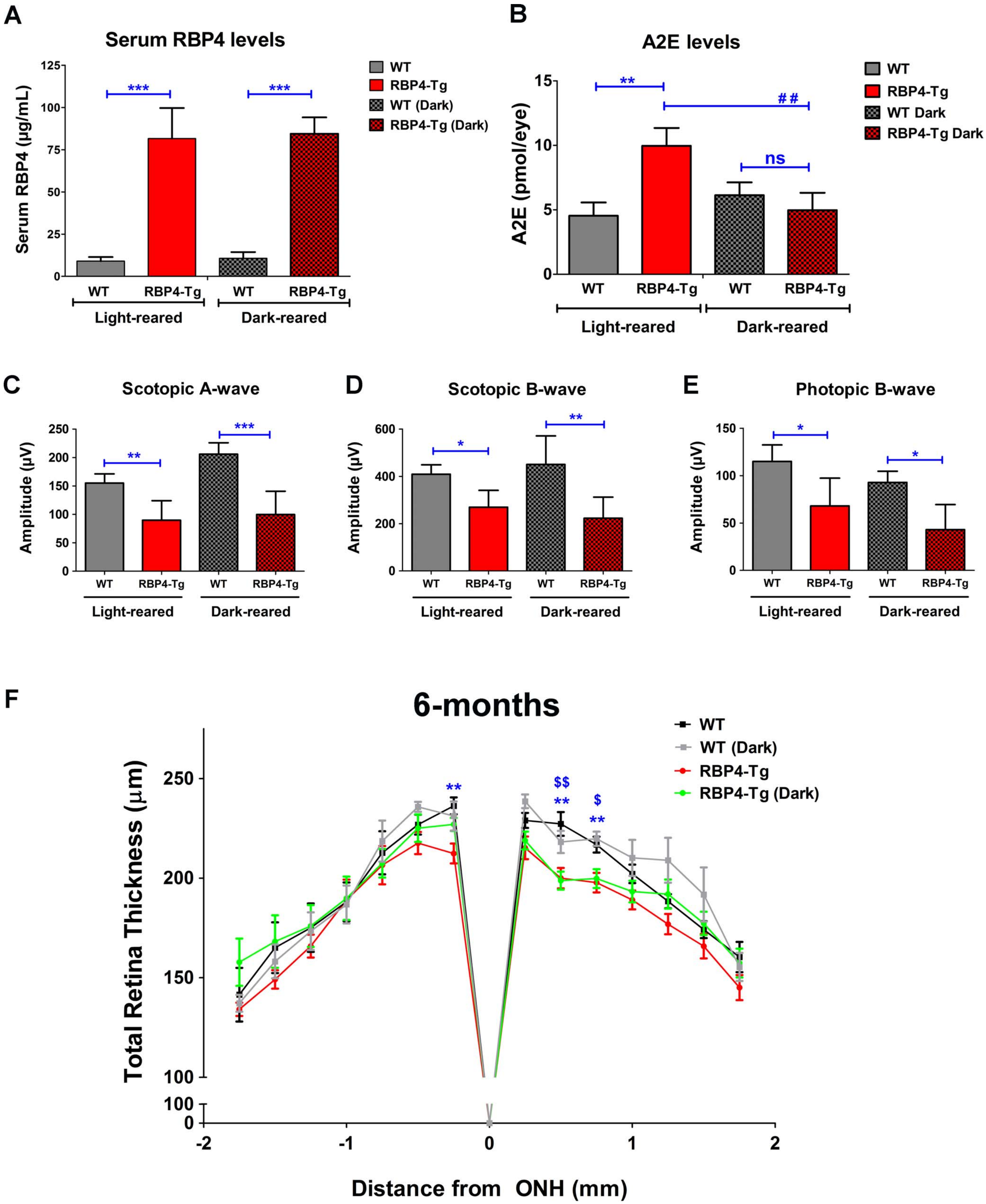

FIGURE 2. Retinal degeneration in *RBP4-Tg* mice is light-independent. Wild-type (WT) and *RBP4-Tg* mice were reared in normal cyclic room light conditions or in a dark room. (**A**) Total RBP4 (mouse and human transgene) levels in mouse serum at 6 months of age. Graph represents the mean ± standard deviation from at least five mice per group. $^{***}P < 0.001$ for *RBP4-Tg* compared to WT by Student's *t*-test. (**B**) Quantification of bis-retinoid A2E in eyecups from 9-month-old mice. Values are means ± SEM from at least three samples (one sample consists of four eyecups pooled from two mice) per group. ns, not significant; $^{**}P < 0.01$ for *RBP4-Tg* compared to WT; $^{\#\#}P < 0.01$ for dark-reared *RBP4-Tg* compared to *RBP4-Tg* reared on normal cyclic light by 1-way ANOVA with Tukey's post hoc test. Note that dark-reared *RBP4-Tg* mice have significantly less A2E than *RBP4-Tg* reared in normal cyclic light, confirming the dark-rearing was successful. (**C**, **D**) Scotopic ERG a-wave (**C**) and b-wave (**D**) amplitudes in mice aged 6

months. Values are means ± standard deviation from at least four mice per group. (**E**) Photopic ERG b-wave amplitudes in mice aged 6 months. Values are means ± standard deviation from at least four mice per group. **P* < 0.05, ***P* < 0.01; ****P* < 0.001 for *RBP4-Tg* compared to WT by 1-way ANOVA with Tukey's post hoc test. (**F**) Quantitative histological analyses of total retina thickness from mice aged 6 months (ONH, optic nerve head). Values are means ± standard deviation from at least five mice per group. ***P* < 0.01 for *RBP4-Tg* compared to WT; $*P* < 0.05, $$*P* < 0.01 for dark-reared *RBP4-Tg* compared to WT by Student's *t*-test.

---

nyl)retinamide)]. Fenretinide can bind and activate retinoic acid receptors (RARs) to elicit off-target effects, whereas A1120 does not bind RARs.[22] Similar to previous studies,[22,23] we found no obvious signs of A1120-induced systemic toxicity, as mice treated with A1120 had normal weight and food consumption and no signs of lethargy. Therefore, although the A1120-treated *RBP4-Tg* mice maintained serum RBP4 levels at or above wild-type level (Fig. 3A), the remaining RBP4 in the serum of A1120-treated mice is likely bound to A1120 and therefore unable to deliver much retinol to the retinal pigment

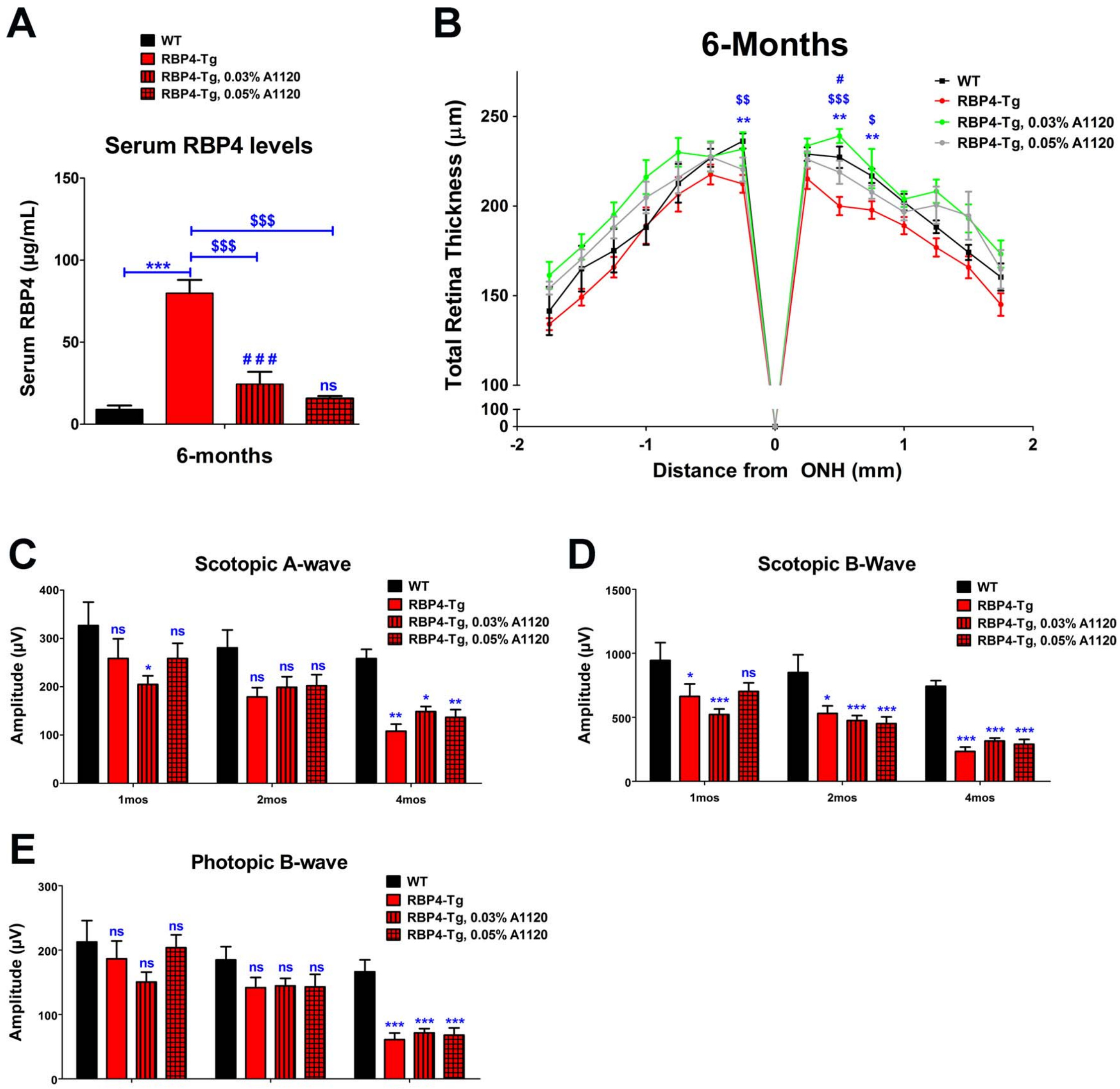

Figure 3. Pharmacologically lowering serum RBP4 levels prevents retinal degeneration, but loss of retinal function remains. (**A**) Total RBP4 (mouse and human transgene) levels in mouse serum at 6 months of age. Graph represents the mean ± standard deviation from at least five mice per group; ****P* < 0.001 for *RBP4-Tg* compared to wild-type (WT); $$$*P* < 0.001 for *RBP4-Tg* mice on A1120 diet compared to *RBP4-Tg* mice on standard diet; ###*P* < 0.001 for *RBP4-Tg* mice on A1120 diet compared to WT by 1-way ANOVA with Tukey's post hoc test. Note that the *RBP4-Tg* on 0.05% A1120 diet had serum RBP4 levels that were not significantly (ns) different from WT mice. (**B**) Quantitative histological analyses of total retina thickness from mice aged 6 months (ONH, optic nerve head). Values are means ± standard deviation from at least eight mice per group. ***P* < 0.01 for *RBP4-Tg* compared to WT; $*P* < 0.05, $$*P* < 0.01, $$$*P* < 0.001 for *RBP4-Tg* on 0.03% A1120 diet compared to *RBP4-Tg* on regular diet; #*P* < 0.05 for *RBP4-Tg* on 0.05% A1120 diet compared to *RBP4-Tg* on regular diet by Student's *t*-test. Note that *RBP4-Tg* mice on either 0.03% or 0.05% A1120 diet had no statistically significant difference from WT mice. (**C**, **D**) Scotopic ERG a-wave (**C**) and b-wave (**D**) amplitudes in mice aged 1 to 4 months. Values are means ± standard deviation from at least six mice per group. (**E**) Photopic ERG b-wave amplitudes in mice aged 1 to 4 months. Values are means ± standard deviation from at least four mice per group. ns, not significant **P* < 0.05, ***P* < 0.01; ****P* < 0.001 for any *RBP4-Tg* group compared to WT by 2-way ANOVA with Bonferroni's post hoc test. Note that there was no statistically significant difference between *RBP4-Tg* on standard diet versus A1120-supplemented diets.

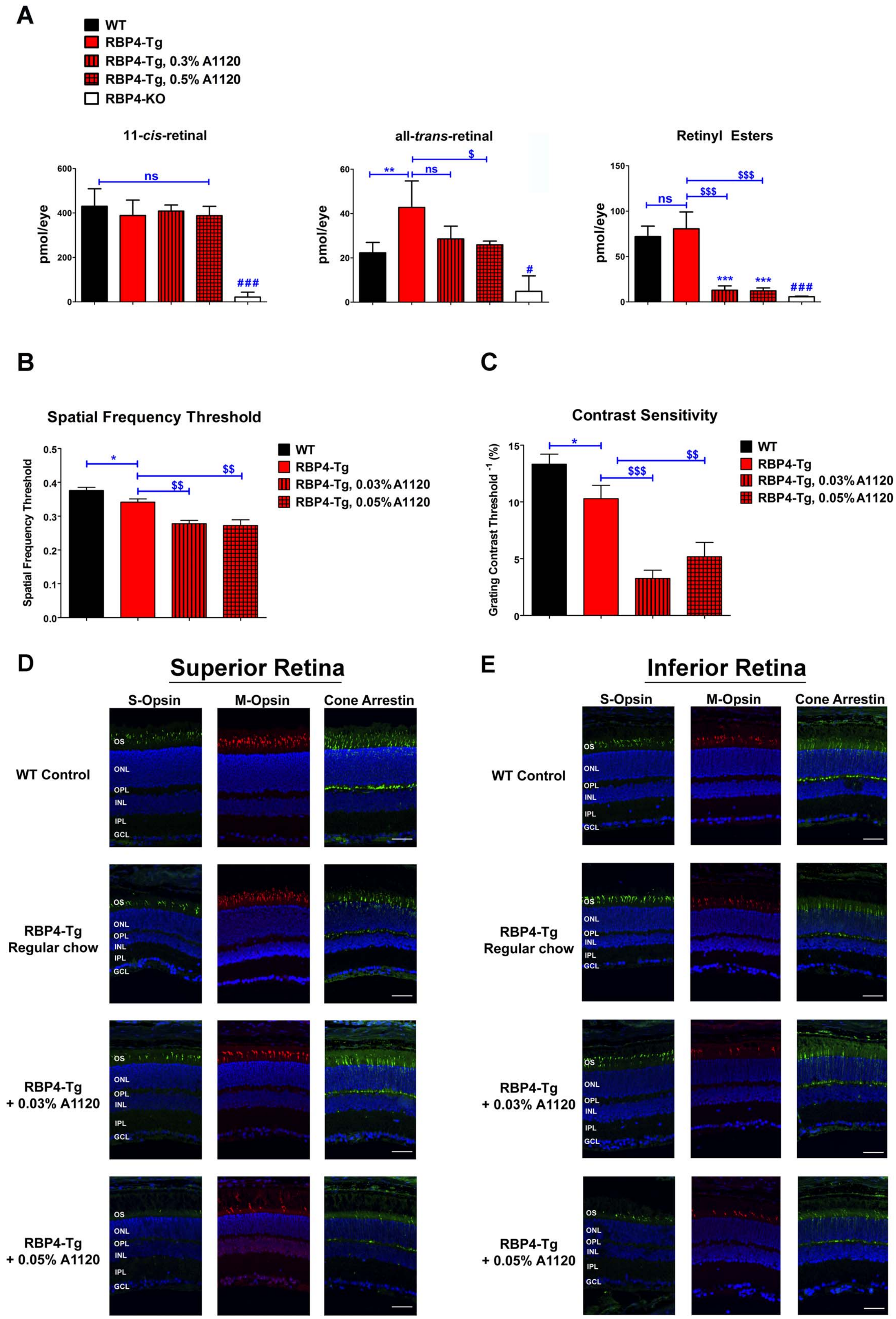

Figure 4. A1120 treatment severely reduces retinyl ester stores in the retina, impairs OKT, and results in loss of cone photoreceptors. (**A**) HPLC-based quantification of visual cycle retinoid isomers in eyes from mice aged 6 months and dark-adapted for 16 hours prior to euthanization. Values are mean ± standard deviation from at least three mice per group. ns, not significant; $^{**}P < 0.01$ for any *RBP4-Tg* group compared to wild-type (WT), \$$P < 0.05$, \$\$\$$P < 0.001$ for *RBP4-Tg* on A1120 diet compared to *RBP4-Tg* on standard diet, #$P < 0.05$, ###$P < 0.001$ for *RBP4-KO* compared to WT by 1-way ANOVA with Tukey's post hoc test. Note that A1120 diet groups have severely reduced retinyl ester levels that are similar to *RBP4-KO* mice. (**B**, **C**) OKT response to changes in spatial frequency (**B**) and grating contrast (**C**) in mice aged 4 months. All OKT analyses were performed

Investigative Ophthalmology & Visual Science

on at least five mice per group; Values are mean ± standard error of the mean; *$P < 0.05$ for *RBP4-Tg* on standard diet compared to WT; $$$P < 0.01$, $$$$P < 0.001$ for *RBP4-Tg* on either A1120 diet compared to *RBP4-Tg* on standard diet by 1-way ANOVA with Tukey's post hoc test. Note that the mice on A1120 diet have very significant and severe reduction in OKT responses compared to *RBP4-Tg* mice on standard diet. (**D**, **E**) Representative images of retinal paraffin sections from 6-month-old mice immunofluorescently labeled for cone S-opsin, cone M-opsin, and cone arrestin as indicated. DAPI counterstaining is shown in *blue*. Images were acquired at 20× magnification, and the *scale bar* indicates 50 μm. The images in panel **D** correspond to the superior (M-opsin rich) retina, and images in panel **E** were taken from the inferior (S-opsin rich) retina. Note that the A1120 diet groups clearly have less staining for both S- and M-opsins, as well as cone arrestin.

epithelium, which explains why A1120-treated mice have depleted retinyl ester stores.

Surprisingly, *RBP4-Tg* mice on A1120 treatment had a very significant worsening of OKT responses (Figs. 4B, 4C). Moreover, we found that A1120-treated *RBP4-Tg* mice had a clear reduction in immunohistochemical labeling of cone M- and S-opsins, as well as cone arrestin (Figs. 4D, 4E), which indicates a loss of cones in A1120-treated mice. The mechanisms underlying the loss of cones and OKT impairment in mice on A1120 diet is unclear, but may be related to the severe depletion of retinyl ester stores in the retina. Cones function in bright light, and have a higher requirement for fast regeneration of visual chromophore to sustain vision.[32–34] Moreover, in absence of visual chromophore, cones degenerate rapidly.[35–38] Interestingly, a recent study in zebrafish suggests that retinyl ester stores are especially important to supply visual chromophore and thereby sustain bright light cone-mediated vision.[39] Thus, we speculate that the loss of cones in A1120-treated mice may be an indirect result of the depletion of retinal retinyl ester stores, although we cannot rule out the possibility that A1120 has distinct and specific cone toxicity.

While it is difficult to directly extrapolate mouse studies to humans, the finding that A1120 treatment results in loss of cone photoreceptors and severely reduces OKT responses, an indirect measurement of visual acuity in rodents, suggests that future studies or plans to use A1120 as a therapeutic in humans should be approached with caution and attempt to identify the minimal dosing that may provide therapeutic benefit without eliciting extreme retinoid depletion, cone toxicity, or other adverse effects. The doses of A1120 used in this study were determined empirically to identify the necessary dosage to reduce serum RBP4 levels in *RBP4-Tg* mice to near wild-type levels. Since *RBP4-Tg* mice have an 8- to 10-fold elevation of serum RBP4 levels (Figs. 2A, 3A), and most clinical studies have found only a 2- to 5-fold elevation in serum RBP4 levels in humans with obesity,[10,11] insulin resistance,[10–15] and type 2 diabetes,[11,15] it is likely that a lower dose of A1120 could be effective to lower serum RBP4 levels without causing adverse effects in humans.

Acknowledgments

The authors thank Mark Dittmar and the staff at the Dean McGee Eye Institute for assisting with establishing and maintaining a room in the animal research facility for dark-rearing mice, and Loredana Quadro, PhD, who kindly provided the *RBP4-Tg* and *RBP4-KO* mice. The authors also thank Konstantin Petrukhin, PhD, for providing A1120 for preliminary studies related to this manuscript.

Supported by an institutional development award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number P20GM104934 (KMF received funding as a promising junior investigator on this grant), and a bridge grant from the Presbyterian Health Foundation (KMF).

Disclosure: **M. Du**, None; **E. Phelps**, None; **M.J. Balangue**, None; **A. Dockins**, None; **G. Moiseyev**, None; **Y. Shin**, None; **S. Kane**, None; **L. Otalora**, None; **J.-X. Ma**, None; **R. Farjo**, None; **K.M. Farjo**, None

References

1. Blomhoff R, Blomhoff HK. Overview of retinoid metabolism and function. *J Neurobiol*. 2006;66:606–630.
2. Kanai M, Raz A, Goodman DS. Retinol-binding protein: the transport protein for vitamin A in human plasma. *J Clin Invest*. 1968;47:2025–2044.
3. Raz A, Shiratori T, Goodman DS. Studies on the protein-protein and protein-ligand interactions involved in retinol transport in plasma. *J Biol Chem*. 1970;245:1903–1912.
4. Vahlquist A, Nilsson SF, Peterson PA. Isolation of the human retinol binding protein by affinity chromatography. *Eur J Biochem*. 1971;20:160–168.
5. Quadro L, Blaner WS, Salchow DJ, et al. Impaired retinal function and vitamin A availability in mice lacking retinol-binding protein. *Embo J*. 1999;18:4633–4644.
6. Kiser PD, Golczak M, Palczewski K. Chemistry of the retinoid (visual) cycle. *Chem Rev*. 2014;114:194–232.
7. Saari JC. Vitamin A metabolism in rod and cone visual cycles. *Annu Rev Nutr*. 2012;32:125–145.
8. Thompson DA, Gal A. Vitamin A metabolism in the retinal pigment epithelium: genes, mutations, and diseases. *Prog Retin Eye Res*. 2003;22:683–703.
9. Travis GH, Golczak M, Moise AR, Palczewski K. Diseases caused by defects in the visual cycle: retinoids as potential therapeutic agents. *Annu Rev Pharmacol Toxicol*. 2007;47:469–512.
10. Aeberli I, Biebinger R, Lehmann R, L'Allemand D, Spinas GA, Zimmermann MB. Serum retinol-binding protein 4 concentration and its ratio to serum retinol are associated with obesity and metabolic syndrome components in children. *J Clin Endocrinol Metab*. 2007;92:4359–4365.
11. Graham TE, Yang Q, Bluher M, et al. Retinol-binding protein 4 and insulin resistance in lean, obese, and diabetic subjects. *N Engl J Med*. 2006;354:2552–2563.
12. Bremer AA, Devaraj S, Afify A, Jialal I. Adipose tissue dysregulation in patients with metabolic syndrome. *J Clin Endocrinol Metab*. 2011;96:E1782–E1788.
13. Kowalska I, Straczkowski M, Adamska A, et al. Serum retinol binding protein 4 is related to insulin resistance and nonoxidative glucose metabolism in lean and obese women with normal glucose tolerance. *J Clin Endocrinol Metab*. 2008;93:2786–2789.
14. Mostafaie N, Sebesta C, Zehetmayer S, et al. Circulating retinol-binding protein 4 and metabolic syndrome in the elderly. *Wien Med Wochenschr*. 2011;161:505–510.
15. Yang Q, Graham TE, Mody N, et al. Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. *Nature*. 2005;436:356–362.
16. Moraes-Vieira PM, Yore MM, Dwyer PM, Syed I, Aryal P, Kahn BB. RBP4 activates antigen-presenting cells, leading to adipose tissue inflammation and systemic insulin resistance. *Cell Metab*. 2014;19:512–526.
17. Norseen J, Hosooka T, Hammarstedt A, et al. Retinol-binding protein 4 inhibits insulin signaling in adipocytes by inducing proinflammatory cytokines in macrophages through a c-Jun N-terminal kinase- and toll-like receptor 4-dependent and

retinol-independent mechanism. *Mol Cell Biol*. 2012;32: 2010–2019.

18. Du M, Otalora L, Martin AA, et al. Transgenic mice overexpressing serum retinol-binding protein develop progressive retinal degeneration through a retinoid-independent mechanism. *Mol Cell Biol*. 2015;35:2771–2789.
19. Quadro L, Blaner WS, Hamberger L, et al. Muscle expression of human retinol-binding protein (RBP). Suppression of the visual defect of RBP knockout mice. *J Biol Chem*. 2002;277: 30191–30197.
20. Prusky GT, Alam NM, Beekman S, Douglas RM. Rapid quantification of adult and developing mouse spatial vision using a virtual optomotor system. *Invest Ophthalmol Vis Sci*. 2004;45:4611–4616.
21. Cioffi CL, Dobri N, Freeman EE, et al. Design, synthesis, and evaluation of nonretinoid retinol binding protein 4 antagonists for the potential treatment of atrophic age-related macular degeneration and Stargardt disease. *J Med Chemistry*. 2014;57:7731–7757.
22. Dobri N, Qin Q, Kong J, et al. A1120, a nonretinoid RBP4 antagonist, inhibits formation of cytotoxic bisretinoids in the animal model of enhanced retinal lipofuscinogenesis. *Invest Ophthalmol Vis Sci*. 2013;54:85–95.
23. Motani A, Wang Z, Conn M, et al. Identification and characterization of a non-retinoid ligand for retinol-binding protein 4 which lowers serum retinol-binding protein 4 levels in vivo. *J Biol Chem*. 2009;284:7673–7680.
24. Du M, Martin A, Hays F, Johnson J, Farjo RA, Farjo KM. Serum retinol-binding protein-induced endothelial inflammation is mediated through the activation of toll-like receptor 4. *Mol Vis*. 2017;23:185–197.
25. Farjo KM, Farjo RA, Halsey S, Moiseyev G, Ma JX. Retinol-binding protein 4 induces inflammation in human endothelial cells by an NADPH oxidase- and nuclear factor kappa B-dependent and retinol-independent mechanism. *Mol Cell Biol*. 2012;32:5103–5115.
26. Halder SK, Matsunaga H, Ishii KJ, Akira S, Miyake K, Ueda H. Retinal cell type-specific prevention of ischemia-induced damages by LPS-TLR4 signaling through microglia. *J Neurochem*. 2013;126:243–260.
27. Kohno H, Chen Y, Kevany BM, et al. Photoreceptor proteins initiate microglial activation via Toll-like receptor 4 in retinal degeneration mediated by all-trans-retinal. *J Biol Chem*. 2013; 288:15326–15341.
28. Qi Y, Zhao M, Bai Y, et al. Retinal ischemia/reperfusion injury is mediated by Toll-like receptor 4 activation of NLRP3 inflammasomes. *Invest Ophthalmol Vis Sci*. 2014;55:5466–5475.
29. Wang H, Shi H, Zhang J, et al. Toll-like receptor 4 in bone marrow-derived cells contributes to the progression of diabetic retinopathy. *Mediators Inflamm*. 2014;2014:858763.
30. Wang YL, Wang K, Yu SJ, et al. Association of the TLR4 signaling pathway in the retina of streptozotocin-induced diabetic rats. *Graefes Arch Clin Exp Ophthalmol*. 2015;253: 389–398.
31. Rhee AC, Cain AL, Hile KL, Zhang H, Matsui F, Meldrum KK. IL-18 activation is dependent on Toll-like receptor 4 during renal obstruction. *J Surg Res*. 2013;183:278–284.
32. Wang JS, Estevez ME, Cornwall MC, Kefalov VJ. Intra-retinal visual cycle required for rapid and complete cone dark adaptation. *Nat Neurosci*. 2009;12:295–302.
33. Wang JS, Kefalov VJ. The cone-specific visual cycle. *Prog Retin Eye Res*. 2011;30:115–128.
34. Sato S, Frederiksen R, Cornwall MC, Kefalov VJ. The retina visual cycle is driven by cis retinol oxidation in the outer segments of cones. *Vis Neurosci*. 2017;34:E004.
35. Kunchithapautham K, Coughlin B, Crouch RK, Rohrer B. Cone outer segment morphology and cone function in the Rpe65-/- Nrl-/- mouse retina are amenable to retinoid replacement. *Invest Ophthalmol Vis Sci*. 2009;50:4858–4864.
36. Rohrer B, Lohr HR, Humphries P, Redmond TM, Seeliger MW, Crouch RK. Cone opsin mislocalization in Rpe65-/- mice: a defect that can be corrected by 11-cis retinal. *Invest Ophthalmol Vis Sci*. 2005;46:3876–3882.
37. Znoiko SL, Rohrer B, Lu K, Lohr HR, Crouch RK, Ma JX. Downregulation of cone-specific gene expression and degeneration of cone photoreceptors in the Rpe65-/- mouse at early ages. *Invest Ophthalmol Vis Sci*. 2005;46:1473–1479.
38. Zhang H, Fan J, Li S, et al. Trafficking of membrane-associated proteins to cone photoreceptor outer segments requires the chromophore 11-cis-retinal. *J Neurosci*. 2008;28:4008–4014.
39. Babino D, Perkins BD, Kindermann A, Oberhauser V, von Lintig J. The role of 11-cis-retinyl esters in vertebrate cone vision. *FASEB J*. 2015;29:216–226.