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Therapeutic potential of AAV2-shmTOR gene therapy in reducing retinal inflammation and preserving endothelial Integrity in age-related macular degeneration

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Age-related macular degeneration (AMD) is a prevalent retinal disorder that leads to central vision loss, mainly due to chronic inflammation. Tumor necrosis factor-alpha (TNF-α) is a critical mediator of inflammatory responses within the retinal environment. This study has investigated TNF-α's influence on inflammatory cytokine production and endothelial barrier integrity in human microglial (HMC3) and endothelial (HUVEC) cells. We found that TNF-α significantly elevated the expression and secretion of interleukin-6 (IL-6) and interleukin-1β (IL-1β) in HMC3 cells and disrupted endothelial tight junctions in HUVECs, as evidenced by weakened ZO-1 staining and compromised barrier function. To mitigate these effects and further investigate the in vitro mechanism of actions in CRG-01's in vivo therapeutic efficacy of anti-inflammation, we employed AAV2-shmTOR, CRG-01, as the candidate for therapeutic vector targeting the mammalian target of the rapamycin (mTOR) pathway. TNF-α-induced IL-6, IL-1β, and NF-κB signaling in HMC3 cells were significantly reduced by AAV2-shmTOR treatment, which may present a promising avenue for the fight against AMD. It also effectively preserved endothelial tight junction integrity in TNF-α-treated HUVECs, providing reassurance about its effectiveness. Furthermore, the supernatant medium collected from AAV2-shmTOR-treated HMC3 cells decreased oxidative stress, protein oxidation, and cytotoxicity in ARPE retinal pigment epithelial cells. These results strongly suggested that CRG-01, the candidate therapeutic vector of AAV2-shmTOR, may have a therapeutic potential to treat AMD-related retinal inflammation.

Keywords Gene therapy, Age-related macular degeneration (AMD), Adeno-associated virus (AAV), ShmTOR, Retinal inflammation, (Vascular endothelial growth factor (VEGF)

Age-related macular degeneration (AMD) is a significant global health concern, particularly among the elderly, as it is one of the leading causes of vision loss worldwide[1,2]. AMD primarily affects the macula, the retina's central region responsible for sharp vision, leading to progressive deterioration and potential blindness[3]. It manifests in the more prevalent dry form and the severe wet form[4]. Dry AMD involves the gradual accumulation of drusen and atrophy of the macula[5,6]. At the same time, wet AMD is characterized by abnormal blood vessel growth and leakage under the retina, causing rapid vision loss[3,7,8]. The pathogenesis of AMD is complex, involving genetic, environmental, and metabolic factors. Inflammation is pivotal in both dry and wet forms[3,9–11]. Persistent low-grade inflammation damages cells in the retina[12,13], while acute inflammation in wet AMD contributes to new blood vessel formation and breakdown of the blood-retina barrier[14,15]. Genetic factors, such as variations in complement factor H (CFH), highlight the role of immune dysregulation in AMD development[16,17].

Current AMD treatments primarily target the wet form by inhibiting vascular endothelial growth factor (VEGF) to control abnormal blood vessel growth. Anti-VEGF therapies like ranibizumab and aflibercept have been effective but require frequent injections and do not cure the disease[18–24]. They are ineffective against dry AMD, which currently has no approved treatments to halt its progression[25]. Additionally, some wet AMD

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patients do not respond to anti-VEGF therapies, necessitating alternative treatments targeting both angiogenesis and inflammation[26–28]. Therefore, targeting inflammatory pathways holds promise for therapeutic intervention in AMD.

Pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 play central roles in mediating this inflammatory response[29], impacting multiple retinal cell types, including microglia, endothelial cells, and RPE cells[14]. Chronic inflammation is a critical factor in the progression of AMD, disrupting the retinal environment and contributing to cumulative damage over time[30–36]. TNF-α, in particular, is a crucial mediator that exacerbates retinal dysfunction by promoting cellular stress, barrier disruption, and oxidative damage. Targeting inflammation and its downstream effects offers a promising approach to mitigating AMD progression and preserving retinal function.

Oxidative stress, characterized by an imbalance between the production of reactive oxygen species (ROS) and the capacity of cellular antioxidant defenses, plays a pivotal role in various pathological processes[37], including angiogenesis. Excess ROS levels can damage cellular components, leading to a pro-angiogenic environment by activating transcription factors such as hypoxia-inducible factor-1α (HIF-1α) and nuclear factor-κB (NF-κB)[38,39]. These factors promote the expression of angiogenic mediators like vascular endothelial growth[40]. In diseases like AMD and diabetic retinopathy (DR), oxidative stress-induced angiogenesis contributes to pathological vascularization, which exacerbates tissue damage and vision loss. Given its significant role, targeting oxidative stress represents a promising strategy for managing abnormal angiogenesis in these diseases.

The mammalian target of the rapamycin (mTOR) pathway is increasingly recognized as regulating angiogenesis and inflammation, making it an attractive target for AMD treatment[41–43]. Moreover, mTOR influences cell growth and survival, and its dysregulation is implicated in diseases like cancer and AMD[41,44–48]. Targeting the mTOR pathway could inhibit abnormal blood vessel growth and chronic inflammation, addressing core AMD pathologies[49]. The mTOR pathway plays a role in oxidative stress regulation, mainly through mTORC1, which promotes ROS production independently of AKT. Studies show that inhibiting mTOR can prevent excessive ROS, normalize levels, and reduce oxidative stress, essential in cancer cells where high ROS supports aggressive proliferation, especially in PI3K-resistant cases[50]. In stem cells, mTOR inhibition helps balance ROS levels, supporting mitochondrial function and cellular health through FOXO3 activity[51]. Thus, targeting mTOR offers a potential therapeutic strategy for diseases driven by oxidative damage, stabilizing ROS and counteracting high ROS's survival advantage.

Our team has developed CRG-01 (AAV2-shmTOR), an adeno-associated virus (AAV)-based gene therapy designed to deliver a small hairpin RNA (shRNA) that silences mTOR expression[49,52–54]. AAV-based therapies offer significant advantages for retinal disorders, including safety, precise targeting, and the potential for prolonged expression[52]. Previous studies have demonstrated the efficacy of AAV2-shmTOR in reducing angiogenesis and inflammation in various retinopathy models, highlighting its applicability in AMD[49,53–55]. By downregulating mTOR signaling, which is implicated in inflammatory and degenerative processes in the retina, CRG-01 aims to suppress inflammation and mitigate retinal cell damage. This approach addresses a critical mechanism underlying AMD progression, making CRG-01 a promising candidate for therapeutic development in this context.

Results

Effects of TNF-α on inflammatory cytokine expression in human microglial HMC3 cells

To evaluate the impact of TNF-α on inflammatory cytokine expression, the human microglial cell line HMC3 was treated with recombinant TNF-α at concentrations of 50, 100, and 200 ng/mL for 24 h. Bright-field microscopy demonstrated that HMC3 cells maintained healthy morphology across all treatment conditions, indicating that TNF-α exposure at these concentrations did not compromise cell viability. This ensured the reliability and validity of subsequent analyses (Supplementary Fig. 1). Immunoblotting analysis demonstrated a significant, dose-dependent increase in protein levels of IL-6 and IL-1β (active-form) compared to untreated controls (Fig. 1A-C). Furthermore, we performed ELISA to measure the secretion of these cytokines in the culture supernatant. Treatment with 100 ng/mL TNF-α significantly elevated the secretion of IL-6 and IL-1β (active-form), compared to PBS-treated controls (Fig. 1D, E). These results indicate that TNF-α enhances the expression of IL-6 and IL-1β (active-form) within HMC3 cells and stimulates their release into the extracellular environment. This suggests a mechanistic role for TNF-α in promoting inflammatory responses in microglial cells, aligning with the study's objective to understand inflammatory pathways in ocular disorders.

Efficient knockdown of mTOR proteins by AAV2-shmTOR in HMC3 cells

To assess the efficiency of viral transduction and mTOR knockdown, HMC3 cells were infected with AAV2-GFP or AAV2-shmTOR at various multiplicities of infection (MOI). Fluorescence microscopy confirmed robust transduction, as indicated by GFP expression in AAV2-GFP and AAV2-shmTOR-infected cells (Fig. 2A). Immunoblotting analysis demonstrated a significant reduction in mTOR protein levels in cells treated with AAV2-shmTOR (Fig. 2B, C). Notably, this reduction was consistent across different MOIs, with even a low MOI (1×10^4 vg/cell) achieving pronounced mTOR knockdown. Additionally, there was a substantial decrease in phosphorylated mTOR (p-mTORS2448) and total mTOR proteins in AAV2-shmTOR-treated cells compared to controls (Fig. 2D, E). These findings validate the efficiency of AAV2-shmTOR in mediating effective mTOR knockdown in HMC3 cells, supporting its potential applicability in targeting inflammatory pathways in retinal diseases.
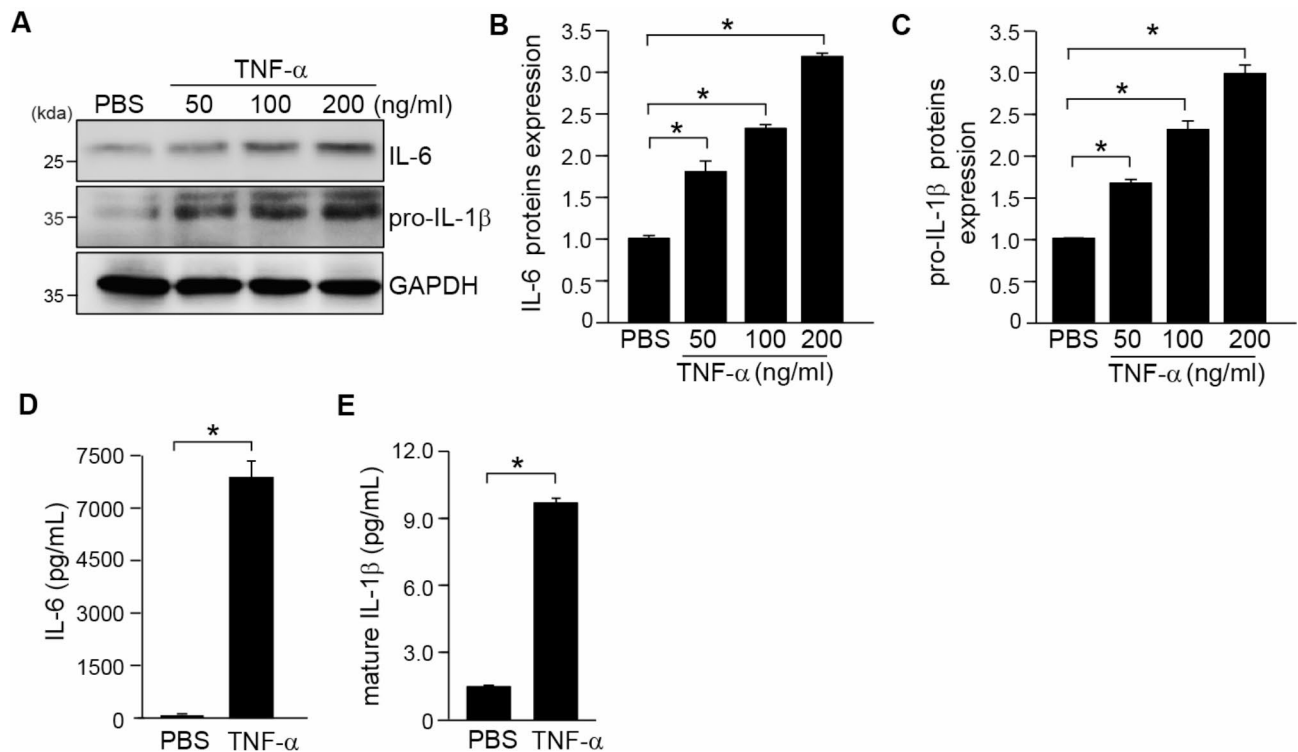
Fig. 1. TNF-α-induced increase in IL-6 and IL-1β expression in human microglial HMC3 cells. (**A**) Immunoblotting for IL-6 and IL-1β (pro-IL-1β) in HMC3 cells, showing increased levels after TNF-α treatment ($n = 3$). (**B**, **C**) Quantifying IL-6 and IL-1β (pro-IL-1β) protein levels from (A), normalized to GAPDH. (**D**, **E**) ELISA measurements of IL-6 (**D**) and mature IL-1β (**E**) protein levels in the supernatant of HMC3 cells 24 h post-TNF-α treatment ($n = 3$). Error bars represent mean ± SEM. *$P < 0.01$, ANOVA with Student-Newman-Keuls post hoc analysis (B, C) or Student's t-test (D, E). IL-6, interleukin-6; IL-1β, interleukin-1 beta; TNF-α, tumor necrosis factor-alpha; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; SEM, standard error of the mean.

AAV2-shmTOR effectively reduces TNF-α-Induced increases in IL-6, IL-1β, and NF-κB signaling in HMC3 cells

To evaluate the efficacy of AAV2-shmTOR in mitigating TNF-α-induced inflammatory responses, we examined its impact on the production of proinflammatory cytokines and NF-κB signaling in HMC3 cells. Bright-field and fluorescence microscopy confirmed that AAV2-shmTOR and AAV2-GFP transduction were efficient and did not compromise cell viability, ensuring the reliability of subsequent analyses (Supplementary Fig. 2). Immunoblotting analysis showed that TNF-α treatment significantly elevated IL-6, IL-1β (pro-IL-1β), and mTOR protein levels in HMC3 cells (Fig. 3A, B). In contrast, treatment with AAV2-shmTOR effectively reduced these increased protein levels compared to control cells treated with AAV2-GFP (Fig. 3A, B).

Furthermore, measuring cytokine levels in the culture supernatant using ELISA confirmed that AAV2-shmTOR significantly decreased TNF-α-induced increases in IL-6 (Fig. 3C) and IL-1β(active-form) (Fig. 3D) 24 h post-treatment. The influence of AAV2-shmTOR on NF-κB signaling was also assessed; TNF-α treatment elevated phosphorylated NF-κB p65 (Ser 536) and total NF-κB p65 levels, indicating heightened signaling activity. However, AAV2-shmTOR significantly reduced phosphorylated and total NF-κB p65 levels, suggesting effective inhibition of NF-κB signaling (Fig. 3E, F). These findings demonstrate that AAV2-shmTOR can substantially dampen TNF-α-induced proinflammatory cytokine production and NF-κB signaling, highlighting its potential role in controlling inflammation in retinal microglial cells.

Conditioned medium from TNF-α-treated HMC3 cells transduced with AAV2-shmTOR reduces oxidative stress and cytotoxicity in ARPE cells.

In AMD, microglial inflammation significantly contributes to disease progression, particularly affecting the retinal pigment epithelium (RPE)[30,57,58]. To investigate the effects of inflammatory conditions on ARPE cells, conditioned media from TNF-α-treated HMC3 cells transduced with AAV2-GFP or AAV2-shmTOR were applied to ARPE cells. The experimental workflow is shown in Fig. 4A, detailing the preparation of the conditioned medium and its application to ARPE cells. Fluorescence microscopy of HMC3 cells 24 h after viral transduction and TNF-α treatment confirmed that AAV2-shmTOR and AAV2-GFP transduction were both highly efficient, as evidenced by robust GFP expression in fluorescence images. Bright-field microscopy revealed healthy cell morphology, indicating that viral transduction and TNF-α treatment did not compromise HMC3 cell viability (Supplementary Fig. 3). These results demonstrate that the HMC3 cells maintained their structural
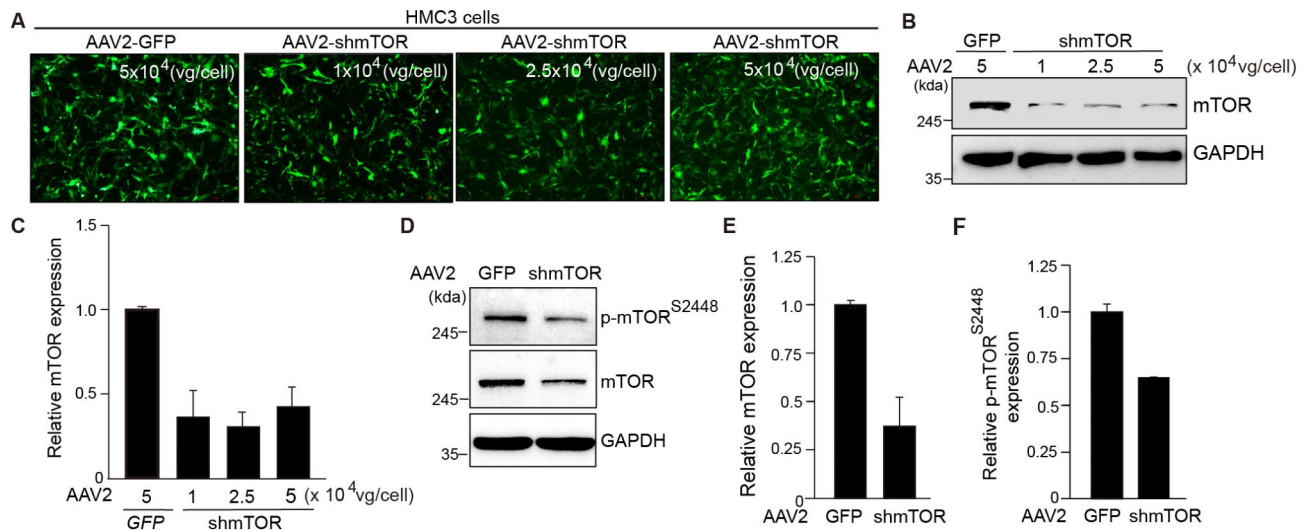
Fig. 2. Efficient AAV2-shmTOR-mediated knockdown of mTOR proteins in human microglial HMC3 cells. (**A**) GFP fluorescent images of HMC3 cells transduced with AAV2-GFP or AAV2-shmTOR at a multiplicity of infection (MOI). (**B**) Immunoblotting for total mTOR in HMC3 cells ($n = 2$). (**C**) Quantification of the immunoblot results from (B) normalized to GAPDH. (**D**) Immunoblotting for phosphorylated mTOR^{S2448} and total mTOR in HMC3 cells ($n = 2$). (**E**, **F**) Quantifying IL-6 and IL-1β protein levels from (D), normalized to GAPDH. AAV2, adeno-associated virus serotype 2; GFP, green fluorescent protein; shmTOR, short hairpin RNA targeting mTOR; mTOR, mechanistic target of rapamycin; p-mTOR^S2448, phosphorylated mTOR at serine 2448; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MOI, multiplicity of infection.

integrity and viability under experimental conditions, ensuring the reliability and consistency of the conditioned medium collected for downstream experiments. Using the OxyBlot assay, we evaluated protein oxidation as a marker of oxidative stress. ARPE cells treated with conditioned medium from AAV2-GFP + TNF-α-transduced HMC3 cells exhibited a significant increase in protein carbonylation, reflecting elevated oxidative stress (Fig. 4B, C). In contrast, ARPE cells treated with conditioned medium from AAV2-shmTOR + TNF-α-transduced HMC3 cells displayed markedly reduced protein carbonylation levels, suggesting a protective effect of AAV2-shmTOR against TNF-α-induced oxidative stress. Bright-field microscopy revealed morphological differences in ARPE cells among the experimental groups. ARPE cells exposed to conditioned medium from the AAV2-GFP + TNF-α group showed signs of cellular stress, such as compromised morphology and cell integrity, compared to the control group. Conversely, cells treated with conditioned medium from the AAV2-shmTOR + TNF-α group exhibited improved morphology and healthier cell conditions (Supplementary Fig. 4), consistent with the reduced oxidative stress observed in the OxyBlot assay. To further validate the protective effects of AAV2-shmTOR, an LDH assay was performed to assess cytotoxicity in ARPE cells. Conditioned medium from AAV2-GFP + TNF-α-treated HMC3 cells resulted in significantly higher LDH activity, indicating increased cytotoxicity. In contrast, conditioned medium from AAV2-shmTOR + TNF-α-treated HMC3 cells significantly reduced LDH activity, further supporting the conclusion that AAV2-shmTOR mitigates TNF-α-induced cytotoxic effects (Fig. 4D). These findings indicate that the conditioned medium from AAV2-shmTOR-treated cells reduces oxidative stress and minimizes cytotoxic effects, highlighting its potential therapeutic capacity in counteracting AMD-associated oxidative damage.

AAV2-shmTOR preserves endothelial tight junction integrity and barrier function in HUVECs exposed to TNF-α

Endothelial tight junction disruption, marked by altered ZO-1 staining and reduced protein expression, is associated with retinal vascular leakage and inflammation in AMD[55,56]. We investigated whether AAV2-shmTOR can protect endothelial tight junctions and barrier function in HUVECs exposed to TNF-α. Immunofluorescence imaging demonstrated that PBS-treated HUVECs, transduced with either AAV2-GFP or AAV2-shmTOR, retained intact tight junctions, as evidenced by apparent ZO-1 staining and distinct nuclear DAPI staining (Fig. 5A, B, E). In contrast, TNF-α-treated HUVECs transduced with AAV2-GFP displayed noticeable morphological damage to tight junctions, indicated by disrupted and weakened ZO-1 staining, reflecting compromised barrier function (Fig. 5C, E). However, AAV2-shmTOR-treated HUVECs exposed to TNF-α exhibited enhanced ZO-1 levels and maintained junction integrity (Fig. 5D, E). Immunoblotting corroborated these observations, showing elevated ZO-1 and decreased mTOR levels in the AAV2-shmTOR group compared to the TNF-α-AAV2-GFP group (Fig. 5F, G). To further examine the protective effects of AAV2-shmTOR on endothelial barrier function, the integrity of the actin cytoskeleton was analyzed in HUVECs exposed to TNF-α. Phalloidin staining revealed well-organized and intact actin filaments in PBS-treated cells transduced with either AAV2-GFP or AAV2-shmTOR (Fig. 5H, I). However, TNF-α treatment led to significant disorganization of the actin cytoskeleton in AAV2-GFP-transduced cells, as evidenced by reduced phalloidin intensity and disrupted
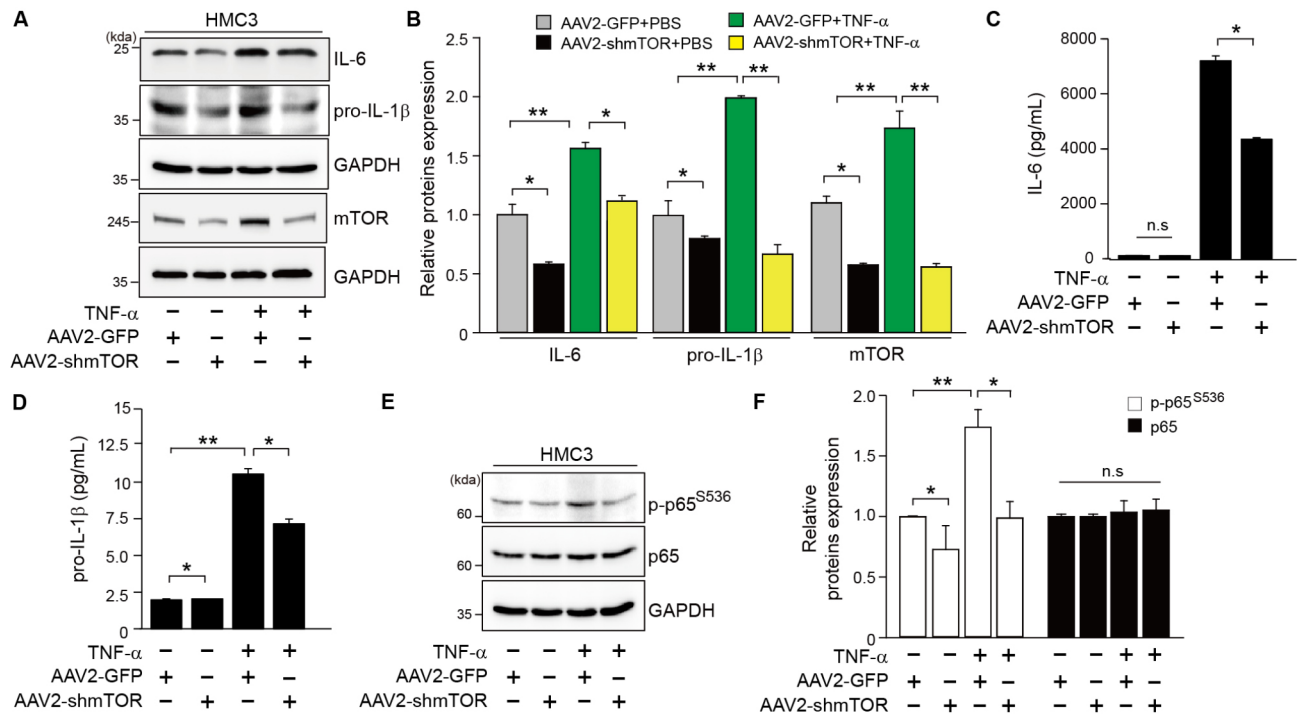
Fig. 3. AAV2-shmTOR inhibits IL-6 and IL-1β protein expression and activation of the NF-κB signaling pathway in human microglial HMC3 cells. (**A**) Immunoblotting analysis for IL-6, IL-1β (pro-IL-1β), and mTOR in HMC3 cells. (**B**) Quantification of the immunoblot results from (A), with protein levels normalized to GAPDH ($n = 3$). (**C**, **D**) ELISA measurements of IL-6 (**C**) and mature IL-1β (**D**) protein levels in the supernatant of HMC3 cells 24 h post-treatment ($n = 3$). (**E**) Immunoblotting for phosphorylated NF-κB p65 (Ser 536) and total NF-κB p65 in HMC3 cells. (**F**) Quantification of the immunoblot results from (E) normalized to GAPDH. Error bars represent mean ± SEM. *$P < 0.01$, ANOVA with Student-Newman-Keuls post hoc analysis. AAV2, adeno-associated virus serotype 2; shmTOR, short hairpin RNA targeting mTOR; IL-6, interleukin-6; IL-1β, interleukin-1 beta; mTOR, mechanistic target of rapamycin; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; p-NF-κB p65 S536, phosphorylated NF-κB p65 at serine 536; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SEM, standard error of the mean.

filament structures (Fig. 5J). In contrast, AAV2-shmTOR-transduced cells retained actin filament integrity under TNF-α exposure, demonstrating minimal cytoskeletal damage (Fig. 5K). These observations suggest that AAV2-shmTOR effectively mitigates TNF-α-induced actin cytoskeleton disruption, crucial for maintaining endothelial barrier function. Quantitative analysis of phalloidin intensity further confirmed these findings, showing significantly higher phalloidin intensity in AAV2-shmTOR-treated cells compared to AAV2-GFP-treated cells under TNF-α exposure (Fig. 5L). These findings underscore AAV2-shmTOR's potential to shield endothelial tight junctions from TNF-α-induced damage, preserving barrier function and potentially mitigating AMD-related vascular issues.

Discussion

The primary objective of this study was to evaluate whether CRG-01 (AAV2-shmTOR), an adeno-associated virus-based gene therapy targeting mTOR signaling, could mitigate both inflammation and oxidative stress in an AMD-like environment, with a particular focus on the inflammatory response induced by TNF-α. Inflammation and oxidative stress are closely linked processes that play pivotal roles in AMD progression, contributing to retinal cell dysfunction and degeneration. Our findings demonstrate that CRG-01 has the potential to modulate critical inflammatory pathways while also addressing oxidative stress, highlighting its therapeutic promise in targeting the multifaceted mechanisms underlying AMD-related retinal damage. By downregulating mTOR signaling, CRG-01 aims to provide comprehensive protection to retinal cells, addressing the cumulative effects of these damaging processes characteristic of AMD.

Our results suggest that CRG-01 can effectively reduce inflammatory responses across these cell types, indicating its potential as a therapeutic agent for AMD. By downregulating mTOR signaling, CRG-01 may attenuate the TNF-α-induced inflammatory response, protecting retinal cells from inflammation-induced damage. This study highlights the importance of targeting multiple pathways in AMD, as our in vitro model suggests that effective AMD treatments may require addressing inflammation at several cellular levels to prevent disease progression.

This study underscores the potential of AAV2-shmTOR as an innovative therapeutic approach for age-related macular degeneration (AMD), addressing critical mechanisms such as inflammation and oxidative stress (Fig. 6).
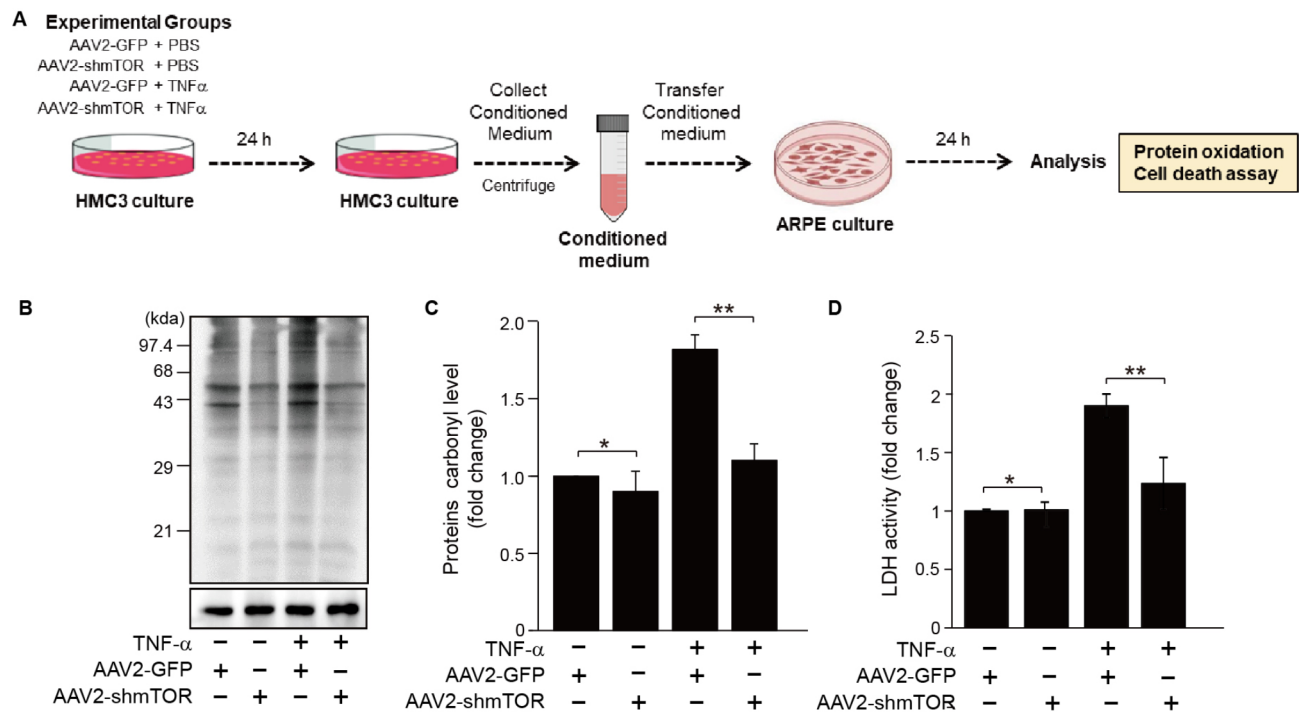
Fig. 4. Conditioned medium derived from AAV2-shmTOR inhibits TNF-α-induced protein oxidation in ARPE cells. (**A**) The schematic diagram is for preparing a conditioned medium and treating ARPE cells. (**B**) Measurements of protein oxidation by OxyBlot in ARPE cells exposed to conditioned medium. (**C**) Quantification of carbonylation levels OxyBlots results from (B). (**D**) Cytotoxicity was measured using LDH activity assay ($n = 3$). Error bars represent mean ± SEM. *$P < 0.01$, Student's t-test. AAV2, adeno-associated virus serotype 2; shmTOR, short hairpin RNA targeting mTOR; TNF-α, tumor necrosis factor-alpha; ARPE, adult retinal pigment epithelial cells; LDH, lactate dehydrogenase; SEM, standard error of the mean.

Current AMD treatments, including anti-VEGF agents like ranibizumab (Lucentis) and aflibercept (Eylea), are effective in managing neovascularization[18–21,28] but do not address chronic inflammation and oxidative stress, which are significant contributors to retinal degeneration and AMD progression. The ability of AAV2-shmTOR to target these underlying factors offers valuable new insights and represents a promising advancement in the development of AMD therapies.

In this study, we used the microglia, endothelial cells, and ARPE cells as an in vitro model, which provides a comprehensive approach to studying AMD, as it involves complex intercellular signaling. Microglia, upon activation, release pro-inflammatory cytokines like IL-1β and IL-6, which disrupt the function of neighboring endothelial and ARPE cells. This creates a chronic inflammatory environment that increases vascular permeability, damages the blood-retina barrier, and exacerbates oxidative stress. Endothelial cells model the BRB's breakdown, while ARPE cells simulate the oxidative and inflammatory damage to retinal support functions, both critical in AMD progression. The combined use of these three cell types more accurately reflects AMD pathology by replicating the cumulative intercellular damage characteristic of the disease, allowing a deeper understanding of AMD mechanisms and providing a platform to test therapies that target multiple pathways.

To simulate the indirect inflammatory effects on retinal pigment epithelial (RPE) cells, we used TNF-α-stimulated microglia-derived conditioned media, which allowed us to investigate paracrine inflammatory signaling in RPE cells. This approach closely reflects the retinal environment in AMD, where activated microglia and compromised BRB integrity can significantly affect RPE cell function, leading to oxidative stress and cellular damage.

Our results demonstrate that AAV2-shmTOR effectively reduces the expression of pro-inflammatory cytokines IL-6 and IL-1β in retinal cells following TNF-α stimulation. Additionally, TNF-α treatment significantly increases the phosphorylation of p65 at Serine 536 (p-p65S536), indicating NF-κB activation, which drives the production of these cytokines[56,57]. TNF-α is a pivotal mediator of inflammation in AMD, known for disrupting endothelial integrity and initiating inflammatory pathways that accelerate disease progression[12,31,58]. Although IL-1β secretion typically requires an initial priming step followed by a secondary stimulus such as ATP, recent evidence suggests microglial cells can also release ATP under specific conditions. For example, previous studies reported ATP release from LPS-stimulated microglial cells[59–61] demonstrated ATP release mediated by intracellular Ca²⁺ upon glutamate stimulation in microglia. These findings support the possibility that TNF-α alone could induce ATP release from microglial cells, facilitating IL-1β secretion observed in our study. Further investigations are necessary to confirm the specific mechanism underlying ATP-mediated IL-1β release in HMC3 microglial cells. By mitigating these cytokines and preserving endothelial tight junction
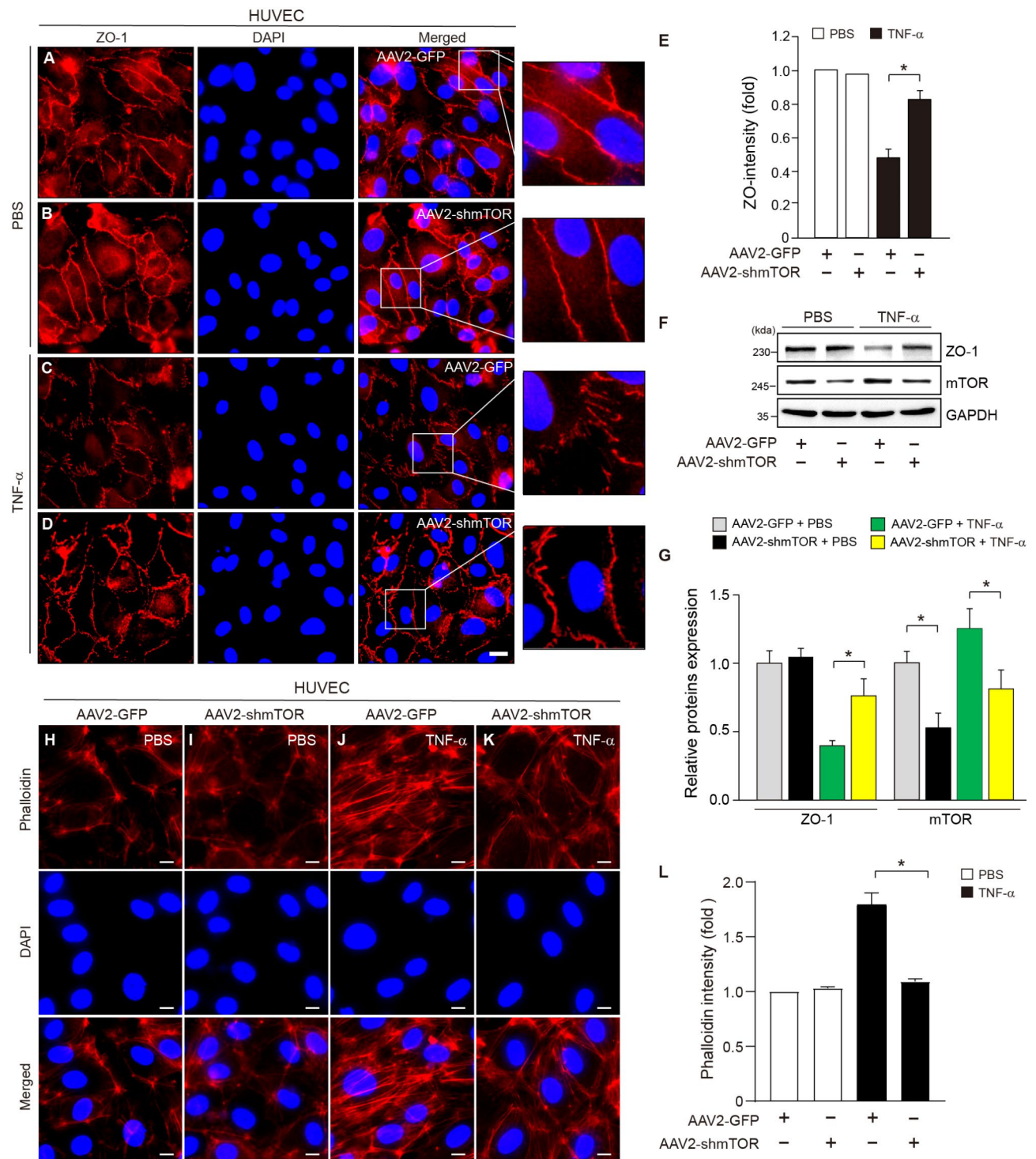
Fig. 5. AAV2-shmTOR protects endothelial tight junctions from TNF-α-induced morphological damage and maintains barrier function in HUVECs. (**A-D**) Immunofluorescence imaging of HUVECs showing cell nuclei (DAPI, blue) and tight junctions (ZO-1, red). Boxed areas in panels C, F, I, and L are shown at higher magnification on the right panels. Scale bar = 50 μm. (**E**) Quantification of ZO-1 staining intensity in HUVEC cells, expressed in arbitrary units (a.u.) ($n = 3$). (**F**) Immunoblotting for ZO-1 and mTOR in HUVEC cells. (**G**) Quantification of the immunoblot results from (F) normalized to GAPDH ($n = 3$). (**H-K**) Immunofluorescence imaging shows cell nuclei (DAPI, blue) and actin filaments (phalloidin, red). Scale bar = 50 μm. (**L**) Quantification of Phalloidin staining intensity in HUVEC cells, expressed in arbitrary units (a.u.) ($n = 3$). Error bars represent mean ± SEM. *$P < 0.05$, unpaired two-tailed Student's t-test. AAV2, adeno-associated virus serotype 2; shmTOR, short hairpin RNA targeting mTOR; TNF-α, tumor necrosis factor-alpha; HUVECs, human umbilical vein endothelial cells; ZO-1, zonula occludens-1; DAPI, 4',6-diamidino-2-phenylindole; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SEM, standard error of the mean.
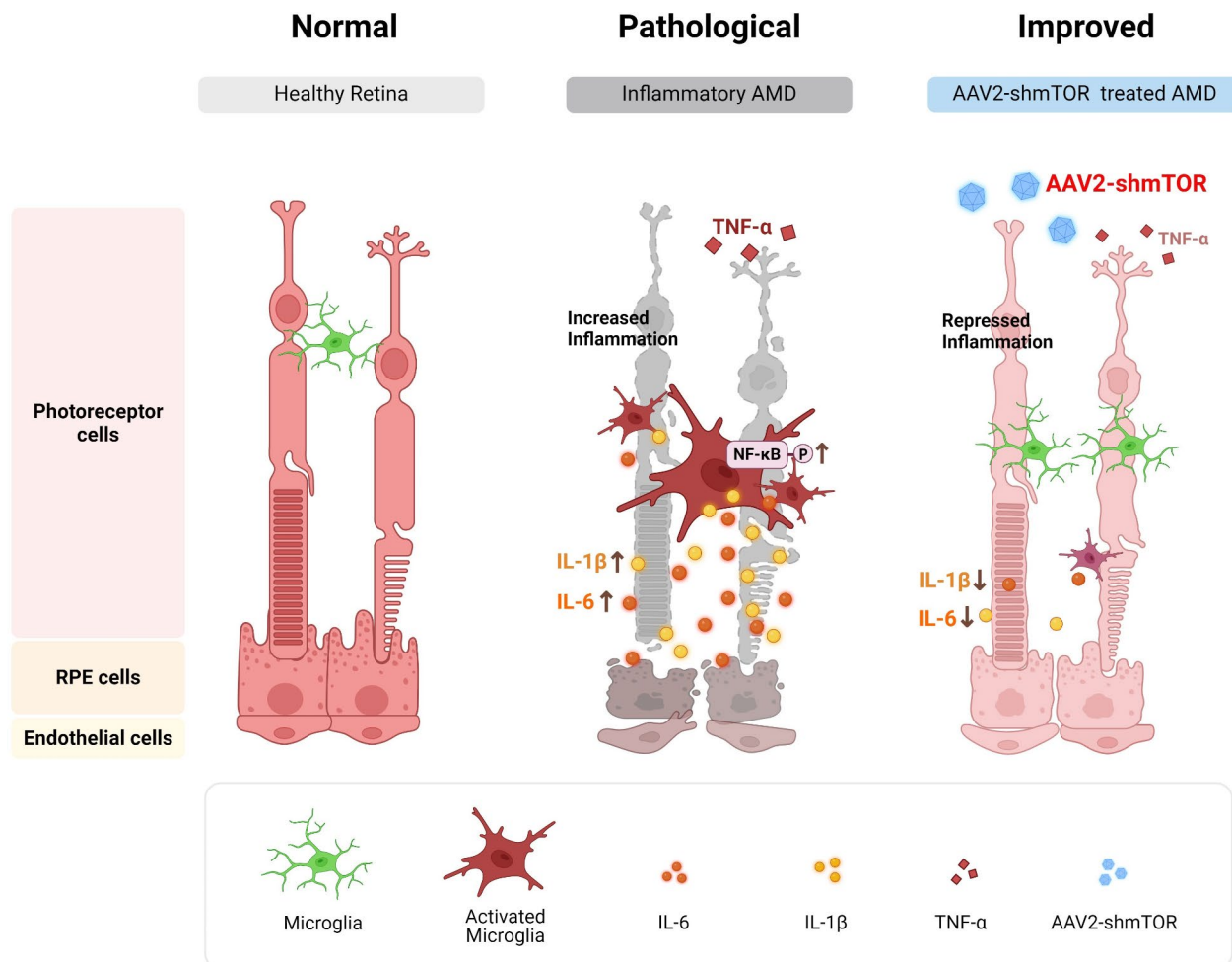
Fig. 6. Schematic Representation of AAV2-shmTOR Therapy in Age-Related Macular Degeneration (AMD). Normal Retina (Left): Illustrates a healthy retina with intact photoreceptor cells, retinal pigment epithelium (RPE), and endothelial cells. Microglia are shown resting, contributing to maintaining homeostasis without significant inflammation. **Pathological Retina (Middle)**: Depicts an inflamed retina affected by AMD, where TNF-α triggers microglia activation, leading to increased secretion of pro-inflammatory cytokines, including IL-1β and IL-6. This inflammatory response activates the NF-κB signaling pathway, resulting in cellular damage and disruption of retinal structure. **AAV2-shmTOR Treated Retina (Right)**: Demonstrates the therapeutic effect of AAV2-shmTOR in reducing retinal inflammation. After treatment, AAV2-shmTOR significantly reduces the expression of IL-1β and IL-6, restoring the structural integrity of retinal cells and reducing inflammatory damage. Green microglia: Resting microglia; Red microglia: Activated microglia; Orange/Yellow dots: IL-1β and IL-6 cytokines; Red diamond: TNF-α; Blue particles: AAV2-shmTOR. AAV2, adeno-associated virus serotype 2; shmTOR, short hairpin RNA targeting mTOR; AMD, age-related macular degeneration; IL-1β, interleukin-1 beta; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha; RPE, retinal pigment epithelium. Data Availability Statement.

integrity, AAV2-shmTOR offers a multi-faceted protective approach for the retina, promoting overall retinal health. Furthermore, our study revealed that conditioned media from AAV2-shmTOR-treated microglial cells significantly decreased oxidative stress and cytotoxicity in retinal pigment epithelial (RPE) cells. This dual action against inflammation and oxidative stress provides a strategic advantage over current anti-VEGF treatments that primarily target angiogenesis but fail to address these additional pathogenic processes.

In summary, the multi-cellular approach used in this study provides a more comprehensive view of AMD pathology, enhancing the relevance of our findings. CRG-01 shows promise in modulating inflammation within this complex cellular landscape, supporting its further investigation as a gene therapy for AMD. Future studies will focus on in vivo assessments to validate these findings and further explore CRG-01's therapeutic potential.

For patients inadequately responsive to traditional anti-VEGF therapies, AAV2-shmTOR presents a promising alternative. Non-responders pose significant clinical challenges, often experiencing disease progression despite treatment[27,28]. This may be due to AMD's complex nature, involving not only neovascularization but also chronic inflammation and oxidative damage[27,28]. By inhibiting the mammalian target of the rapamycin (mTOR) pathway—a key regulator of cellular stress responses, inflammation, and tissue degeneration—AAV2-

shmTOR simultaneously tackles multiple disease mechanisms[43,45,55,58,62–65]. Inhibiting mTOR effectively reduces both angiogenesis and inflammatory responses, offering broader therapeutic benefits for AMD patients unresponsive to VEGF inhibition alone[55,62,66]. In addition to these benefits, comparing AAV2-shmTOR with recent advancements in alternative AMD treatments can highlight its unique advantages. Understanding how AAV2-shmTOR differentially engages inflammatory pathways or preserves RPE cell health compared to other therapies underscores its potential as a comprehensive treatment strategy. Further research into the molecular mechanisms by which AAV2-shmTOR reduces inflammation and oxidative stress could lead to optimized designs and applications, potentially increasing its efficacy or minimizing side effects.

A significant benefit of AAV-based gene therapy over anti-VEGF treatments is its potential for lasting effects from a single administration. Anti-VEGF therapies require frequent intraocular injections, which are burdensome for patients and caregivers and associated with risks such as increased intraocular pressure[24,67–69]. AAV-based therapies deliver therapeutic genes to retinal cells, allowing for sustained protein expression over months or even years, which reduces the need for repeated interventions and thus improves patient compliance[70,71].

The use of AAV vectors in gene therapy has gained traction due to their favorable safety profile and efficacy. Compared to other viral vectors such as adenovirus, lentivirus, and retrovirus, AAV is non-pathogenic and presents a lower risk of harmful immune reactions or integration into the host genome, making it a safer candidate for long-term therapy, especially in sensitive tissues like the retina[72,73]. To address concerns about immunogenicity and potential toxicity, we conducted a 13-week preclinical toxicity study of AAV2-shmTOR in non-human primates. While this data is still being prepared for publication, preliminary findings are promising, showing no significant signs of toxicity in major organs like the eyes, brain, and liver. An initial transient T cell immune response was observed post-administration, but this quickly subsided with no long-term effects, suggesting that AAV2-shmTOR is effective and safe for AMD gene therapy.

Expanding AAV2-shmTOR's application beyond AMD to other retinal diseases characterized by inflammation and oxidative stress, such as diabetic retinopathy and retinitis pigmentosa, could broaden its therapeutic scope[74,75]. The pathological similarities among these conditions highlight AAV2-shmTOR's potential to benefit a broader patient population through targeted action on shared disease processes.

In conclusion, AAV2-shmTOR represents a promising treatment option for AMD, particularly for patients who do not respond adequately to anti-VEGF therapies. By targeting vital pro-inflammatory cytokines like IL-6, IL-1β, and TNF-α, AAV2-shmTOR addresses critical drivers of AMD progression, including inflammation and oxidative stress. This multifaceted approach differentiates it from current treatments that focus solely on angiogenesis. Comprehensive clinical studies are essential to confirm the efficacy and safety of AAV2-shmTOR and to optimize its use across AMD and other retinal diseases. The potential for long-lasting effects of AAV-based gene therapy could significantly reduce treatment burdens, enhance patient compliance, and provide a holistic solution to retinal degeneration.

Materials and methods
Chemicals and reagents
Recombinant Human TNF-alpha protein (210-TA-020/CF) was purchased from R&D Systems (Minneapolis, MN). The following primary antibodies were used in this study: phospho-mTOR (S2448; ab109268, Abcam, Cambridge, UK; 1:1000), mTOR (2983 S; Cell Signaling Technology, Danvers, MA; 1:2000), NF-κB (8242 S; Cell Signaling Technology; 1:1000), phospho-NF-κB (Ser536; 3033 S, Cell Signaling Technology; 1:500), IL-6 (MBS9606748, MyBioSource, San Diego, CA; 1:1000), IL-1β (P420B, Invitrogen, Waltham, MA; 1:500), ZO-1 (33–9100; Invitrogen, USA; 1:1000), Phalloidin-TRITC (P1951, Sigma, St. Louis, MO; 1:1000), GAPDH (sc-166574, Santa Cruz Biotechnology, Dallas, TX; 1:2000), secondary antibody Alexa Fluor 594 (A32742, Invitrogen, USA; 1:1000).

Virus vector development
We have previously described the generation of rAAV2-shmTOR-SD and rAAV2-shCon-SD from the precursor plasmids pAAV-shmTOR-GFP and pAAV-shCon-GFP. In brief, the mTOR-inhibiting shRNA sequence (5'-GAAUGUUGACCAAUGCUAU-3') and a control shRNA sequence (5'-AUUCUAUCACUAGCGUGAC-3') were inserted into a self-complementary AAV2 vector. These sequences were driven by an H1 promoter, with stuffer DNA derived from the human UBE3A gene added for structural purposes. All viral vectors used in this study were supplied by CdmoGen Co., Ltd., located in Cheongju, Korea.

Cell culture and infection
HMC3 cells (CRL-3304; ATCC, Manassas, VA) were obtained from ATCC (Manassas, VA) and cultured in Minimum Essential Medium Eagle with 1 mM sodium pyruvate and 1.5 mg/mL sodium bicarbonate (LM007-54; Welgene Inc., Gyeongsan, Korea) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. ARPE-19 cells (CRL-2302; ATCC) were sourced from ATCC and maintained in Dulbecco's Modified Eagle's Medium (DMEM)/Nutrient Mixture F-12 (DMEM/F12, LM002-04; Welgene) supplemented with 10% FBS and 1% penicillin-streptomycin. HUVECs (C-12200; PromoCell, Germany) were purchased from PromoCell (Germany) and cultured in EBM-2 Endothelial Cell Growth Basal Medium (CC-3156; Lonza) supplemented with FBS, hydrocortisone, hFGF-B, hEGF, and Heparin from the EGM-2 Endothelial SingleQuots Kit (CC-4176; Lonza). Experiments were conducted in dishes coated with 0.1% gelatin (LS023-01; Welgene, Inc., Gyeongsan, Korea). All cells were maintained at 37℃ in a humidified atmosphere with 5% CO2. We used cells at passage numbers between 5 and 10 to ensure consistency and minimize variability in cellular responses. The cells were seeded into appropriate dishes 6 h before virus infection. The cells were seeded into appropriate dishes 6 h before virus infection. The virus was diluted in complete media to the specified infection titer for each

experiment. Unless otherwise specified, infections were performed with the respective viral vector at 1×10^4 vg/cell. The diluted virus was added dropwise to each well, and the plates were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

Immunocytochemistry

HUVECs were seeded onto a 4-well glass slide (154526; Lab-Tek® II, Thermo Fisher Scientific, USA) at a density of 8×10^4 cells per well. After infection with either the AAV2-shmTOR or AAV2-GFP vector for 72 h, the cells were treated with 50 ng/mL of TNF-α for 6 h. The cells were then fixed with 4% paraformaldehyde and washed with PBS. Permeabilization was performed using 0.1% Triton X-100 and 0.5% BSA for 15 min, followed by incubation in 5% normal goat serum blocking solution (S-1000-20; Vector Laboratories, USA) in PBST for 30 min. After overnight incubation at 4°C with ZO-1 primary antibody, the cells were washed with 0.5% BSA in PBS, followed by incubation at room temperature for 2 h with the secondary antibody Alexa Fluor 594. For F-actin staining, the cells were treated with Phalloidin-TRITC for 1 h at room temperature. The cells were counterstained with DAPI (H-1200; Vector Laboratories, USA) for nuclear visualization. Fluorescence microscopy was performed, and images were captured using a Ts2-FL microscope (Nikon, Tokyo, Japan). Negative controls were included in our immunohistochemical experiments to ensure the specificity and accuracy of staining. We omitted the primary antibody for the negative controls while keeping all other conditions constant. This allowed us to confirm that any observed staining was specific to the target antigen and not due to nonspecific binding.

Western blotting

HMC3 cells were seeded in 6-well plates at a density of 2.0×10^5 cells per well and infected with either AAV2-shmTOR or AAV2-GFP virus. After 72 h, cells were treated with 200 ng/mL TNF-α in serum-free MEM for 24 h. Cells were washed with PBS and lysed in RIPA buffer (9806, Cell Signaling Technology) containing phenylmethanesulfonyl fluoride (PMSF; 93482, Sigma-Aldrich, MO, USA). Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, MA). Equal amounts of protein were loaded onto an SDS-PAGE gel, separated, and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% bovine serum albumin (BSA; A7030, Sigma-Aldrich) in Tris-buffered saline containing Tween 20 (TBST) at room temperature for 1 h and incubated overnight at 4 °C with the respective primary antibodies. After washing with TBST, membranes were incubated with secondary antibodies for 1 h. Detection was performed using a chemiluminescence system (LuminoGraph II, ATTO, Tokyo, Japan), and band intensity was quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).

ELISA assay

After 72 h of infection with AAV2-shmTOR or AAV2-GFP, cells were treated with 200 ng/mL of TNF-α for 24 h (IL-6 measurement) or 72 h (IL-1β measurement). The supernatant was collected and centrifuged at 1000 x RCF (g) for 5 min to remove detached cells and debris. IL-6 and IL-1β concentrations were measured using the Human IL-6 Quantikine ELISA Kit (D6050B, R&D Systems) and the Human IL-1β/IL-1F2 Quantikine ELISA Kit (DLB50, R&D Systems), respectively, following the manufacturer's protocol.

Protein oxidation

HMC3 cells were infected with AAV2-shmTOR or AAV2-GFP for 72 h and treated with TNF-α for 48 h. A conditioned medium was collected from these cells to incubate ARPE-19 cells for 72 h. After incubation, ARPE-19 cells were lysed with RIPA buffer supplemented with PMSF, and the protein concentration was measured using the Pierce™ BCA Protein Assay Kit. Equal amounts of protein (3 μg/μL) were used for further analysis. The OxyBlot Protein Oxidation Detection Kit (S7150, Temecula, CA) was used following the manufacturer's instructions to detect protein carbonyl groups introduced by oxidative reactions. Briefly, 5 μL of each protein sample was denatured with 5 μL of 12% SDS and derivatized with 10 μL of DNPH (2,4-dinitrophenylhydrazine) solution for 15 min. After derivatization, 7.5 μL of neutralization solution was added, and the samples were subjected to immunoblot analysis.

LDH cytotoxicity assay

According to the manufacturer's instructions, LDH activity was measured using the CyQUANT™ LDH Cytotoxicity Assay (C20300, Invitrogen). Briefly, 50 μL of supernatant was collected from ARPE-19 cells incubated with HMC3 conditioned medium and mixed with 50 μL of the Reaction Mixture. After incubating for 30 min, Stop Solution was added, and absorbance was measured at 490 nm (A490) and 680 nm (A680) using a microplate spectrophotometer (Epoch, BioTek, VT, USA). Cytotoxicity was calculated with the following formula:

% cytotoxicity = (compound − treated LDH activity–spontaneous LDH activity) / (maximum LDH activity–spontaneous LDH activity) $\times$ 100.

Statistical analysis

ImageJ (version 1.53a, https://imagej.nih.gov/ij/) was used to quantify band intensities from immunoblots. Statistical significance was evaluated using an unpaired two-tailed Student's t-test, one-way ANOVA followed by Student–Newman–Keuls post hoc analysis, or two-way ANOVA. Quantitative data are presented as means ± SEM, and differences were considered significant at $p < 0.05$. All experiments were repeated at least three times.

Data availability

The datasets generated and analyzed during the current study are included in this published article and its supplementary information files. Any additional data can be requested from the corresponding author upon reasonable request.

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Author contributions

J.K., S.Y.M., H.G.K., and H.J.K. conducted the in vitro experiments and analyzed the resulting data. S.H.S.L., J.S.C., and K.P. reviewed and edited the manuscript, offering technical advice regarding the experimental design. K.P. and S.Y.W. were responsible for designing the study, analyzing the data, and writing the manuscript. All authors have read and approved the final version of the manuscript for publication.

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Declarations

Competing interests
The authors declare no competing interests.

Ethics approval and consent to participate
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