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TAK1 blockade as a therapy for retinal neovascularization

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ABSTRACT

Retinal neovascularization, or pathological angiogenesis in the retina, is a leading cause of blindness in developed countries. Transforming growth factor-β-activated kinase 1 (TAK1) is a mitogen-activated protein kinase kinase kinase (MAPKKK) activated by TGF-β1 and other proinflammatory cytokines. TAK1 is also a key mediator of proinflammatory signals and plays an important role in maintaining vascular integrity upon proinflammatory cytokine stimulation such as TNFα. However, its role in pathological angiogenesis, particularly in retinal neovascularization, remains unclear. Here, we investigate the regulatory role of TAK1 in human endothelial cells responding to inflammatory stimuli and in a rat model of oxygen-induced retinopathy (OIR) featured retinal neovascularization. Using TAK1 knockout human endothelial cells that subjected to inflammatory stimuli, transcriptome analysis revealed that TAK1 is required for activation of NFκB signaling and mediates its downstream gene expression related to endothelial activation and angiogenesis. Moreover, pharmacological inhibition of TAK1 by 5Z-7-oxozeaenol attenuated angiogenic activities of endothelial cells. Transcriptome analysis also revealed enrichment of TAK1-mediated NFκB signaling pathway in the retina of OIR rats and retinal neovascular membrane from patients with proliferative diabetic retinopathy. Intravitreal injection of 5Z-7-oxozeaenol significantly reduced hypoxia-induced inflammation and microglial activation, thus attenuating aberrant retinal angiogenesis in OIR rats. Our data suggest that inhibition of TAK1 may have therapeutic potential for the treatment of retinal neovascular pathologies.

1. Introduction

Retinal neovascularization is a severe complication of a number of vascular retinopathies including proliferative diabetic retinopathy, central retinal vein occlusion, and retinopathy of prematurity [1]. Retinal vascular abnormalities are observed in these diseases, resulting in ischemia, subsequent hypoxia-induced neovascularization, and leukostasis, which are principal causes of vision loss or blindness [2]. Retinal neovascularization shares similar characteristics to pathological angiogenesis elsewhere in the body, exhibiting features including

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hypoxia, endothelial proliferation and migration, elevated vascular permeability and inflammation; all processes in which vascular endothelial growth factor (VEGF) plays a crucial role [3]. Recently, the advent of ocular anti-VEGF therapies has been a significant advance in mitigating the risk of vision loss associated with retinal neovascularization. Despite this success, there is increasing evidence that some patients do not respond well to anti-VEGF treatments [4]. Therefore, it is necessary to seek other therapeutic targets.

The inflammatory response in retina during ischemic retinopathy plays critical roles in retinal neovascularization [5]. While retinal hypoxia may serve a protective role by promoting retinal vascular remodeling, it can also induce several pathological conditions, such as retinal neovascularization, due to the resulting excessive inflammatory response. Several studies have reported increased expression of various proinflammatory cytokines, chemokines, pro-angiogenic factors and adhesion molecules in patients with retinal neovascularization, such as tumour necrosis factor α (TNFα), interleukin-1 (IL1), chemokine (C-X-C motif) ligand 8 (CXCL8; known as IL-8), vascular endothelial growth factor (VEGF), as well as intercellular adhesion molecule-1 (ICAM-1 and VCAM-1) [6–10]. TNFα was found to suppress the expression of tight junction proteins and is required for VEGF-mediated hyperpermeability in endothelial cells and the breakdown of the blood-retinal barrier in patients with diabetic retinopathy (DR) [11,12]. Interleukins are also primary mediators of inflammation that can promote angiogenesis via induction of proangiogenic factors such as VEGF [4]. Moreover, TNFα and IL-1 stimulates expression of ICAM-1, CXCL8 and VEGF in endothelial cells and microglia cells, both of which promote progression of retinal neovascularization [13,14]. A key role for inflammatory mediators is highlighted by their growing use as prognostic markers of the severity of proliferative diabetic retinopathy (PDR) [5]. In line with these findings, our previous study analyzing retinal transcriptome data from OIR rats and patients with proliferative diabetic retinopathy also pinpointed that immune responses and inflammation are the major biological processes during ischemia-induced retinal neovascularization [15].

Transforming growth factor-β-activated kinase 1 (TAK1), a member of the mitogen-activated protein kinase (MAPK) family, is a critical serine/threonine kinase in several cellular signaling pathways. Such pathways can be activated by diverse proinflammatory stimuli including TNFα, IL1, transforming growth factor beta (TGFβ), or toll-like receptor (TLR) ligands. The engagement of TAK1 in turn activates downstream signaling pathways, including nuclear factor kappa B (NFκB) and MAPK p38, extracellular signal-regulated kinase (ERK), and c-Jun kinase (JNK) signaling, modulating inflammatory responses and cell survival [16–18]. TAK1 inhibition has been shown to reduce TNFα and CXCL8 secretion in leukocytes [19] and to suppress prostaglandin-endoperoxide synthase 2 (PTGS2; known as cyclooxygenase-2, COX-2), ICAM-1, and VEGF expression in cancer cells through NFκB pathway [20]. Expression of TAK1 provides protection for endothelial cells against TNFα-induced apoptosis under inflammatory conditions [21]. Indeed, TAK1 deletion causes embryonic lethality as the capacity to prevent TNFα-induced endothelial cell death and vessel regression is lost. Moreover, TAK1 is also important for supporting TNFα-independent vascular formation and endothelial migration. These broad roles for TAK1 suggest that endothelial TAK1 inhibition may be a useful alternate anti-angiogenesis target in retinal disease [21,22].

We hypothesized that TAK1 plays a crucial role in pathological angiogenesis by mediating the endothelial activation under inflammatory conditions and TAK1 inhibition can suppress retinal neovascularization. To specifically test the hypothesis and delineate the potential molecular insight of TAK1 mediates common pathways shared between inflammation and angiogenesis in endothelial cells, we generated the TAK1 knockout human endothelial cells. Using this line and subjected to TNFα, our data confirmed that TAK1 is required to activate NFκB signaling and induce transcription of its downstream genes, such as *CXCL8, VEGF, PTGS2* and *ICAM-1*, which is associated with endothelial activation and angiogenesis. Angiogenic assay further revealed that pharmacological inhibition of TAK1 by 5Z-7-oxozeaenol attenuated angiogenic activities of endothelial cells. Intravitreal injection of 5Z-7-oxozeaenol significantly reduced inflammation and microglial activation, thus attenuating aberrant retinal angiogenesis in oxygen-induced retinopathy (OIR) rats. Our findings demonstrate that TAK1 is an important regulator of angiogenesis in retinal endothelial cells that collectively contribute to retinal neovascularization.

2. Materials and methods

2.1. Cell culture

Telomerase-immortalized human microvascular endothelial cell (passage 5–10; TIME; CRL-4025™) and primary human retinal microvascular endothelial cell (passage 3–5; HRMEC; ACBRI 181V) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and Cell Systems (Kirkland, WA, USA). Endothelial cells were cultured in EBM™ Plus Basal Medium supplied with EGM™ Plus SingleQuots™ supplements (CC-5035; Lonza, Basel, Switzerland). All cell lines were maintained in a humidified incubator at 37 °C and 5 % CO_2. Cell lines were mycoplasma free tested by MycoAlert™ Mycoplasma Detection Kit (LT07; Lonza).

2.2. Generation of TAK1-KO TIME Cell Line

Three sgRNAs targeting the exonic regions of the human TAK1 gene (Table S1. Key Resources table) were designed on CHOPCHOP (https://chopchop.cbu.uib.no). The annealed oligos ordered from the Integrated DNA Technologies (IDT; Coralville, IA, USA) representing the various sgRNAs were cloned into the BsmBI sites (R0580S; New England Biolabs; Ipswich, MA, USA) of the lentiCRISPRv2 plasmid (52961; Addgene; Watertown, MA, USA). The plasmid was validated by Sanger sequencing with 3500 Series Genetic Analyzers (Thermo Fisher Scientific; Waltham, MA, USA). HEK293FT cells (passage 10; R70007; Life Technologies Australia, Mulgrave, VIC, Australia) were plated at 1×10^7 cells per T75 flask one day prior to transfection. Lentivirus was made by co-transfecting lentiCRISPRv2 (15 μg), pCMV Δ8.91 (15 μg) and pMDG plasmids (1.5 μg) (RNAiCore; Academia Sinica, Taipei, Taiwan) in HEK293FT cells using Lipofectamine 2000 (11668019; Life Technologies Australia). Media was changed 5 h post transfection. The virus-contained media was harvested and filtered through a 0.45 μm PDVF syringe filter (SLHV033RS; Merck Millipore; Burlington, MA, USA) to remove cell debris 48 h post transfection. Lentivirus aliquots were precipitated by 5x PEG-it (LV810A-1; System Biosciences; Palo Alto, CA, USA) at 4 °C overnight and centrifugated at 1500*g* at 4 °C for 30 min. After discard of supernatant, the lentivirus was resuspended within opti-MEM and ready to use. Wild type TIME cells (passage 5; 7.5×10^5) were plated at T75 flask before transduction. When grow cells with 50 % confluence, they were transduced by TAK1 sgRNA lentiCRISPRv2 lentivirus with polybrene (8 μg/mL) (H9268; Sigma-Aldrich; St. Louis, MO, USA) for 72 h. Medium containing 0.5 μg/mL puromycin (P8833; Sigma-Aldrich) was then replaced to select stable clones for another 72 h then subsequently cultured as the stable TAK1 KO cell lines.

2.3. Single colony selection of TAK1-KO cell lines

Once the selected TAK1 KO cell lines (passage 2) reached 80 % confluence, the cells were trypsinized from the stable cell pool by TrypLE Express Enzyme (1X) (12605028; Life Technologies Australia) and braked up any clumps into individual cells. The TAK1-KO cells were then diluted to density of 5 cells/mL and resuspended with 100ul of which in each well in 96-well plate. After expanded for 7–14 days, picked up the colonies that come from single cell and subsequently cultured as the single colony selected TAK1-KO cell line in culture dishes by trypsinization.

2.4. Identification of TAK1 indels in TAK1-KO lines

Genomic DNA was isolated from wild type and TAK1-KO TIME cell lines using Quick-DNA Microprep Kit (D3021; Zymo Research; Irvine, CA, USA) according to the manual. Extracted genomic DNA was used as a PCR template to amplify the target of the TAK1 sgRNA using sequence specific primers (Table S1). Briefly, PCR reactions were made up to 25 μL comprising 12.5 μL Q5 Hot Start High-Fidelity 2X Master Mix (M0494; New England Biolabs), 1.25 μL of forward and reverse primers and diluted the gDNA, under thermocycling conditions of 98 °C initial denaturation for 30 s, and 30 cycles of 98 °C denaturation for 10 s, 65 °C annealing for 30 s, and 72 °C extension for 30 s with a 72 °C final extension for 2 min. PCR amplification product was validated using electrophoresis using 1 % agarose gel (A6361; Sigma-Aldrich). The gDNA was cleaned and concentrated by Quick-DNA Microprep Kit (D3021; Zymo Research). Following the first-round PCR amplification, a second round of sequencing PCR was performed. The second-round PCR products were subsequently purified using Agencourt AMPure XP (A63881; Beckman Coulter; Brea, CA, USA) 1.8X paramagnetic bead cleanup. The PCR samples were denatured at 98 °C for 5 min and sequenced by Sanger sequencing.

2.5. RNA-seq analysis

Analysis was undertaken in triplicate on total RNAs (1 μg) from normoxic and OIR rats at P18 or from TIME cells (passage 4–7) treated with TNFα (10 ng/mL for 10 min), prepared as per the manufacturers' instructions. cDNA libraries from animal tissues were sequenced (50 bp single end reads) by Australian Genome Research Facility (AGRF, Melbourne, VIC, Australia) using an Illumina Hiseq-2500 RNA-seq. cDNA libraries from cells were sequenced (50 bp pair end reads) by GENEWIZ (Suzhou, China) using an Illumina Novaseq PE150 platform. The adapter sequences and the low-quality reads in the raw fastq files of single-end or pair-end reads were removed and dropped by Trimgalore 0.4.4 (Babraham Bioinformatics). Filtering parameters were set as follows: -q 25 –length 50 -e 0.1 –stringency 5. The trimmed reads were subjected to alignment with default settings using STAR 2.5.3. Aligned RNA-seq data was counted over gene exon using featureCounts (subread 1.6.4). Genes were annotated as per the Rattus_norvegicus.Rnor_6.0.104 and Gencode Version 33 annotation file, respectively.

2.6. Gene set variation analysis (GSVA)

Pathway analyses were performed on the REACTOME subset of curated gene sets described in the molecular signature database, exported using the MSigDB database (v7.4). To assign pathway activity estimates to individual samples, we applied the GSVA using standard settings with the GSVA package (v1.40.1). To examine differential activities of pathways between groups of samples, we applied the Limma package (v3.48.3) to compare the activity scores. Differential activities of pathways were calculated for each group, and t-values of the results of significantly differential pathways (padj < 0.05) were visualized using heatmaps and horizontal bar charts.

2.7. Western blotting

A standard immunoblotting protocol was used. Briefly, protein was extracted from cells or retinas with Pierce RIPA buffer (89900; Life Technologies Australia) supplied with protease inhibitor cocktail (11697498001; Roche Diagnostics, Basel, Switzerland). Equal amounts of cell lysates from different groups were subjected to Western blotting analysis with specific antibodies against IκB, phospho-NFκB p65, NFκB p65, phospho-p44/42 MAPK (Erk1/2), p44/42 MAPK (Erk1/2), phospho-TAK1, TAK1, phospho-SAPK/JNK, SAPK/JNK, phospho-p38 MAPK, p38 MAPK (4814, 3033, 4764, 9101, 4695, 9339, 4505, 9255, 9252, 9211, 8690, respectively; Cell Signalling Technology; Danvers, MA, USA), and β-actin (MAB1501; Merck Millipore). The quantitative densitometry was analyzed using Image Pro Plus software (Media Cybernetics, Rockville, MA, USA).

2.8. RNA isolation and quantitative polymerase chain reaction (qPCR)

Total cells or tissue RNA was isolated using Zymo Quick-RNA MiniPrep kit (R1055; Zymo Research, Irvine, CA) according to the manufacturer's instructions. The quantity of RNA was evaluated with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Wilmington, DE, USA). Total RNAs (100 ng) was reverse transcribed to cDNA using a High-Capacity cDNA Reverse Transcription kit (4368814; Life Technologies Australia). Quantitative PCR was performed using TaqMan fast advanced master mix (4444553; Applied Biosystems, Foster City, CA, USA) and gene TaqMan probe sets to detect gene expression (Table S1). The expression levels of target genes were normalized to the levels of rat beta-actin (Rn00821065_g1) or human GAPDH (Hs99999905_m1). Subsequently, the ΔΔCt method was used to evaluate relative expression level (fold change) in each condition versus the corresponding control condition.

2.9. Animals and ethics statement

All animal experiments were conducted in accordance with guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Ethics Committees of St Vincent's Hospital (004/16) and the University of Tasmania (A0017311). Pregnant Sprague-Dawley rats (female, 12–14 weeks old) were supplied by the Animal Resources Centre (Murdoch, WA, Australia) and Cambridge Farm Facility of the University of Tasmania (Cambridge, TAS, Australia). Animals were housed in standard cages with a temperature/humidity-controlled and a 12-h light (50 lux illumination)/12-h dark (<10 lux illumination) cycle environment. Food and water were available ad libitum. Rats were randomly allocated to treatment groups such that littermates were distributed equally between groups.

2.10. Rat model of oxygen-induced retinopathy and intravitreal injection of 5Z-7-oxozeaenol

The oxygen-induced retinopathy model was induced in rats as previously described [23]. Briefly, Sprague-Dawley litters (within 12 h of birth; P0) and their nursing mothers were exposed to daily cycles of 80 % O_2 for 21 h and room air for 3 h in a custom-built and humidity-controlled (> 70 %) chamber until P14. At P14, animals were returned to room air until P16 or P18, when they were sacrificed to harvest retinae. An ProOx 110 oxygen controller (BioSpherix; Parish, NY, USA) was used to regulate and monitor the oxygen level.

At P14, intravitreal injections were performed to administer 5Z-7-oxozeaenol visualizing with a microsurgical microscope. Rats were anesthetized with isoflurane (1.5–2 mL/min). Under surface anaesthesia, a puncture in the superior temporal quadrant of the limbus was made by a 30-gauge needle. A hand-pulled glass micropipette (BioStrategy; Tullamarine, VIC, Australia) connected to a 10 μL Hamilton syringe (Hamilton Company; Reno, NV, USA) was inserted into the vitreous cavity, and one microliter of balanced salt solution containing 1 % DMSO (as vehicle), low (18 ng) or high dose (90 ng) of 5Z-7-oxozeaenol was intravitreally injected into the eye. A total of 63 neonatal rats (from 7 litters) were used in the in vivo study. Animals were monitored until full recovery and then returned to the oxygen chamber. Any issues with the injection, including large backflow upon removal of the needle and hemorrhaging of external or internal vessels, were noted, and those eyes were excluded from the analysis.

2.11. Immunohistochemistry and vessel quantification

The rat retinal flat-mount were collected at P18 followed by retina dissection, staining overnight with isolectin GS-IB$_4$ Alexa Fluor™ 488 conjugate (5 μg/mL; I21411, Life Technologies Australia), anti-TAK1 (1:100; NBP1–87819, Novus Biologicals; Centennial, CO, USA), or anti-Iba1 (1:300; 019–19741, FUJIFILM Wako Chemicals; Richmond, VA, USA), and then incubated with the appropriate secondary antibodies. The images were captured by FV3000 confocal laser scanning microscope. The avascular (vaso-obliteration) and neovascularized areas were quantified by two blinded assessors using Adobe Photoshop (San Jose, CA, USA) [24]. Deep vessel area was analyzed by AngioTool software (National Institutes of Health, Bethesda, MA, USA) [25].

2.12. Statistical analysis

All data are expressed as mean ± SEM. Statistical analyses were performed with Graphpad Prism 7 (GraphPad; San Diego, CA, USA) using student t-test for unpaired data, one-way or two-way ANOVA wherever appropriate followed by Tukey's or Bonferroni's multiple comparisons test. P values < 0.05 were considered statistically significant.

2.13. Data and resource availability statement

The RNA-Seq data of retina of control and OIR rats have been deposited in Gene Expression Omnibus (GEO) under GEO: GSE104588. To access gene expression in retinas from patients with retinal neovascularization due to proliferative diabetic retinopathy, we obtained the RNA-seq raw counts and TPM matrices from GEO: GSE102485. We specifically compared samples from patients with retinal neovascular proliferative membranes due to type II diabetes (GSM2739349, GSM2739350 and GSM2739351) and those from normal controls (GSM2739364, GSM2739365 and GSM2739366).

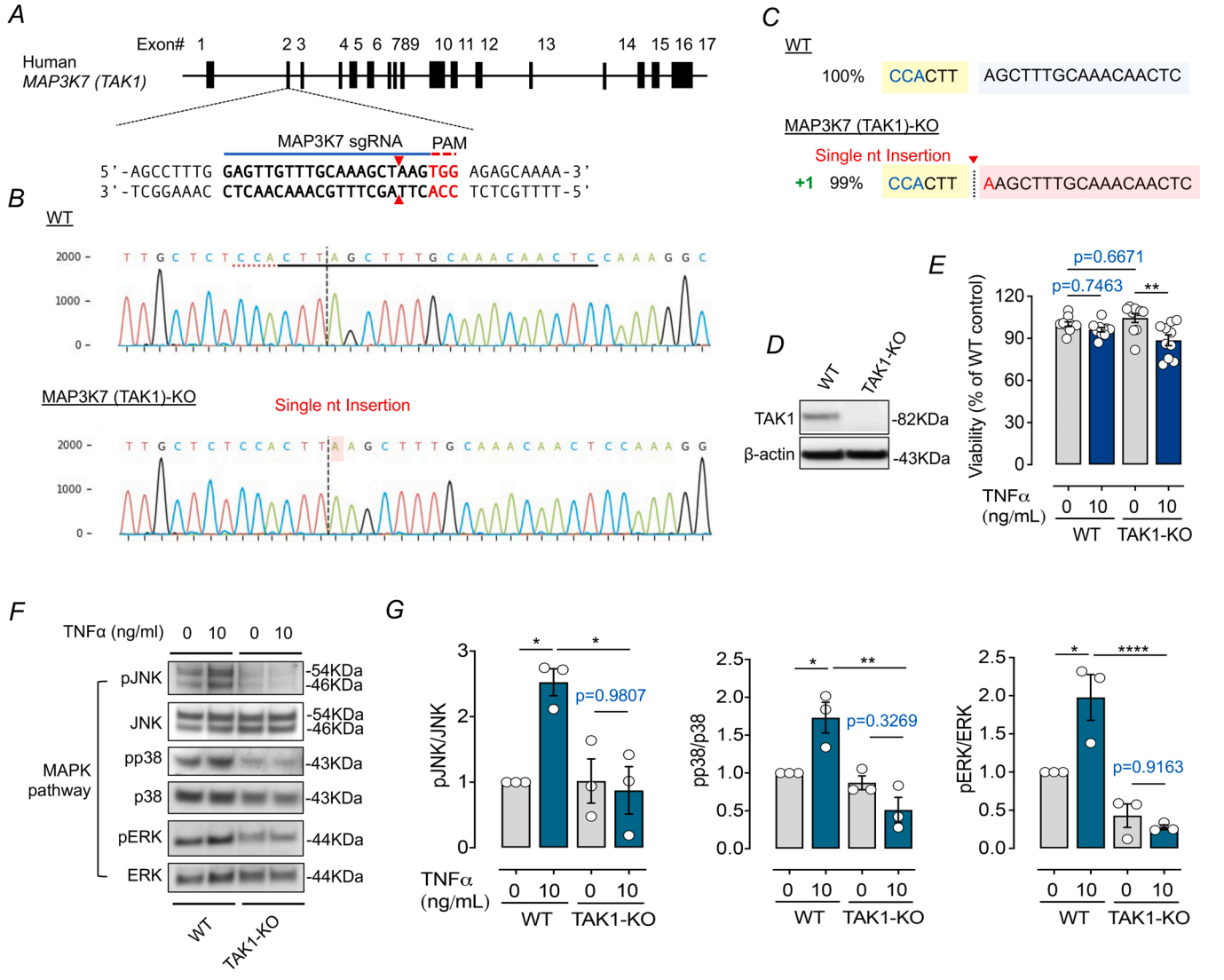

Fig. 1. Generation of CRISPR/Cas9-mediated *MAP3K7 (TAK1)* knockout (TAK1-KO) in telomerase-immortalized human microvascular endothelial cells (TIMEs). (A) Genome of *MAP3K7 (TAK1)*. A sgRNA was designed to target exon 2 of the *MAP3K7 (TAK1)* gene. (B) As compared with wild-type (WT) TIMEs, genomic DNA sequences revealed a single homogenous TAK1-KO TIMEs were established after CRISPR/Cas9 gene editing. (C) Sanger sequencing confirmed that TAK1-KO TIMEs was a single nucleotide insertion, which created as premature Stop Condon in the exon 2 region of *MAP3K7 (TAK1)*. (D) Western blot characterization of TAK1 deletion in TAK1-KO TIMEs. (E) WT or TAK1-KO TIMEs treated with vehicle or TNFα (10 ng/mL) for 48 h, followed by the MTT cell viability assay (n = 10 from 3 independent experiments). (F-G) Western blot characterization of proteins and corresponding quantitative analysis of protein expression in MAPK signaling pathways in TAK1-KO TIMEs (n = 3 from 3 independent experiments). All data were presented as means ± SEM. Statistical analysis was conducted by one-way ANOVA and Tukey's multiple comparison test. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$.

The original Western Blot images and the details of statistical analysis for this study are available in the supplementary information. Other original datasets will also be available to the research community upon reasonable request.

3. Results

3.1. Established human microvascular endothelial cells with TAK1 deletion

To evaluate the role of TAK1 in the endothelial inflammatory response and angiogenesis, we generated *MAP3K7 (TAK1)*-knockout (KO) human telomerase-immortalized microvascular endothelial (TIME) cells (TAK1-KO) using CRISPR/Cas9. The TIME cells were transduced with lentiCRISPRv2-TAK1 sgRNA lentivirus (Fig. 1A), followed by puromycin selection. The transduced cells were further subjected to single colony selection to obtain a monoclonal cell line, termed TAK1-KO. Sanger sequencing results showed that TAK1-KO cells had a single nucleotide insertion, resulting in the premature stop codon in the exon 2 region of *MAP3K7 (TAK1)* (Fig. 1B and C). Western blot analysis demonstrated that there were no detectable levels of TAK1 protein in TAK1-KO cells (Fig. 1D). MTT assay showed that *TAK1* deletion did not

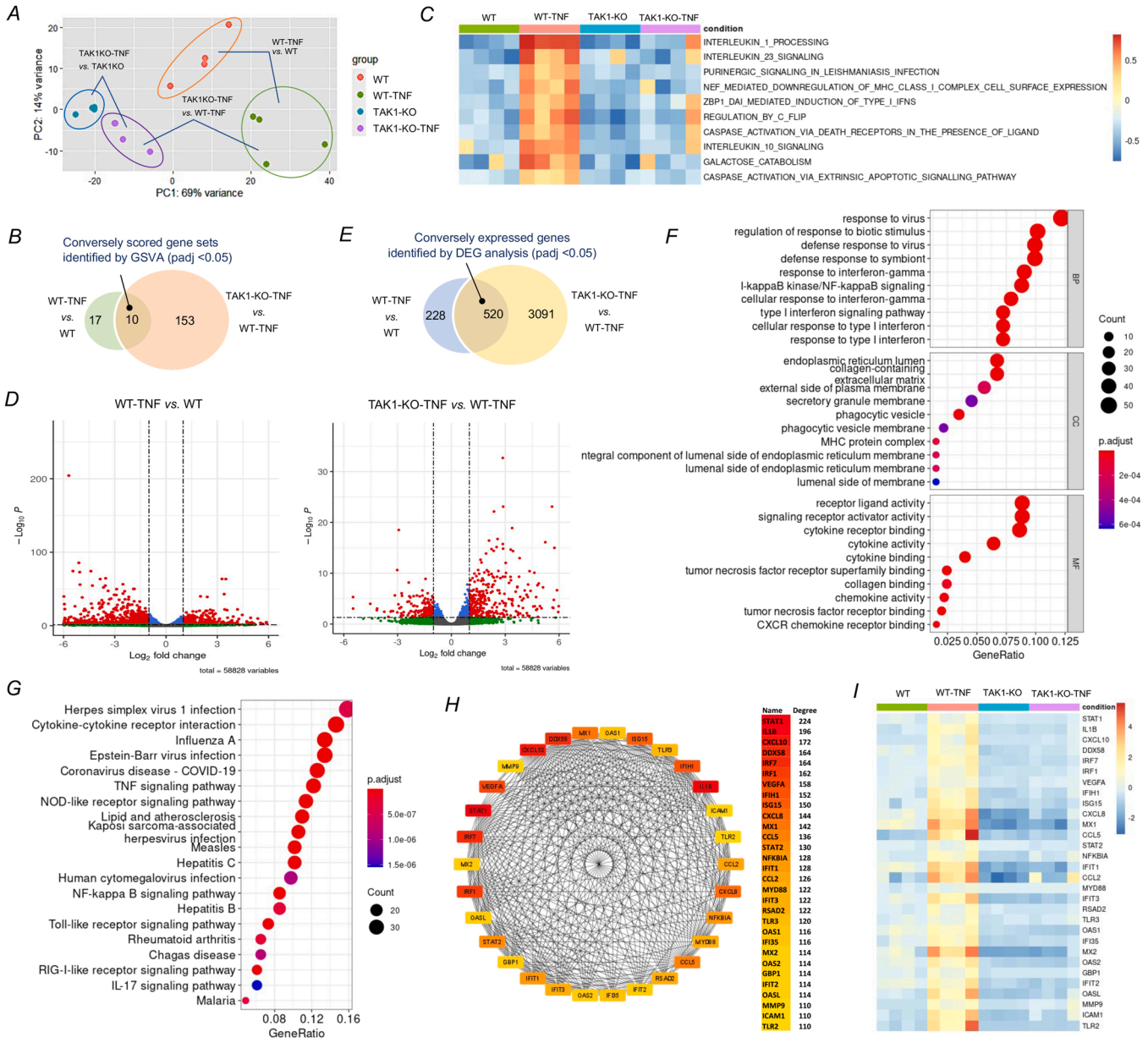

Fig. 2. TAK1 mediates NFκB signalling pathway in TIMEs upon inflammatory insults. (A) Principal components analysis (PCA) of RNA-seq data of wild-type (WT) TIMEs, TAK1 knockout (TAK1-KO) TIMEs and TNFα-treated both WT and TAK1-KO TIMEs (WT-TNF and TAK1-KO-TNF) (n = 4 from 4 independent experiments). (B) Venn diagram of differentially scored gene sets between two data sets showing intersection of 10 gene sets, analyzed by GSVA. (C) Heatmap of identified 10 gene sets in B for each sample in different groups. (D) Volcano plots show genes that were significantly changed in both WT and TAK1-KO TIMEs when stimulated by TNFα. Red dots refer to genes showing a log2|fold change| > 1 and FDR < 0.05. Labels indicate significantly up-regulated genes in each data set. (E) Venn diagram of differentially expressed genes between two data sets showing intersection of 520 genes. (F) Gene Ontology and (G) KEGG enrichment analysis of 520 genes identified in E. 520 genes were imported to Cytoscape to identify hub genes. (H) Top 30 hub genes were identified by Cytoscape plugin cytoHubba. (I) Heatmap of expression of 30 hub genes identified in H for each sample in different groups. Genes highlighted are associated with NFκB signaling pathway. BP: biological process; CC: cellular component; MF: molecular function.

affect cell viability compared to wild-type cells. However, cell viability was decreased slightly when cells were exposed to the proinflammatory cytokine TNFα (Fig. 1E), which is consistent with the previous report [21].

To assess the effects on the activation of the classic signaling mediated by TAK1, MAPKs signaling, caused by *TAK1* knockout, we characterized the signaling in the TAK1-KO cells induced by TNFα using western blot. Our data showed that TNFα treatment significantly increased phosphorylation of JNK, p38, and ERK in MAPK signaling in wild-type cells, an effect not observed in TAK1-KO cells (Fig. 1F and G). Together, these results confirmed the successful generation of TAK1-KO microvascular endothelial cells.

3.2. Transcriptome profiling reveals key signaling and hub genes mediated by TAK1 in endothelial cells upon proinflammatory stimuli

To identify the inflammatory associated angiogenesis signaling mediates by TAK1 in human microvascular endothelial cells, we generated the transcriptome profile of wild-type (WT), TAK1-KO cells and TNFα-treated both cell lines (WT-TNF and TAK1-KO-TNF) by RNA sequencing. Gene expression was assessed initially with principal component analysis for all groups combined (Fig. 2A). This demonstrated a clear separation between groups, indicating effects induced by both *TAK1* knockout and TNFα treatments. We used Gene Set Variation Analysis (GSVA), a non-parametric, unsupervised method for estimating variation of gene set enrichment across the samples based on the gene expression [26], to identify gene pathways associated with TAK1 and inflammatory inducer, TNFα. Overlapping significantly enriched gene sets (padj <0.05) between dataset generated from WT-TNF vs. WT and dataset generated from TAK1-KO-TNF vs. WT-TNF resulted in 10 gene sets that identified in both data sets (Fig. 2B). A heatmap revealed these data sets were associated with interleukin (IL)-mediated inflammation, such as IL-1, IL23 and IL10, and were conversely scored (positive score in WT-TNF but negative score in TAK1-KO-TNF) in each data set, suggesting a regulatory role of TAK1 in inflammation (Fig. 2C).

We further identified and compared the differentially expressed genes (DEGs, padj < 0.05) between the two data sets above mentioned (Fig. 2D), resulting in 520 genes that were conversely expressed in each data set (Fig. 2E; Table S2). Gene ontology (GO) enrichment analysis revealed that these 520 DEGs were primarily associated with NFκB signaling in the biological process, extracellular matrix external side of plasma membrane and MHC protein complex in cellular function, and cytokine/chemokine activity in molecular function (Fig. 2F). Interestingly, KEGG enrichment analysis (Fig. 2G) also demonstrated several pathways, including NFκB signaling pathway (Fig. S1) and TNF signaling pathway (Fig. S2). Since canonical NFκB signaling is important for a sustained inflammatory response by endothelial cells and angiogenesis, we further characterized NFκB signaling in the TAK1-KO cells induced by TNFα using western blot. TNFα treatment significantly increased TAK1 phosphorylation in wild-type cells, an effect not observed in TAK1-KO cells (Fig. S3A and B). IκB degradation and NFκB p65 phosphorylation are key events in NFκB signaling [27]. TNFα treatment significantly induced NFκB p65 phosphorylation and IκB degradation in wild-type cells, while no such changes were detected in TAK1-KO cells (Fig. S3C). Likewise, TAK1 deletion effectively inhibited expression of NFκB mediated proinflammatory and proangiogenic genes, including *VEGF-A, CXCL8*, *ICAM-1* and *PTGS2* (Fig. S3D) [28,29]. Together, these DEGs enrichment results further suggested that TAK1 is a key regulator in inflammation and is principally involved in NFκB signaling in endothelial cells upon inflammatory stimulation.

To further pinpoint hub genes most relevant to TAK1, we applied Cytoscape plugin cytoHubba to rank the 520 DEGs according to the degree of interactions between genes, a topological analysis method to assess the essentiality of genes [30]. This analysis resulted in 30 top scored genes (Fig. 2H), 6 of which are involved in NFκB signaling, including *IL1B*, DExD/H-box helicase 58 (*DDX58*), *CXCL8*, NFκB inhibitor alpha (*NFKBIA*), myeloid differentiation primary response 88 (*MYD88*) and *ICAM-1*. Of note, a few identified hub genes were also classic genes related to inflammation and angiogenesis, such as *VEGFA* and matrix metallopeptidase 9 (*MMP9*) [28]. A heatmap further demonstrated that expression of these genes was evidently higher in WT-TNF compared to TAK1-KO-TNF cells (Fig. 2I), suggesting that their expression is potentially mediated by TAK1 in endothelial cells upon inflammation.

3.3. TAK1 inhibition by 5Z-7-oxozeaenol suppresses angiogenic activities

Inflammation and angiogenesis, subsequent to hypoxia, are two of the main factors that contribute to retinal neovascularization. During the disease, inflammation and angiogenesis establish a strict cross talk, with inflammation promoting angiogenesis. Therefore, pharmacological targeting the inflammatory mediator such as TAK1 could be beneficial for managing retinal neovascularization. A few studies demonstrated that TAK1 is activated upon different proinflammatory stimuli such as TNFα and mediates downstream signaling associated with both angiogenesis and inflammation via NFκB pathway [18]. We therefore assessed the effect of a selective inhibitor of TAK1, 5Z-7-oxozeaenol, a resorcylic acid lactone derived from a fungus [29], on the downstream factors of NFκB pathway related to both angiogenesis and inflammation upon TNFα stimulation in human retinal microvascular endothelial cells (HRMECs). Consistent with the inhibition of NFκB activity observed in TAK1-KO cells, 5Z-7-oxozeaenol effectively inhibited the expression of TNFα-induced proinflammatory and proangiogenic genes in a dose-dependent manner, including *ICAM-1* and *CXCL8*, *VEGF-A* as well as *PTGE2* (Fig. 3A). Similar results were found in TAK1 knockdown HRMECs using TAK1 siRNA (siTAK1) (Fig. S4A and B).

We further examined the role of TAK1 in response to inflammatory insults in various endothelial functions related to angiogenesis through *in vitro* assays, including cell proliferation, scratch migration and tube formation assay in HRMECs. 5Z-7-oxozeaenol showed a significant inhibitory effect on HRMEC proliferation for 48 h ($p < 0.0001$; Fig. 3B). Furthermore, cells treated with 5Z-7-oxozeaenol in the presence or absence of TNFα showed > 20 % reduction in cell migration compared to controls or cells treated with TNFα ($p < 0.01$; Fig. 3C). In addition, 5Z-7-oxozeaenol effectively diminished TNFα-induced tube formation activity in HRMECs (Fig. 3D). Together, these results indicated that pharmacological inhibition of TAK1 effectively represses inflammation-mediated angiogenic signaling and reduces endothelial cell angiogenic activity.

3.4. Increased TAK1 expression in the neovascular tufts of a rat model of retinal neovascularization

We subsequently used a rat model of OIR featuring retinal neovascularization to study the role of TAK1 in pathological neovascularization *in vivo*. Neonatal rats were subjected to daily cycles of 80 % oxygen for 21 h and ambient air for 3 h from postnatal day 0 (P0) to P14 to induce vaso-obliteration. Rats were returned to room air at P14, resulting in maximal preretinal neovascularization at P18 (Fig. 4A). A significant increase in TAK1 expression at both RNA and protein levels was observed in OIR retina at P18 compared with controls (Fig. 4B and C). Immunohistochemistry results demonstrated that TAK1 expression was uniquely expressed in areas of neovascular tufts in the OIR retina (Fig. 4D), suggesting its active may involve in pathological angiogenesis. Nevertheless, TAK1 is not necessarily expressed in endothelial cell. To gain further insight into the role of TAK1 activation at the peak of retinal neovascularization, we performed retinal transcriptome analyses of OIR and control rats at P18 using RNA sequencing. GSVA-based analysis with the transcriptome dataset revealed positive enrichment of several TAK1 upstream pathways in OIR rats, including IL1, TNF, B-cell receptor

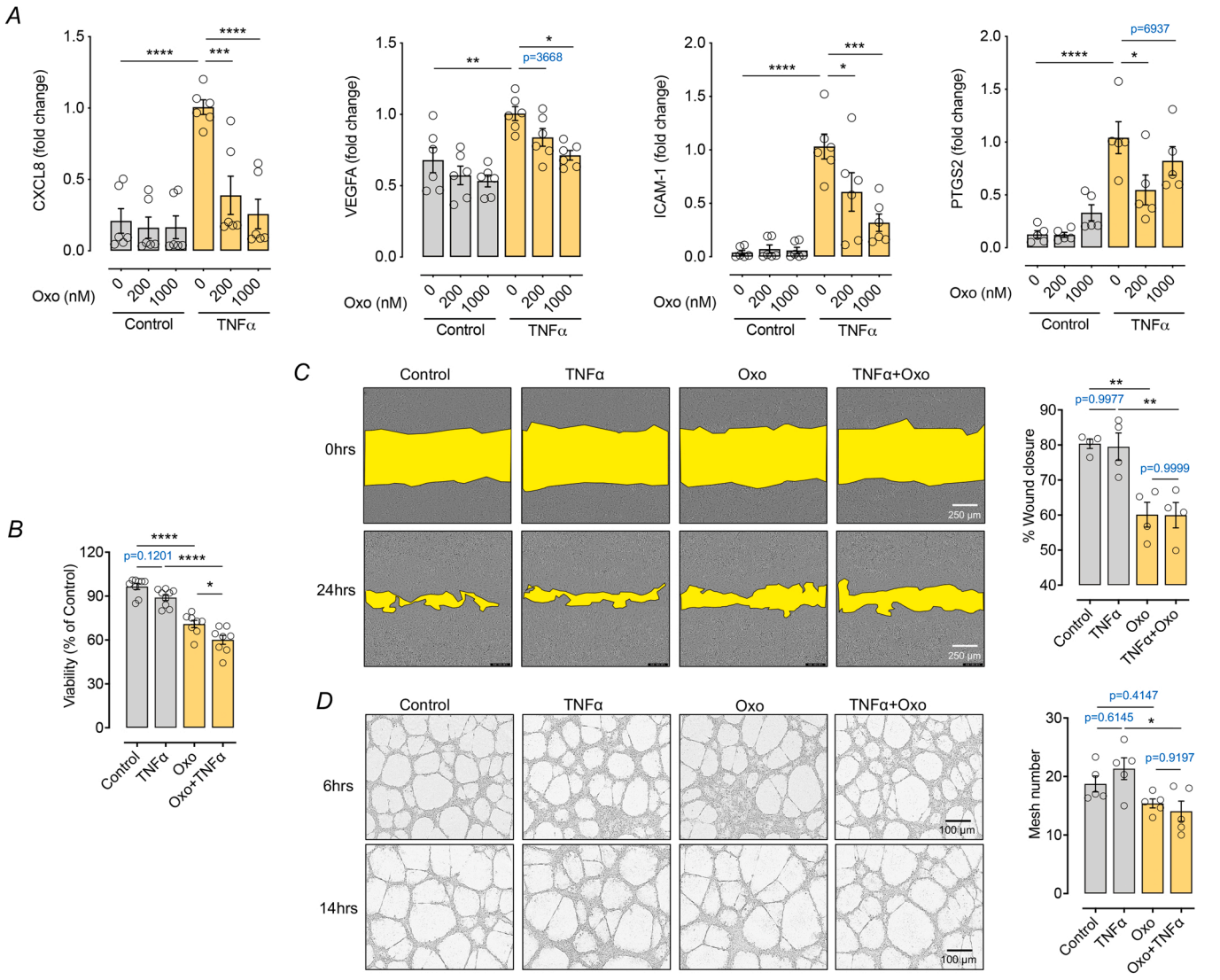

Fig. 3. 5Z-7-oxozeaenol suppresses NFκB signaling pathway and angiogenic activities in HRMECs. (A) 5Z-7-oxozeaenol inhibited expression level of *ICAM-1*, *CXCL8*, *VEGF-A* and *PTGS2* in a dose-responding manner in HRMECs stimulated by TNFα, assessed by qPCR (n = 5–6 from 3 independent experiments). (B) HRMECs stimulated by TNFα (10 ng/mL for 10 min) were treated with vehicle or 5Z-7-Oxozeaenol at 1000 nM for 48 h, followed by the cell viability assay (n = 8 from 2 independent experiments). (C) A wound healing assay was performed to evaluate the migration activity in HRMECs upon TNFα stimulation in the presence or absence of 5Z-7-oxozeaenol (1000 nM). Representative images of the wound area were taken immediately after scraping and 24 h after 5Z-7-oxozeaenol treatment. Quantitative analysis was determined by healing distance normalised to the original wound distance (n = 4 from 4 independent experiments). (D) Tube formation was performed in HRMECs upon TNFα stimulation in the presence or absence of 5Z-7-oxozeaenol (1000 nM). Representative images were taken 6 and 14 h post tube formation. Quantitative analysis was conducted by counting the lumen number (n = 5 from 5 independent experiments). All data were presented as means ± SEM. Statistical analysis was conducted by one-way ANOVA and Tukey's multiple comparison test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Oxo: 5Z-7-oxozeaenol.

(BCR) and Toll-like receptor (TLR) as well as the pathway directly related to TAK1, "TAK1 activates NFκB by phosphorylation and activation of inhibitor of κB (IκB) kinase (IKK) complex" (Fig. 4E). Moreover, there was a negative enrichment of genes associated with phototransduction and neuronal systems in OIR rats. Of note, certain canonical pathways related to angiogenesis were positively enriched in the OIR model, such as VEGF-A/VEGFR2, PDGF pathway (Fig. 4E). Interestingly, we also observed similar positively enriched pathways, particularly TAK1 activation via NFκB pathway, in retinal neovascular membrane from patients with proliferative diabetic retinopathy compared to healthy human retina (Fig. 4F). These data suggest the reliability of our prediction of TAK1-mediated hub genes that contribute to retinal neovascularization.

3.5. Pharmacological Inhibition of TAK1 alleviates retinal neovascularization in OIR rats

We next assessed whether TAK1 signaling blockade can alleviate retinal neovascularization by intravitreal administration of low (18 ng) or high doses (90 ng) of 5Z-7-oxozeaenol in OIR rats at P14, followed by retinal whole mount at P18 (Fig. 5A). Quantitative analysis showed that retinal neovascularization was significantly suppressed by 51.3 % and 57.1 % in OIR rats that received low (18 ng) and high doses (90 ng) of 5Z-7-oxozeaenol, respectively, compared to OIR rats that received vehicle only (Fig. 5B; Fig. S5). No significant difference in retinal vaso-obliteration was found among these groups (Fig. 5C). We also examined the development of deep capillary beds to assess the effects of TAK1 inhibition in physiological vessel growth [31]. Normal control rats

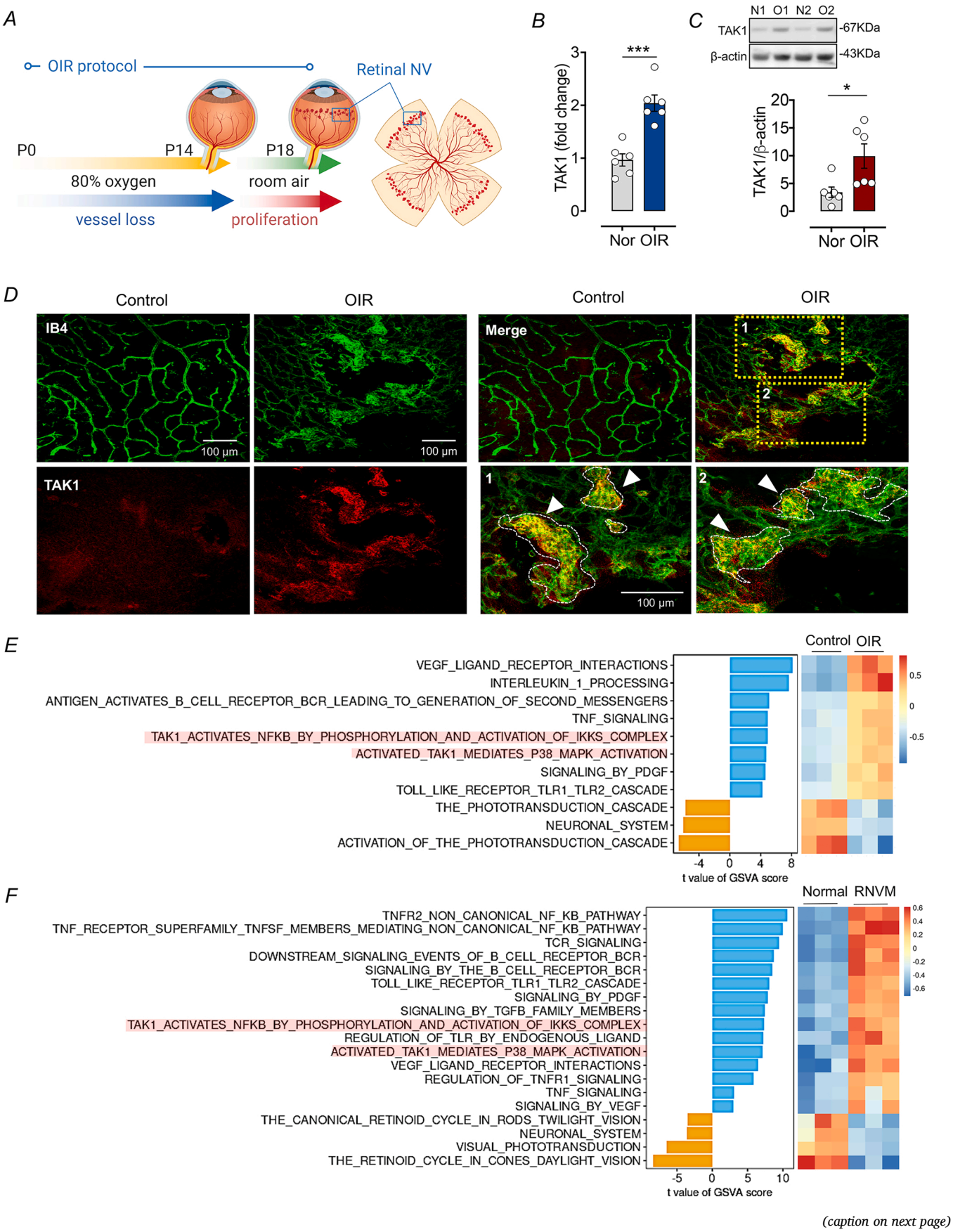

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Fig. 4. TAK1 is involved in pathological retinal angiogenesis in a rat model of oxygen-induced retinopathy (OIR) via NFκB signaling pathway. (A) Schematic diagram of the Sprague-Dawley rat model of OIR. Neonatal rats were exposed to 80 % of oxygen for 21 h and room air for 3 h per day from postnatal day 0 (P0) to P14. Rats were returned to room air from P14 to P18. Retinal neovascularization was induced after rats returned to room air. (B) qPCR analysis of *TAK1* expression in OIR at P18 as compared with the normoxic controls (Nor) (n = 6 per group). (C) Western blot analysis of TAK1 expression in OIR at P18 compared with the normoxic controls (n = 6 per group). Representative immunoblotting images from 3 independent samples of normoxic control (N1 and N2) and OIR group (O1 and O2) were shown. (D) Immunohistochemistry results indicated increased expression of TAK1 in the neovascular tufts. Vasculature were visualized by isolectin GS-IB4 labelling (green) and counterstained with TAK1 (red) in the retinal whole mount. Arrowheads indicate vessel tufts. Scale bar: 100 μm. (E) GSVA indicated that most of TAK1 upstream pathways including TNF, IL1, BCR, TLR, and VEGF pathways were positively enriched in OIR at P18. Positive engagement of TAK1-activated NFκB and p38 MAPK pathways as well as negative impact on retinal neural functions in OIR were also identified. (F) GSVA indicated that most of TAK1 upstream pathways including TNF, IL1, TCR, BCR, TLR, and VEGF pathways were positively enriched in the retinal neovascular membrane from patients with proliferative diabetic retinopathy compared to retina from healthy controls. Positive engagement of TAK1-activated NFκB and p38 MAPK pathways as well as negative impact on retinal neural functions in the retinal neovascular membrane from patients with proliferative diabetic retinopathy were also identified. Group data are shown as means ± SEM. Statistical analysis was undertaken with two-tailed unpaired t-test; $^{*}P < 0.05$, $^{***}P < 0.001$.

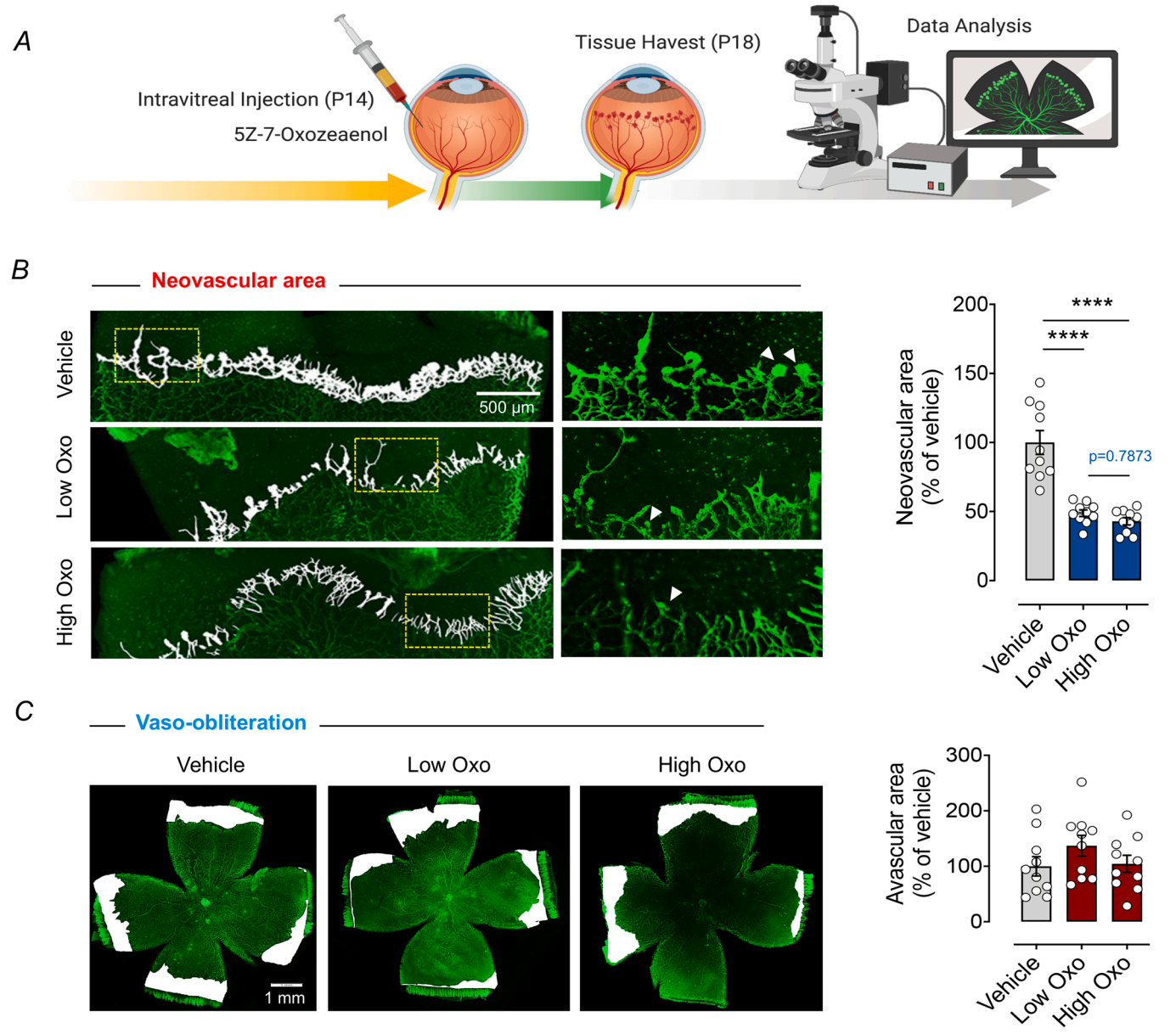

Fig. 5. 5Z-7-oxozeaenol alleviates retinal neovascularization in oxygen-induced retinopathy (OIR) rats. (A) Experimental protocol for 5Z-7-oxozeaenol treatment in the OIR rats. A single intravitreal injection of vehicle, low (18 ng) or high (90 ng) dose of 5Z-7-oxozeaenol was given to OIR rats at P14, followed by quantitative analysis of retinal neovascularization in OIR rats at P18. (B) Retinal neovascularization is highlighted in white, and insets show selected areas at high magnification. Arrowheads indicate vessel tufts. Retinal neovascularization was quantified from 10 individual retinas per group. Scale bar: 500 μm. (C) Vaso-obliteration was simultaneously evaluated with the same samples analyzed in B. The white areas represent the avascular area in the retina. No significant difference was observed between groups (n = 10). Scale bar: 1 mm. Oxo: 5Z-7-oxozeaenol.

developed a complete deep layer vasculature, while such development was severely disrupted in OIR rats (Fig. S6). We found that 5Z-7-oxozeaenol treatment did not improve the damage the formation of deep capillary beds in OIR retina, which is likely attributed to suppression of proliferative vessels by TAK1 inhibition. Moreover, the rats receiving low or high doses of 5Z-7-oxozeaenol did not show any abnormalities in the retinal vasculature under normoxic conditions (Fig. S7).

The response of endothelial cells to inflammation and NF-κB activation is characterized by the induction of adhesion molecules promoting binding and transmigration of leukocytes, while simultaneously increasing their angiogenic potential. Paracrine factors such as VEGF and CXCL8 from NFκB activated microvascular endothelial also elicits angiogenic responses. We therefore assess the effect of TAK1 inhibition on the expression of NF-κB activated paracrine factors, as well as binding of leukocytes to the retinal vasculatures during the development of retinal neovascularization. OIR rats were treated with low and high doses of 5Z-7-oxozeaenol at P14 and the retinae were harvested for qPCR and histological analysis at P16, an earlier harvest timepoint due to short half-life of 5Z-7-oxozeaenol [32], and P18, respectively (Fig. 6A). qPCR results showed that the expression of inflammatory and angiogenic genes, including *Tnfα*, *Vegf*-A and *Icam-1*, was significantly decreased in the retina of OIR rats that had received low (18 ng) or high doses (90 ng) of 5Z-7-oxozeaenol compared to OIR rats that received vehicle only, suggesting that 5Z-7-oxozeaenol can mitigate the production of NF-κB activated paracrine factors (VEGF-A) during the progression of retinal neovascularization (Fig. 6B). We subsequently evaluated activities of retinal microglial in the context of retinal neovascularization. Co-immunostaining of isolectin GS-IB4 (marker for endothelial cells) and Iba1 (marker of microglial cells) illustrated that increased number of microglial cells accumulated around the

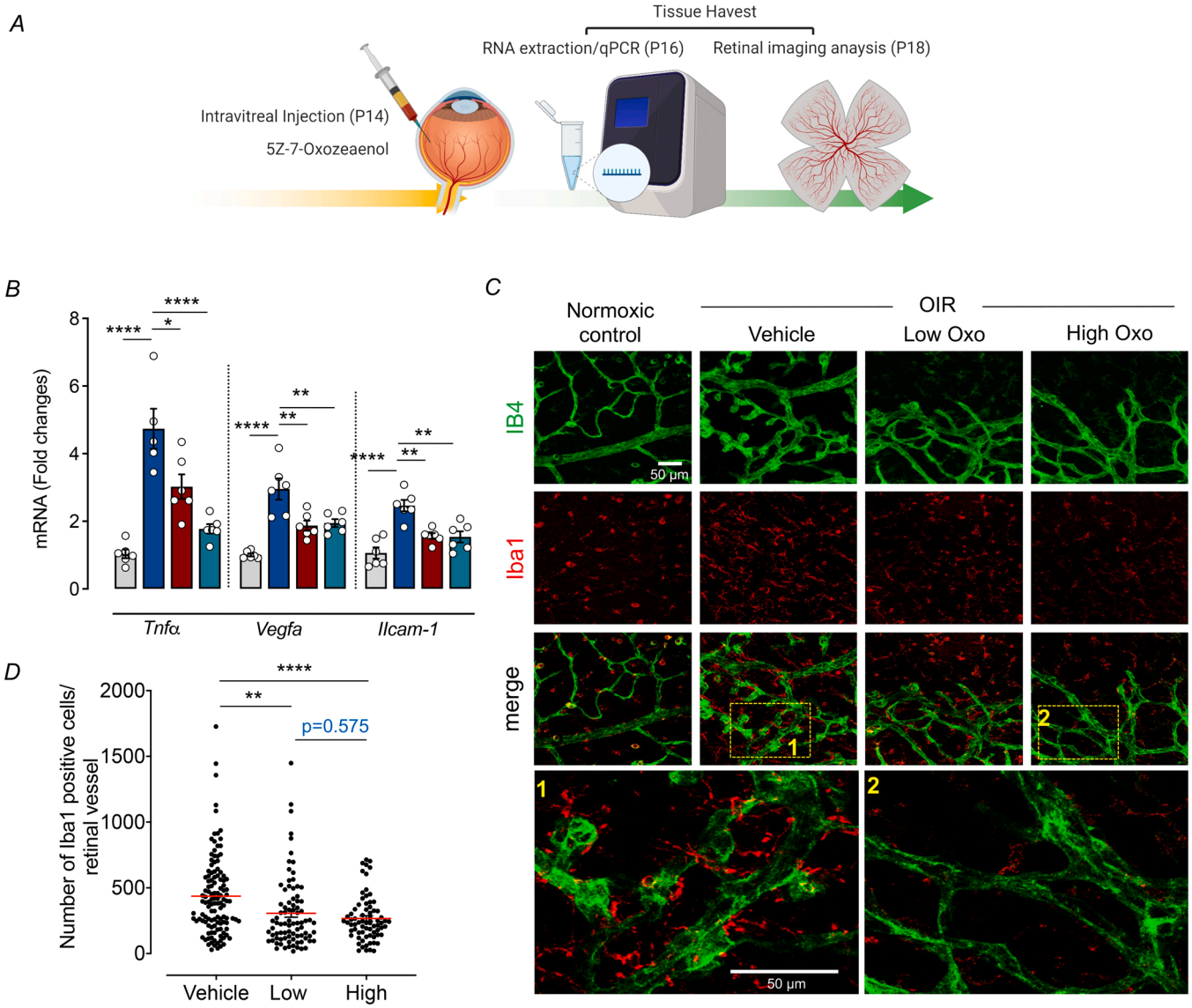

Fig. 6. 5Z-7-oxozeaenol decreased inflammation and microglial activation in the retina of OIR rats. (A) Experimental protocol for 5Z-7-oxozeaenol treatment in the OIR rats. A single intravitreal injection of vehicle, low (18 ng) or high (90 ng) dose of 5Z-7-oxozeaenol was given to OIR rats at P14, and retina was collected for qPCR and immunostaining at P16 and P18, respectively. (B) Intravitreal injection of 5Z-7-oxozeaenol inhibited *Tnfα*, *Vegfa* and *Icam-1* gene expression in OIR rats at P14, assessed by qPCR (n = 6 per group). (C) Representative images of microglial adhesion to the abluminal vascular surface in the retina of OIR rats received 5Z-7-oxozeaenol. Vessels were visualized with isolectin GS-IB4 staining (green), and distribution of microglia was immunolabeled with Iba-1 (red). Scale bar: 50 μm. (D) Quantitative analysis of microglial activation OIR rats received 5Z-7-oxozeaenol (n = 7–12 per group; 10 images from each retina were counted. Group data are shown as means ± SEM. Statistical analysis was undertaken with one-way ANOVA and Tukey's multiple comparison test; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Oxo: 5Z-7-oxozeaenol.

neovascular tufts, while both low and high doses of 5Z-7-oxozeaenol significantly decreased the number of the microglial cells and attenuated the neovascular tufts in OIR rats (Fig. 6C and D), indicating that inhibition of TAK1 may have therapeutic potential for the treatment of retinal neovascular pathologies.

4. Discussion

TAK1 is a key modulator involved in a range of cellular functions including the immune responses [16], cell survival and death [18], angiogenesis [22], fibrosis [33] and tumour metastasis [34]. It is activated in response to various stimuli such as proinflammatory cytokines, hypoxia, oxidative stress, metabolism, DNA damage, Wnt and osmotic shock [16,35–37]. Whether TAK1 is involved in angiogenesis in the retina, much of the molecular mechanisms by which this occurs, has not been studied. Here, we delineate TAK1 plays a central role in regulating inflammatory-related angiogenic signaling via the NFκB pathway in human retinal endothelial cells. Pharmacological antagonism of TAK1 signaling by 5Z-7-oxozeaenol attenuates angiogenic activities of retinal endothelial cells and suppresses aberrant retinal angiogenesis in OIR rats. Thus, inhibition of TAK1 may serve as a potential therapeutic alternative for the treatment of retinal neovascular pathologies.

Here, we provided evidence that genetic deletion of *TAK1* in human microvascular endothelial cells prevents inflammatory stimuli from inducing sequential phosphorylation of upstream kinases mainly in NFκB pathway and suppressed expression of downstream genes that drive inflammation and angiogenesis. We also predicted several hub genes in the NFκB pathway that mediated by *TAK1* in human endothelial cells upon inflammatory stimuli. GSVA results revealed that TAK1 activation via NFκB pathway along with other inflammatory signaling, such as IL1 signaling and TNF signaling, as well as angiogenic signaling, such as VEGF ligand receptor interactions, were also positively active in the retina of OIR rats compared to controls, further indicating TAK1 involvement in inflammation and angiogenesis. Interestingly, we observed similar positively enriched pathways, particularly TAK1 activation via NFκB pathway, in retinal neovascular membrane from patients with proliferative diabetic retinopathy compared to healthy human retina. In addition, TAK1 inhibition by 5Z-7-oxozeaenol in OIR rats significantly reduced cytokines expression and adhesion of microglia to retinal endothelial cells. These data together suggest a significant role for TAK1 in mediating crosstalk between inflammatory and angiogenic pathways mainly through NFκB pathway, both of which are crucial in pathological angiogenesis [38].

TAK1 is a central regulator of cell survival and death [18]. 5Z-7-oxozeaenol acts as an inhibitor of TAK1, and it forms a covalent complex with TAK1 that impedes both the kinase and ATPase activity of TAK1 following bi-phase kinetics [39]. We demonstrated that 5Z-7-oxozeaenol treatment significantly decreased expression of *CXCL8* and *ICAM-1*, both of which are hub genes we predicted associated with TAK1-mediated NFκB pathway, in human endothelial cells upon stimulation of TNFα, one of the major cytokines increased in proliferative eye disease in human and retinal neovascularization in animal models [40]. 5Z-7-oxozeaenol treatment also suppressed angiogenic activities, including cell proliferation, migration and tube formation *in vitro*. Furthermore, intravitreal injection of 5Z-7-oxozeaenol suppressed retinal neovascularization and diminished elevated expression of TNFα and VEGF-A in the retina of OIR rats, without causing degeneration of the normal vasculature. These data suggest that TAK1 inhibition by 5Z-7-oxozeaenol may more specifically attenuate active angiogenesis with less risk of damage to normal endothelial cells. However, further studies are required to understand the effects of repeated and long-term 5Z-7-oxozeaenol treatment on the normal vasculature and the process of revascularization in OIR. Interestingly, TAK1 inhibition has effects on other retinal cell types, such as retinal pigment epithelial (RPE) cells. A study showed TAK1 inhibitor LYTAK1 can suppress the proliferation and migration of RPE cells and prevented TGF-β1–induced epithelial-mesenchymal transition by decreasing the levels of fibronectin and α-smooth muscle actin, the hallmarks of fibrosis in proliferative vitreoretinopathy [41,42]. Moreover, TAK1 inhibition by 5Z-7-oxozeaenol is known to block proinflammatory signaling by selectively inhibiting TAK1 MAPKKK [29]. In an animal model of early brain injury, 5Z-7-oxozeaenol was shown to inhibit subarachnoid haemorrhage-induced phosphorylation of p38 and JNK, the nuclear translocation of NFκB p65 and degradation of IκB, ultimately reducing neuronal apoptosis and early brain injury [43]. Similarly, another study demonstrated that 5Z-7-oxozeaenol alleviated experimental autoimmune encephalomyelitis by reducing the levels of proinflammatory cytokines in splenocytes and the central nervous system, thus reducing the number of activated microglia and blocking MAPKs signaling pathways [44]. Overactive microglia can damage surrounding cells, via a range of detrimental effects including release of proinflammatory cytokines, reactive nitrogen species, reactive oxygen, and proteolytic enzymes [45]. In fact, the induction of ICAM-1 has been suggested to be related to increased expression of activated microglia in a rat model of induced inflammatory pain in the brain [46]. We also observed diminished adhesion of microglia to endothelial cells in OIR rats receiving 5Z-7-oxozeaenol treatment and reduced expression of ICAM-1, a downstream gene in TAK1-mediated NFκB pathway, suggesting that 5Z-7-oxozeaenol alleviates inflammation in the retina, in part by reducing activation of microglia via suppression ICAM-1.

5Z-7-oxozeaenol has been previously examined in several animal studies focusing on brain injury [47–49]. One study demonstrated that 5Z-7-oxozeaenol has neuroprotective function in a mouse model of stroke [47]. However, others suggest long-term TAK1 inhibition may not be protective in neurodegenerative disorders [50]. Chronic TAK1 inhibition resulted in apoptosis of several normal cells, including keratinocytes, hepatocytes, and hematopoietic cells, raising safety concerns [51–53]. We demonstrated that TAK1 inhibition was capable of simultaneously reducing proinflammatory cytokines and proangiogenic cytokines in the retina of OIR rats. While 5Z-7-oxozeaenol efficiently blocks TAK1, it also targets other human kinases due to potentially stemming from cross-reactivity, that possesses an analogous cysteine [54]. It is worth noting that 5Z-7-oxozeaenol has also been shown to inhibit VEGFR2 kinase [55], which may affect angigenesis. Indeed, our transcriptomic data showed that 5Z-7-oxozeaenol treatment resulted in the downregulation of several downstream pathways of VEGF-VEGFR2 signaling, in particular Akt/PKB and p38 (Fig. 7A), both of which are well known for being involved in angiogenesis [56,57]. Western blot further confirmed a significant reduction of phosphorylation of Akt and p38 in endothelial cells treated by 5Z-7-oxozeaenol upon recombinant human VEGF-A protein (rVEGF-A) stimuli, independent of TAK1 (Fig. 7B–D). Thus, our data suggest that 5Z-7-oxozeaenol may at least in part affect VEGF-mediating signalings such as Akt and p38 that ultimately cause anti-angiogenic effects. However, whether 5Z-7-oxozeaenol reduces the phosphorylation of Akt and p38 in endothelial cells directly through inhibition of VEGFR2 kinases or other kinase pathways still needs further verification.

Whilst encouraging, further studies with chronic dosing of 5Z-7-oxozeaenol are clearly needed to confirm its safety particularly on specificity of targets. Additionally, inhibition of TAK1 leads to a decrease in macromolecular permeability of the barrier in brain endothelial cells under the inflammatory stimuli [58], suggesting TAK1 inhibition might have beneficial effects on endothelial barrier function. Future studies should assess the effects on vascular permeability by TAK1 inhibition in OIR models. There are increasing reports showing that a significant subset of patients with retinal vascular diseases, such as neovascular age-related macular dystrophy and proliferative diabetic retinopathy, fail to respond to anti-VEGF therapy [59,60]. Other inflammatory cytokines and chemokines other than VEGF as well as immune cell types have been indicated to play important roles in retinal vascular diseases. Our recent study using deconvolution analysis of retinal transcriptomic data demonstrated that the proportion of M2 macrophages, a major immune cell type in inflammation, is significantly increased in the retina

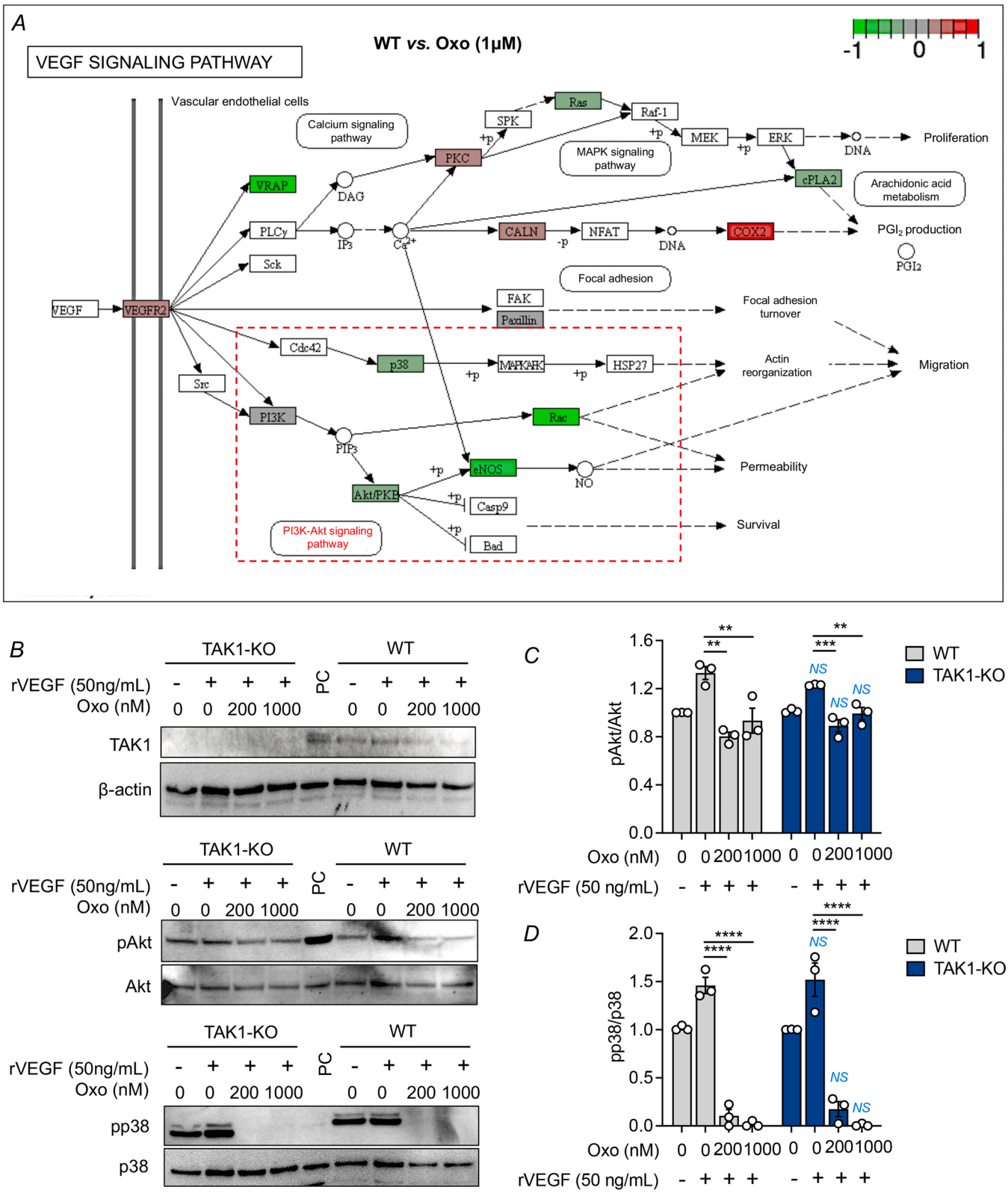

Fig. 7. VEGF signaling is targeted by 5Z-7-oxozeaenol. (A) Diagrams for the significantly changed VEGF signaling pathway (KEGG) plotted using pathview [61]. Intensity of colour represents gene Log2(fold change) with downregulation in green and upregulation in red. Pathway maps are displayed with copyright permission from KEGG. (B) TAK1 knockout (TAK1-KO) or wild-type (WT) TIMEs were preincubated with or without 5Z-7-oxozeaenol (200 or 1000 nM) for 30 min and then stimulated by recombinant human VEGF-A protein (rVEGF-A,50 ng/mL) for 30 min. The expression of TAK1 and the phosphorylation of Akt or p38 proteins were assessed using western blot (n = 3 from 3 independent experiments). PC: HEK293 cell lysate. (C-D) Quantitative analysis of phosphorylation of Akt and p38 in cells subjected to 5Z-7-oxozeaenol treatment and rVEGF-A stimuli. Group data are shown as means ± SEM. Statistical analysis was undertaken with one-way ANOVA and Tukey's multiple comparison test; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. *NS*, no statistically difference (WT vs. TAK1-KO at different doses of 5Z-7-oxozeaenol upon rVEGF-A stimulation). Oxo: 5Z-7-oxozeaenol.

of OIR rats and patients with PDR [15]. It would be interesting to understand if TAK1 inhibition or deletion has an effect on the proportion of M2 macrophages in OIR models. Accumulating evidence demonstrates that TAK1 plays a key role in the TNFα-induced endothelial cell activities [21], suggesting a potential therapeutic effect by targeting TAK1 for retinal vascular diseases.

A limitation of this study is that we did not examine the retinal functionality and integrity of existing vasculature in OIR rats intravitreally received 5Z-7-oxozeaenol, although 5Z-7-oxozeaenol significantly attenuates retinal neovascularization in this animal model. Secondly, we did not examine the mechanism by which 5Z-7-oxozeaenol diminishes activation of microglial cells in OIR rats, apart from targeting ICAM-1, at least in part resulting in suppression of retinal neovascularization. Third, there were only two doses (18 ng and 90 ng) of 5Z-7-oxozeaenol included for the animal studies where both doses showed significant inhibitory effects on retinal neovascularization. However, a comprehensive dose-dependent experiment should be performed to confirm specific selectivity of 5Z-7-oxozeaenol for TAK1 and to define the minimal dose of 5Z-7-oxozeaenol that is effective to inhibit retinal neovascularization. Lastly, our study found that other angiogenic signals, such as VEGF-mediated Akt and p38 pathways, were affected by 5Z-7-oxozeaenol and independent of TAK1, which may also contribute to the anti-angiogenic effect. Thus, we can not exclude that the inhibition of VEGF sgnlaing or other angiogenic pathways by 5Z-7-oxozeaenol also directly contributes to the suppression of retinal angiogenesis.

5. Conclusions

In summary, the present study identified TAK1 as a mediator in inflammatory and angiogenic signaling pathways, both playing central roles in retinal neovascularization. We demonstrated that TAK1 mediates NFκB signaling pathway in human endothelial cells upon inflammatory stimuli. Using a well-studied selective TAK1 inhibitor, 5Z-7-oxozeaenol, we showed that pharmacological inhibition of TAK1 effectively alleviated retinal neovascularization in *in vivo* models in rats. These findings provide new insights into the molecular and cellular mechanisms by which TAK1 is involved in hypoxia-induced inflammation and pathological angiogenesis, thus highlighting a potential alternative therapeutic approach to manage retinal neovascular pathology in eye diseases.

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Ethics approval

All animal experiments were conducted in accordance with guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Ethics Committees of St Vincent's Hospital (004/16), the University of Melbourne (13–044UM), and the University of Tasmania (A0017311).

CRediT authorship contribution statement

Jiang-Hui Wang: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Project administration. **Fan-Li Lin:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Project administration. **Jinying Chen:** Formal analysis, Investigation, Data curation, Writing – review & editing. **Linxin Zhu:** Investigation. **Yu-Fan Chuang:** Investigation. **Leilei Tu:** Investigation, Funding acquisition. **Chenkai Ma:** Methodology, Investigation, Writing – review & editing. **Damien Ling:** Investigation. **Alex W. Hewitt:** Resources. **Ching-Li Tseng:** Investigation, Resources. **Manisha H. Shah:** Investigation. **Bang V. Bui:** Methodology, Formal analysis, Investigation, Writing – review & editing. **Peter van Wijngaarden:** Conceptualization, Methodology, Writing – review & editing. **Gregory J. Dusting:** Conceptualization, Resources, Writing – review & editing, Funding acquisition. **Peng-Yuan Wang:** Resources, Writing – review & editing, Funding acquisition, Supervision. **Guei-Sheung Liu:** Conceptualization, Methodology, Formal analysis, Resources, Data curation, Writing – original draft, Project administration, Funding acquisition, Supervision.

Declaration of Competing Interest

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

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. Supporting data and source data can also be found in the supplementary files.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2022.106617.

References

[1] K.A. Neely, T.W. Gardner, Ocular neovascularization: clarifying complex interactions, Am. J. Pathol. 153 (3) (1998) 665–670.
[2] A. Usui-Ouchi, E. Aguilar, S. Murinello, M. Prins, M.L. Gantner, P.E. Wright, R. B. Berlow, M. Friedlander, An allosteric peptide inhibitor of HIF-1alpha regulates hypoxia-induced retinal neovascularization, Proc. Natl. Acad. Sci. USA 117 (45) (2020) 28297–28306.
[3] P.A. Campochiaro, Retinal and choroidal neovascularization, J. Cell Physiol. 184 (3) (2000) 301–310.
[4] M.A. Singer, D.S. Kermany, J. Waters, M.E. Jansen, L. Tyler, Diabetic macular edema: it is more than just VEGF, F1000Res 5 (2016).
[5] Y. Qazi, S. Maddula, B.K. Ambati, Mediators of ocular angiogenesis, J. Genet. 88 (4) (2009) 495–515.
[6] R.B. Goldberg, Cytokine and cytokine-like inflammation markers, endothelial dysfunction, and imbalanced coagulation in development of diabetes and its complications, J. Clin. Endocrinol. Metab. 94 (9) (2009) 3171–3182.
[7] N. Demircan, B.G. Safran, M. Soylu, A.A. Ozcan, S. Sizmaz, Determination of vitreous interleukin-1 (IL-1) and tumour necrosis factor (TNF) levels in proliferative diabetic retinopathy, Eye 20 (12) (2006) 1366–1369.
[8] M. Capitao, R. Soares, Angiogenesis and inflammation crosstalk in diabetic retinopathy, J. Cell Biochem. 117 (11) (2016) 2443–2453.
[9] P. Murugeswari, D. Shukla, A. Rajendran, R. Kim, P. Namperumalsamy, V. Muthukkaruppan, Proinflammatory cytokines and angiogenic and anti-angiogenic factors in vitreous of patients with proliferative diabetic retinopathy and eales' disease, Retina 28 (6) (2008) 817–824.
[10] W. Stevenson, S.F. Cheng, M.H. Dastjerdi, G. Ferrari, R. Dana, Corneal neovascularization and the utility of topical VEGF inhibition: ranibizumab (Lucentis) vs bevacizumab (Avastin), Ocul. Surf. 10 (2) (2012) 67–83.

[11] C.A. Aveleira, C.M. Lin, S.F. Abcouwer, A.F. Ambrosio, D.A. Antonetti, TNF-alpha signals through PKCzeta/NF-kappaB to alter the tight junction complex and increase retinal endothelial cell permeability, Diabetes 59 (11) (2010) 2872–2882.
[12] M. Clauss, C. Sunderkotter, B. Sveinbjornsson, S. Hippenstiel, A. Willuweit, M. Marino, E. Haas, R. Seljelid, P. Scheurich, N. Suttorp, M. Grell, W. Risau, A permissive role for tumor necrosis factor in vascular endothelial growth factor-induced vascular permeability, Blood 97 (5) (2001) 1321–1329.
[13] S. Yoshida, A. Yoshida, T. Ishibashi, Induction of IL-8, MCP-1, and bFGF by TNF-alpha in retinal glial cells: implications for retinal neovascularization during post-ischemic inflammation, Graefes Arch. Clin. Exp. Ophthalmol. 242 (5) (2004) 409–413.
[14] A.M. Joussen, V. Poulaki, N. Mitsiades, B. Kirchhof, K. Koizumi, S. Dohmen, A. P. Adamis, Nonsteroidal anti-inflammatory drugs prevent early diabetic retinopathy via TNF-alpha suppression, FASEB J. 16 (3) (2002) 438–440.
[15] J.H. Wang, S. Kumar, G.S. Liu, Bulk gene expression deconvolution reveals infiltration of M2 macrophages in retinal neovascularization, Invest. Ophthalmol. Vis. Sci. 62 (14) (2021) 22.
[16] S. Sato, H. Sanjo, K. Takeda, J. Ninomiya-Tsuji, M. Yamamoto, T. Kawai, K. Matsumoto, O. Takeuchi, S. Akira, Essential function for the kinase TAK1 in innate and adaptive immune responses, Nat. Immunol. 6 (11) (2005) 1087–1095.
[17] J.H. Shim, C. Xiao, A.E. Paschal, S.T. Bailey, P. Rao, M.S. Hayden, K.Y. Lee, C. Bussey, M. Steckel, N. Tanaka, G. Yamada, S. Akira, K. Matsumoto, S. Ghosh, TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo, Genes Dev. 19 (22) (2005) 2668–2681.
[18] S.R. Mihaly, J. Ninomiya-Tsuji, S. Morioka, TAK1 control of cell death, Cell Death Differ. 21 (11) (2014) 1667–1676.
[19] S.A. Scarneo, A. Mansourati, L.S. Eibschutz, J. Totzke, J.R. Roques, D. Loiselle, D. Carlson, P. Hughes, T.A.J. Haystead, Genetic and pharmacological validation of TAK1 inhibition in macrophages as a therapeutic strategy to effectively inhibit TNF secretion, Sci. Rep. 8 (1) (2018) 17058.
[20] K.B. Harikumar, B. Sung, S.T. Tharakan, M.K. Pandey, B. Joy, S. Guha, S. Krishnan, B.B. Aggarwal, Sesamin manifests chemopreventive effects through the suppression of NF-kappa B-regulated cell survival, proliferation, invasion, and angiogenic gene products, Mol. Cancer Res. 8 (5) (2010) 751–761.
[21] H. Naito, T. Iba, T. Wakabayashi, I. Tai-Nagara, J.I. Suehiro, W. Jia, D. Eino, S. Sakimoto, F. Muramatsu, H. Kidoya, H. Sakurai, T. Satoh, S. Akira, Y. Kubota, N. Takakura, TAK1 prevents endothelial apoptosis and maintains vascular integrity, Dev. Cell 48 (2) (2019) 151–166, e7.
[22] S. Morioka, M. Inagaki, Y. Komatsu, Y. Mishina, K. Matsumoto, J. Ninomiya-Tsuji, TAK1 kinase signaling regulates embryonic angiogenesis by modulating endothelial cell survival and migration, Blood 120 (18) (2012) 3846–3857.
[23] J. Chen, F.L. Lin, J.Y.K. Leung, L. Tu, J.H. Wang, Y.F. Chuang, F. Li, H.H. Shen, G. J. Dusting, V.H.Y. Wong, L. Lisowski, A.W. Hewitt, B.V. Bui, J. Zhong, G.S. Liu, A drug-tunable Flt23k gene therapy for controlled intervention in retinal neovascularization, Angiogenesis 24 (1) (2021) 97–110.
[24] K.M. Connor, N.M. Krah, R.J. Dennison, C.M. Aderman, J. Chen, K.I. Guerin, P. Sapieha, A. Stahl, K.L. Willett, L.E. Smith, Quantification of oxygen-induced retinopathy in the mouse: a model of vessel loss, vessel regrowth and pathological angiogenesis, Nat. Protoc. 4 (11) (2009) 1565–1573.
[25] E. Zudaire, L. Gambardella, C. Kurcz, S. Vermeren, A computational tool for quantitative analysis of vascular networks, PLoS One 6 (11) (2011), e27385.
[26] S. Hanzelmann, R. Castelo, J. Guinney, GSVA: gene set variation analysis for microarray and RNA-seq data, BMC Bioinforma. 14 (2013) 7.
[27] S. Ghosh, M. Karin, Missing pieces in the NF-kappaB puzzle, Cell 109 (Suppl.) (2002) S81–S96.
[28] G. Christoffersson, E. Vagesjo, J. Vandooren, M. Liden, S. Massena, R.B. Reinert, M. Brissova, A.C. Powers, G. Opdenakker, M. Phillipson, VEGF-A recruits a proangiogenic MMP-9-delivering neutrophil subset that induces angiogenesis in transplanted hypoxic tissue, Blood 120 (23) (2012) 4653–4662.
[29] J. Ninomiya-Tsuji, T. Kajino, K. Ono, T. Ohtomo, M. Matsumoto, M. Shiina, M. Mihara, M. Tsuchiya, K. Matsumoto, A resorcylic acid lactone, 5Z-7-oxozeaenol, prevents inflammation by inhibiting the catalytic activity of TAK1 MAPK kinase kinase, J. Biol. Chem. 278 (20) (2003) 18485–18490.
[30] C.H. Chin, S.H. Chen, H.H. Wu, C.W. Ho, M.T. Ko, C.Y. Lin, cytoHubba: identifying hub objects and sub-networks from complex interactome, BMC Syst. Biol. 8 (Suppl 4) (2014) S11.
[31] S.F. Hackett, S. Wiegand, G. Yancopoulos, P.A. Campochiaro, Angiopoietin-2 plays an important role in retinal angiogenesis, J. Cell Physiol. 192 (2) (2002) 182–187.
[32] O. Iriondo, Y. Liu, G. Lee, M. Elhodaky, C. Jimenez, L. Li, J. Lang, P. Wang, M. Yu, TAK1 mediates microenvironment-triggered autocrine signals and promotes triple-negative breast cancer lung metastasis, Nat. Commun. 9 (1) (2018) 1994.
[33] J. Li, C. Liang, Z.K. Zhang, X. Pan, S. Peng, W.S. Lee, A. Lu, Z. Lin, G. Zhang, W. N. Leung, B.T. Zhang, TAK1 inhibition attenuates both inflammation and fibrosis in experimental pneumoconiosis, Cell Discov. 3 (2017) 17023.
[34] L. Yang, S. Joseph, T. Sun, J. Hoffmann, S. Thevissen, S. Offermanns, B. Strilic, TAK1 regulates endothelial cell necroptosis and tumor metastasis, Cell Death Differ. 26 (10) (2019) 1987–1997.
[35] L. Dai, C. Aye Thu, X.Y. Liu, J. Xi, P.C. Cheung, TAK1, more than just innate immunity, IUBMB Life 64 (10) (2012) 825–834.
[36] Y. Onodera, T. Teramura, T. Takehara, K. Shigi, K. Fukuda, Reactive oxygen species induce Cox-2 expression via TAK1 activation in synovial fibroblast cells, FEBS Open Biol. 5 (2015) 492–501.
[37] M. Xie, D. Zhang, J.R. Dyck, Y. Li, H. Zhang, M. Morishima, D.L. Mann, G.E. Taffet, A. Baldini, D.S. Khy, M.D. Schneider, A pivotal role for endogenous TGF-beta-activated kinase-1 in the LKB1/AMP-activated protein kinase energy-sensor pathway, Proc. Natl. Acad. Sci. USA 103 (46) (2006) 17378–17383.
[38] M. Ono, Molecular links between tumor angiogenesis and inflammation: inflammatory stimuli of macrophages and cancer cells as targets for therapeutic strategy, Cancer Sci. 99 (8) (2008) 1501–1506.
[39] J. Wu, F. Powell, N.A. Larsen, Z. Lai, K.F. Byth, J. Read, R.F. Gu, M. Roth, D. Toader, J.C. Saeh, H. Chen, Mechanism and in vitro pharmacology of TAK1 inhibition by (5Z)-7-Oxozeaenol, ACS Chem. Biol. 8 (3) (2013) 643–650.
[40] N. Kociok, S. Radetzky, T.U. Krohne, C. Gavranic, A.M. Joussen, Pathological but not physiological retinal neovascularization is altered in TNF-Rp55-receptor-deficient mice, Invest. Ophthalmol. Vis. Sci. 47 (11) (2006) 5057–5065.
[41] Z. Chen, Y. Mei, H. Lei, R. Tian, N. Ni, F. Han, S. Gan, S. Sun, LYTAK1, a TAK1 inhibitor, suppresses proliferation and epithelialmesenchymal transition in retinal pigment epithelium cells, Mol. Med. Rep. 14 (1) (2016) 145–150.
[42] Z. Dvashi, M. Goldberg, O. Adir, M. Shapira, A. Pollack, TGF-beta1 induced transdifferentiation of rpe cells is mediated by TAK1, PLoS One 10 (4) (2015), e0122229.
[43] D. Zhang, H. Yan, H. Li, S. Hao, Z. Zhuang, M. Liu, Q. Sun, Y. Yang, M. Zhou, K. Li, C. Hang, TGFbeta-activated kinase 1 (TAK1) Inhibition by 5Z-7-oxozeaenol attenuates early brain injury after experimental subarachnoid hemorrhage, J. Biol. Chem. 290 (32) (2015) 19900–19909.
[44] L. Lu, X. Zhang, H. Tong, W. Zhang, P. Xu, S. Qu, Central Administration of 5Z-7-oxozeaenol protects experimental autoimmune encephalomyelitis mice by inhibiting microglia activation, Front. Pharmacol. 8 (2017) 789.
[45] T.D. Merson, M.D. Binder, T.J. Kilpatrick, Role of cytokines as mediators and regulators of microglial activity in inflammatory demyelination of the CNS, Neuromol. Med. 12 (2) (2010) 99–132.
[46] J.D. Huber, C.R. Campos, K.S. Mark, T.P. Davis, Alterations in blood-brain barrier ICAM-1 expression and brain microglial activation after lambda-carrageenan-induced inflammatory pain, Am. J. Physiol. Heart Circ. Physiol. 290 (2) (2006) H732–H740.
[47] B.J. White, S. Tarabishy, V.R. Venna, B. Manwani, S. Benashski, L.D. McCullough, J. Li, Protection from cerebral ischemia by inhibition of TGFbeta-activated kinase, Exp. Neurol. 237 (1) (2012) 238–245.
[48] M. Ivshina, I.M. Alexandrov, A. Vertii, S. Doxsey, J.D. Richter, CPEB regulation of TAK1 synthesis mediates cytokine production and the inflammatory immune response, Mol. Cell Biol. 35 (3) (2015) 610–618.
[49] D.J. Ireland, M.W. Kemp, Y. Miura, M. Saito, J.P. Newnham, J.A. Keelan, Intra-amniotic pharmacological blockade of inflammatory signalling pathways in an ovine chorioamnionitis model, Mol. Hum. Reprod. 21 (5) (2015) 479–489.
[50] M. Neubert, D.A. Ridder, P. Bargiotas, S. Akira, M. Schwaninger, Acute inhibition of TAK1 protects against neuronal death in cerebral ischemia, Cell Death Differ. 18 (9) (2011) 1521–1530.
[51] M. Tang, X. Wei, Y. Guo, P. Breslin, S. Zhang, S. Zhang, W. Wei, Z. Xia, M. Diaz, S. Akira, J. Zhang, TAK1 is required for the survival of hematopoietic cells and hepatocytes in mice, J. Exp. Med. 205 (7) (2008) 1611–1619.
[52] K. Bettermann, M. Vucur, J. Haybaeck, C. Koppe, J. Janssen, F. Heymann, A. Weber, R. Weiskirchen, C. Liedtke, N. Gassler, M. Muller, R. de Vos, M.J. Wolf, Y. Boege, G.M. Seleznik, N. Zeller, D. Erny, T. Fuchs, S. Zoller, S. Cairo, M. A. Buendia, M. Prinz, S. Akira, F. Tacke, M. Heikenwalder, C. Trautwein, T. Luedde, TAK1 suppresses a NEMO-dependent but NF-kappaB-independent pathway to liver cancer, Cancer Cell 17 (5) (2010) 481–496.
[53] E. Omori, S. Morioka, K. Matsumoto, J. Ninomiya-Tsuji, TAK1 regulates reactive oxygen species and cell death in keratinocytes, which is essential for skin integrity, J. Biol. Chem. 283 (38) (2008) 26161–26168.
[54] L. Tan, D. Gurbani, E.L. Weisberg, D.S. Jones, S. Rao, W.D. Singer, F.M. Bernard, S. Mowafy, A. Jenney, G. Du, A. Nonami, J.D. Griffin, D.A. Lauffenburger, K. D. Westover, P.K. Sorger, N.S. Gray, Studies of TAK1-centered polypharmacology with novel covalent TAK1 inhibitors, Bioorg. Med. Chem. 25 (4) (2017) 1320–1328.
[55] P.Y. Dakas, S. Barluenga, F. Totzke, U. Zirrgiebel, N. Winssinger, Modular synthesis of radicicol A and related resorcylic acid lactones, potent kinase inhibitors, Angew. Chem. Int. Ed. Engl. 46 (36) (2007) 6899–6902.
[56] T. Kitamura, N. Asai, A. Enomoto, K. Maeda, T. Kato, M. Ishida, P. Jiang, T. Watanabe, J. Usukura, T. Kondo, F. Costantini, T. Murohara, M. Takahashi, Regulation of VEGF-mediated angiogenesis by the Akt/PKB substrate girdin, Nat. Cell Biol. 10 (3) (2008) 329–337.
[57] K. Issbrucker, H.H. Marti, S. Hippenstiel, G. Springmann, R. Voswinckel, A. Gaumann, G. Breier, H.C. Drexler, N. Suttorp, M. Clauss, p38 MAP kinase–a molecular switch between VEGF-induced angiogenesis and vascular hyperpermeability, FASEB J. 17 (2) (2003) 262–264.
[58] C. Derada Troletti, R.D. Fontijn, E. Gowing, M. Charabati, B. van Het Hof, I. Didouh, S.M.A. van der Pol, D. Geerts, A. Prat, J. van Horssen, G. Kooij, H.E. de Vries, Inflammation-induced endothelial to mesenchymal transition promotes brain endothelial cell dysfunction and occurs during multiple sclerosis pathophysiology, Cell Death Dis. 10 (2) (2019) 45.
[59] M. Suzuki, N. Nagai, K. Izumi-Nagai, H. Shinoda, T. Koto, A. Uchida, H. Mochimaru, K. Yuki, M. Sasaki, K. Tsubota, Y. Ozawa, Predictive factors for non-response to intravitreal ranibizumab treatment in age-related macular degeneration, Br. J. Ophthalmol. 98 (9) (2014) 1186–1191.
[60] M.T. Bolinger, D.A. Antonetti, Moving past Anti-VEGF: novel therapies for treating diabetic retinopathy, Int. J. Mol. Sci. 17 (9) (2016).
[61] W. Luo, C. Brouwer, Pathview: an R/Bioconductor package for pathway-based data integration and visualization, Bioinformatics 29 (14) (2013) 1830–1831.