

# Inhibiting NF- $\kappa$ B Signaling Activation Reduces Retinal Neovascularization by Promoting a Polarization Shift in Macrophages

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**PURPOSE.** Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) signaling is involved in regulating tumor angiogenesis and metastasis; however, the exact mechanism of action in retinal neovascularization (RNV) remains unclear. The purpose of this study was to determine the role and underlying mechanism of NF- $\kappa$ B in regulating RNV in retinal neovascularization mice.

**METHODS.** Expression levels of NF- $\kappa$ B signaling were detected by immunofluorescence staining and western blotting in retinas of oxygen-induced retinopathy (OIR) mice. OIR mice were treated with either pyrrolidinedithiocarbamate (PDTC), a NF- $\kappa$ B signaling inhibitor, or PBS, and retinal flat-mounts were performed to quantify the area of RNV and the recruitment of retinal macrophages by immunofluorescence staining. Macrophage polarization detected by flow cytometric analysis and the expression of macrophage polarization-associated genes were evaluated by immunofluorescence staining, quantitative RT-PCR, and western blotting.

**RESULTS.** Expression levels of phosphorylated I $\kappa$ B $\alpha$  (p-I $\kappa$ B $\alpha$ ) and p-p65 increased in OIR mice. Inhibiting NF- $\kappa$ B signaling activation by PDTC significantly reduced RNV. After treatment with PDTC, a reduction in the quantity of macrophages was observed: M1 polarized macrophages decreased, and M2 polarized macrophages increased; the expression of M1 macrophage-associated cytokines decreased and M2 macrophage-associated cytokines increased in the retinas of OIR mice.

**CONCLUSIONS.** Blocking activation of NF- $\kappa$ B signaling reduces RNV by promoting polarization of M1 macrophages to M2 macrophages in OIR mice.

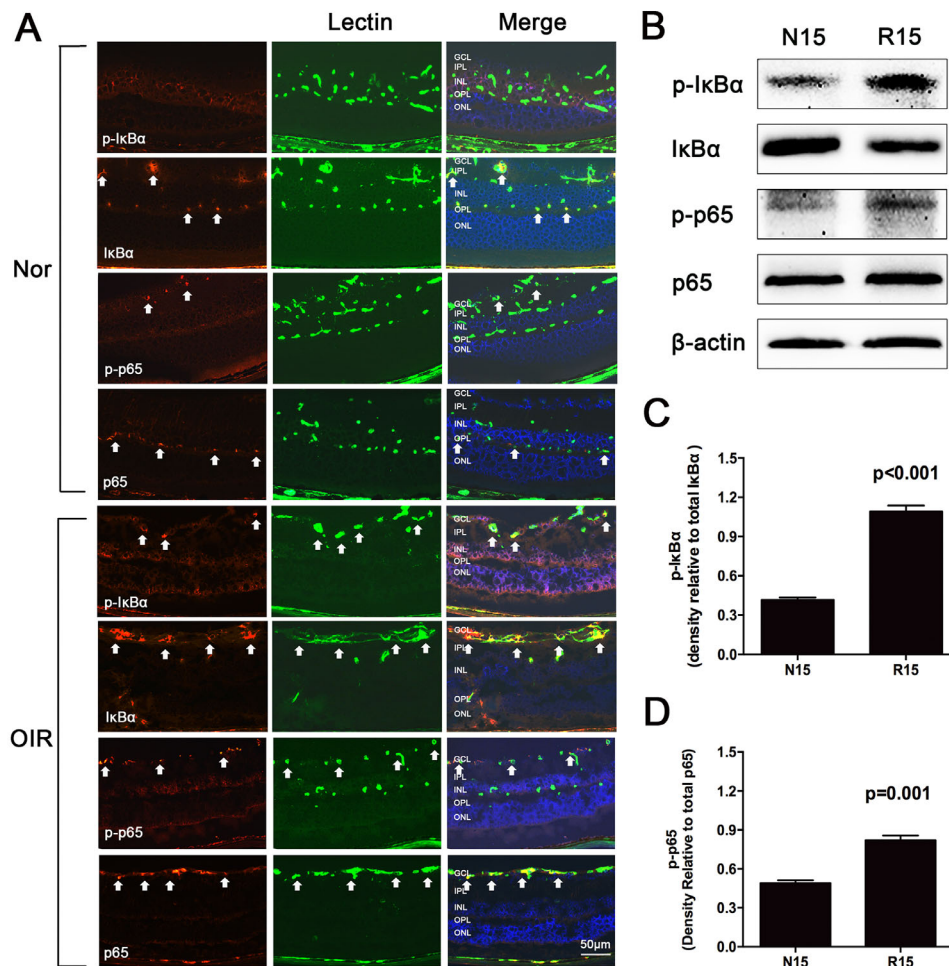
**Keywords:** NF- $\kappa$ B, retinal neovascularization, M1, M2 macrophage polarization

Retinal neovascularization (RNV) is a common pathological process involved in a number of blinding eye diseases, including proliferative diabetic retinopathy, retinopathy of prematurity (ROP), and retinal vein occlusion (RVO).<sup>1</sup> VEGF antagonists have become an important means to treat RNV-associated diseases in addition to traditional methods such as vitrectomy, laser photocoagulation, and photodynamic therapy. However, the cost, safety, and reduced efficacy in some patients suggest that we need to further study the pathogenesis of RNV and find alternative or adjuvant therapies.<sup>2-4</sup>

Recent studies have demonstrated that macrophages play a crucial role in regulating RNV. Macrophages have a powerful phagocytic function and are also involved in directional migration and secretion of a large number of cytokines related to neovascularization.<sup>5-7</sup> Depending on the stimulat-

ing signals of the local microenvironment, macrophages can differentiate into either inflammation-promoting, microbial-killing M1 macrophages or inflammation-inhibiting, tissue-repairing M2 macrophages. M1 and M2 macrophage expression has been demonstrated in ROP, but their roles in RNV have not been well studied.<sup>6,7</sup>

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) is a member of the Rel protein family and a widespread, heterodimeric transcription factor.<sup>8</sup> Under normal circumstances, NF- $\kappa$ B binds to inhibitors of NF- $\kappa$ B: I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$ , thereby inhibiting migration from the cytoplasm to the nucleus.<sup>9,10</sup> Various factors such as viruses and hypoxia can activate I $\kappa$ B kinase (IKK)- $\alpha$  and IKK- $\beta$ , leading to phosphorylation of I $\kappa$ B. Phosphorylated I $\kappa$ B (p-I $\kappa$ B) subsequently induces the disintegration of the cytoplasmic complex, resulting in the release of the NF- $\kappa$ B



**FIGURE 1.** The expression of p-IκBα, IκBα, p-p65, and p65 was examined by immunofluorescence staining and western blot assays in retinas of OIR mice. (A) OIR mice (n = 3) and normal mice (n = 3) were euthanized at P18, their eyes were removed and fixed, and 10-μm-thick frozen sections were generated. Immunofluorescence staining for p-IκBα, total IκBα, p-p65, total p65 (red), and FITC-*Griffonia simplicifolia* Isolectin B4 (a marker for vascular endothelial cell) was performed. Arrows indicate the positive area of each marker. (B–D) Western blot was performed to evaluate the expression levels of p-IκBα, total IκBα, p-p65, and total p65 at P15 in retinas of mice with OIR (n = 3) and normal controls (n = 3). Expression levels of β-actin were used as the endogenous control. Data are expressed as mean ± SEM from three independent experiments, and Student's *t*-test was used for statistical analysis. The layers of the retina in cross-sections are shown: ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), and outer nuclear layer (ONL).

p65 subunit. Free NF-κB p65 translocates to the nucleus, and NF-κB p-p65 activates various transcription factors.<sup>11,12</sup> Targeting NF-κB results in downregulation of macrophage-produced pro-inflammatory cytokines. As a result, NF-κB has become a significant therapeutic target in several chronic inflammatory diseases.<sup>13–16</sup> In previous studies, the use of pyrrolidinedithiocarbamate (PDTC), an inhibitor of NF-κB, significantly reduced RNV, but the specific regulatory mechanism remains unknown.<sup>17</sup> Therefore, the purpose of this study was to further investigate the role of NF-κB in the regulation of RNV and its potential interaction with macrophages in oxygen-induced retinopathy (OIR) mice.

**METHODS**

**Mice and Ethics Statement**

Specific-pathogen-free C57BL/6 mice were used for the purpose of this study (Charles River Laboratories, Wilming-

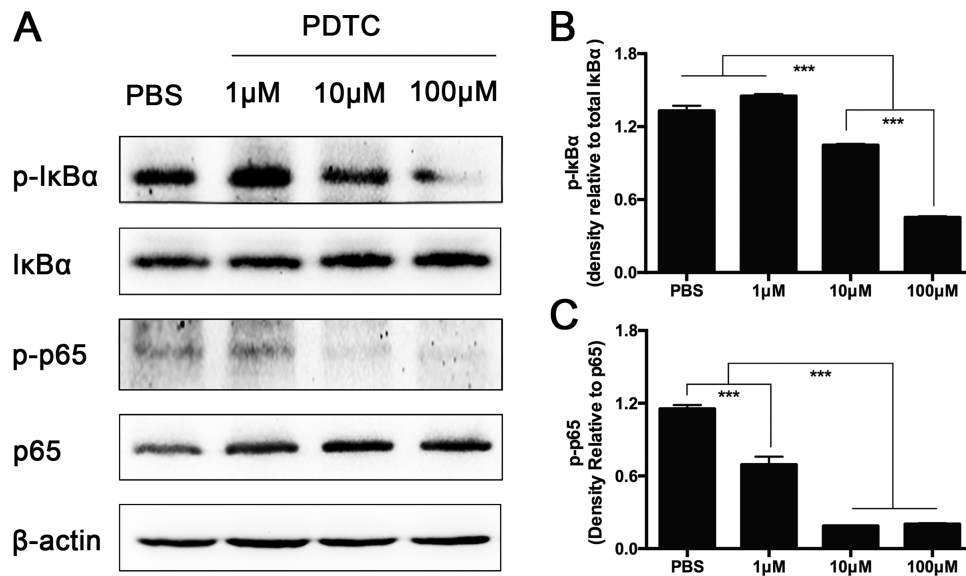
ton, MA, USA). All of the animal procedures were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Guide of the Laboratory Animals Care and Use Committee at Shanghai Jiao Tong University School of Medicine.

**Mouse Model of Oxygen-Induced Retinopathy**

For the mouse model of oxygen-induced retinopathy, 7-day-old C57BL/6 mice and their nursing mothers were exposed to 75% ± 3% oxygen for 5 days and then returned to room air at postnatal day 12 (P12) as previously described.<sup>18,19</sup>

**Immunofluorescence Staining**

Eyes from OIR and unexposed control mice were fixed and prepared for 10-μm-cross-sectioning. After fixation with 4% paraformaldehyde, permeabilization with 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) and blocking



**FIGURE 2.** PDTC inhibited the activation of NF- $\kappa$ B signaling in the retinas of OIR mice. At P12, OIR mice were randomly divided into four groups ( $n = 3$  mice for each group) and received an intravitreal injection of either 1  $\mu$ L of PBS or 1  $\mu$ L of different concentrations of PDTC (1  $\mu$ M, 10  $\mu$ M, or 100  $\mu$ M). At P15, retinal total protein was isolated and quantified by western blot for p-I $\kappa$ B $\alpha$ , total I $\kappa$ B $\alpha$ , p-p65, and total p65 (A). The results are shown as the mean  $\pm$  SEM from three independent experiments (B, C) ( $***P < 0.001$ ). The Student–Newman–Keuls method was used for statistical analysis.

with 5% bovine serum albumin, cross-sections were respectively incubated with rabbit anti-p-I $\kappa$ B $\alpha$ , rabbit anti-I $\kappa$ B $\alpha$  (Abcam, Cambridge, MA, USA), rabbit anti-p-p65, rabbit anti-p65, rabbit anti-inducible nitric oxide synthase (iNOS) (Cell Signaling Technology, Inc., Danvers, MA, USA), and rabbit anti-Arginase-1 (Arg-1) (GeneTex, Irvine, CA, USA) and then incubated with Alexa Fluor 555 anti-rabbit IgG (Cell Signaling Technology) plus *Griffonia simplicifolia* Isolectin B4 (GSA-Lectin)-labeled fluorescein isothiocyanate (FITC; Vector Laboratories, Inc., Burlingame, CA, USA), PE-F4/80 (eBioscience, Vienna, Austria) plus FITC-GSA-Lectin (Vector Laboratories), or PE-F4/80 plus Alexa Fluor 488 anti-rabbit IgG (Cell Signaling Technology). After incubation with 4',6-diamidino-2-phenylindole (Beyotime, Shanghai, China), cross-sections were photographed using fluorescence microscopy (Nikon Instruments, Inc., Melville, New York, USA).

### Retinal Flat-Mounts

In this study, P12 OIR mice received an intravitreal injection of 1  $\mu$ L of either PDTC in different concentrations (1  $\mu$ M, 10  $\mu$ M, or 100  $\mu$ M) or PBS as previously described.<sup>6</sup> At P18, eyes were fixed in 4% paraformaldehyde solution for 4 hours and dissected, and the retinas were removed. Retinas were incubated with PE-F4/80 plus FITC-GSA-Lectin for 40 minutes in the dark.<sup>16</sup> Retinas were mounted in fluorescence mounting medium (Dako, Glostrup, Denmark) on glass slides and photographed using fluorescence microscopy. Images were analyzed by Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

### Western Blotting

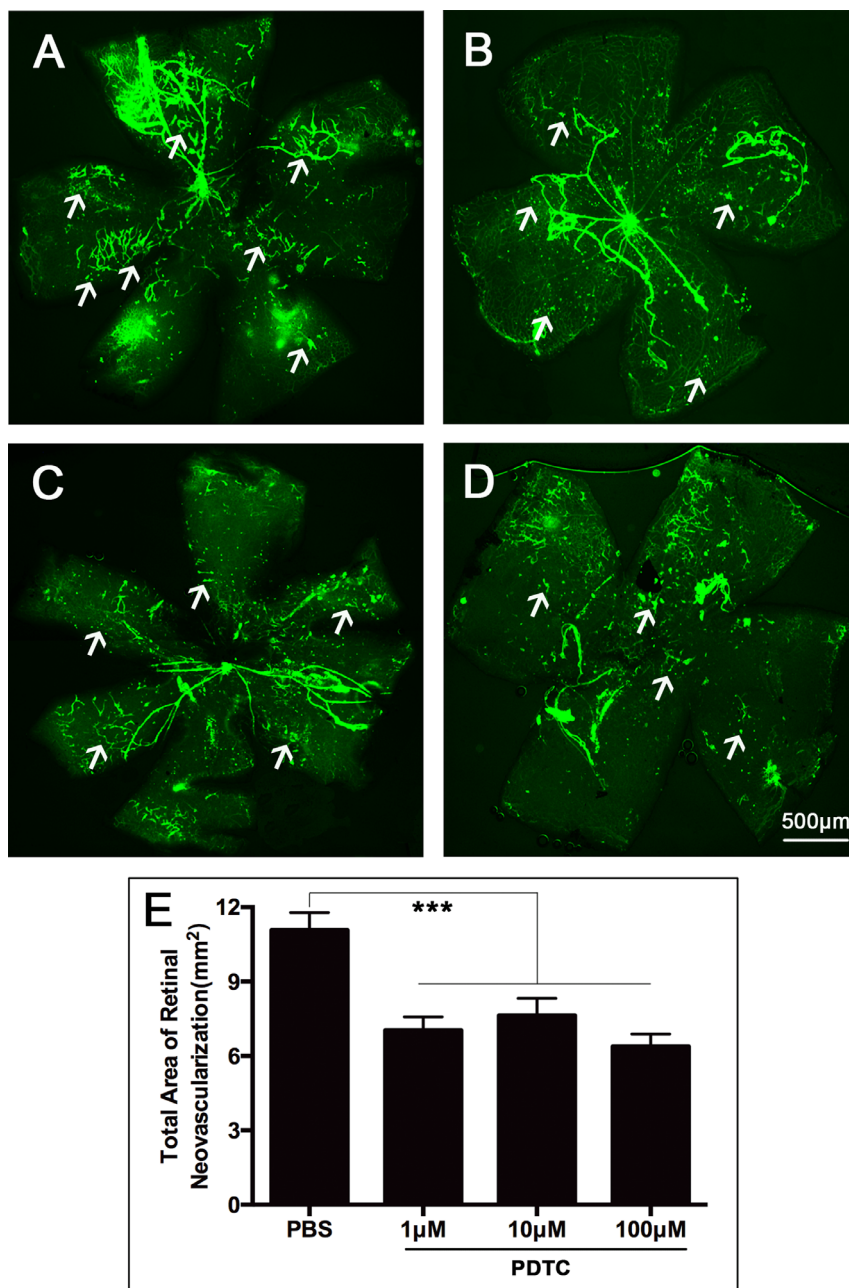
Proteins were extracted from the retinas of C57BL/6 mice at specific time points and adjusted for protein content

(40  $\mu$ g). Protein samples were then mixed with 5 $\times$  SDS sample buffer (Beyotime), separated by SDS polyacrylamide gel electrophoresis, and transferred onto polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Bedford, MA, USA). After being blocked in 5% nonfat dried milk for 2 hours at room temperature, the membranes were incubated overnight at 4°C with primary antibodies. Primary antibodies used in this study included rabbit anti-I $\kappa$ B $\alpha$ , rabbit anti-p-I $\kappa$ B $\alpha$ , rabbit anti-p65, rabbit anti-p-p65, rabbit anti-iNOS, mouse anti- $\beta$ -actin (Cell Signaling Technology), rabbit anti-interleukin-1 $\beta$  (IL-1 $\beta$ ), rabbit anti-Toll-like receptor (TLR)2 (Abcam), goat anti-CD206, rabbit anti-transforming growth factor- $\beta$  (TGF- $\beta$ ), rabbit anti-TLR4 (Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-Arg-1 (GeneTex), goat anti IL-1RA, goat anti-monocyte chemoattractant protein1 (MCP-1; R&D Systems, Minneapolis, MN, USA). Membranes were washed and incubated for 2 hours with secondary antibodies conjugated with horseradish peroxidase (Cell Signaling Technology). We used enhanced chemiluminescence (ECL) western blotting detection solutions (EMD Millipore) to display the immunoreactive bands.

### Quantitative RT-PCR

Total RNA of retinas was isolated, and 2  $\mu$ g of total RNA of each sample was reverse transcribed into cDNA (Roche, Basel, Switzerland). RT-PCR was performed using SYBR Green Mix (Roche) and an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) in a 20- $\mu$ L volume. The housekeeping gene cyclophilin A was used to normalize the relative expression of target genes. The relative quantification of different groups was calculated using the  $2^{-\Delta\Delta CT}$  method. The PCR primers were cyclophilin A, 5'-CAGACGCC ACTGTGCTTT-3' (sense), 5'-TGTCTTTGGAACITTTGTCTG CAA-3' (antisense); IL-1 $\beta$ , 5'-TGCCACCTTTTGACAGTGATG-





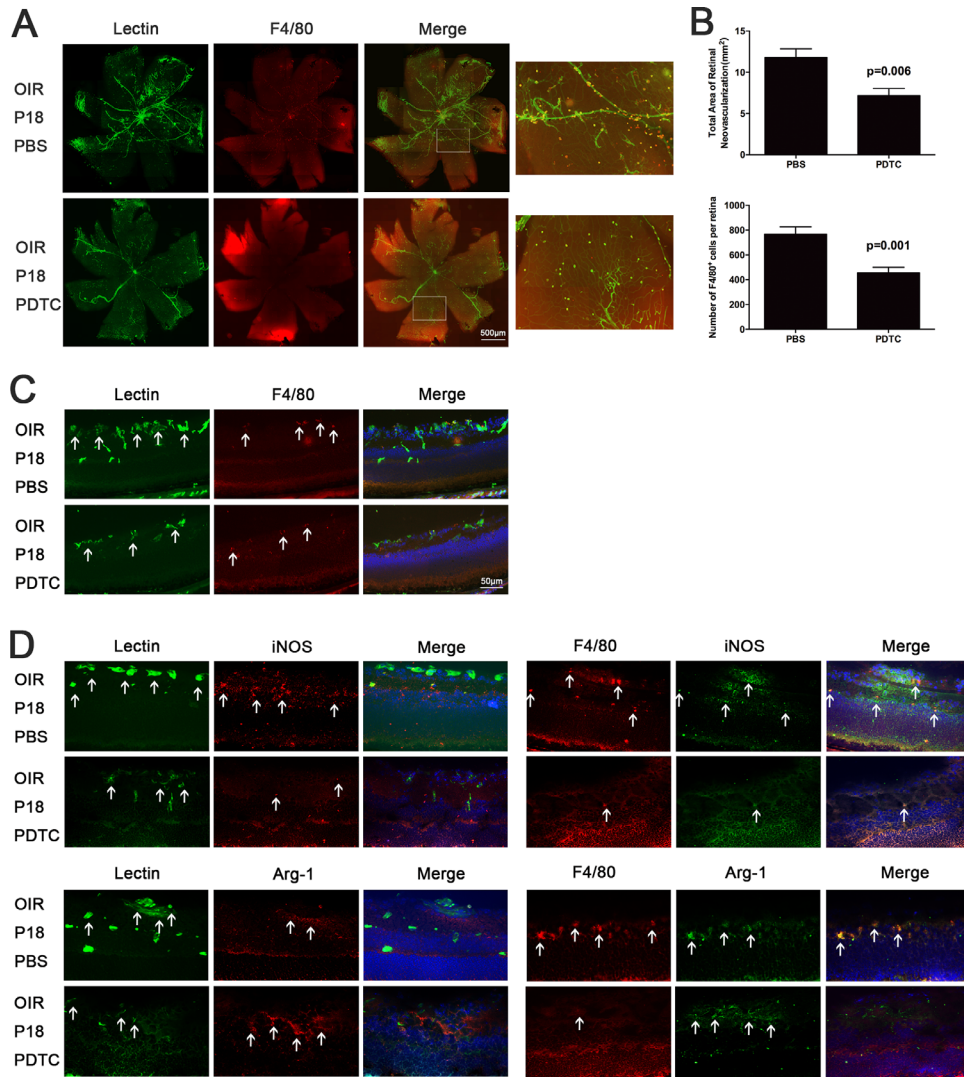
**FIGURE 3.** Blocking activation of NF-κB signaling by PDTC reduced RNV in OIR mice. At P12, OIR mice received intravitreal injection of either (A) 1 μL of PBS (n = 14 eyes) or 1 μL of PDTC at different concentrations: (B) 1 μM, n = 14 eyes; (C) 10 μM, n = 18 eyes; and (D) 100 μM, n = 14 eyes. At P18, whole retinas were stained with FITC-lectin and then flat-mounted. Arrows denote new vessels in retinas of OIR mice. Data are shown as mean ± SEM (E) and were analyzed by the Student–Newman–Keuls method (\*\*P < 0.001).

3' (sense), 5'-AAGGTCCACGGGAAAGACAC-3' (antisense); MCP-1, 5'-CTCGGACTGTGATGCCTTAAT-3' (sense), 5'-TAAATGCAAGGTGTGGATCCA-3' (antisense); iNOS, 5'-CCCTCAATGGTTGGTACATGG-3' (sense), 5'-ACATTGATCTCCGTGACAGCC-3' (antisense); TLR4, 5'-TCAGAGCCGTGGGTGTATCTT-3' (sense), 5'-GCTTTCTTGGGCTTCCTCTT-3' (antisense); TLR2, 5'-GTGTCTGGAGTCTGCTGTGC-3' (sense), 5'-GCTTCTTGGGCTTCCTCTT-3' (antisense); CD206, 5'-GGAATCAAGGGCACAGAGTTA-3' (sense), 5'-ATTGTGGAGCAGATGGAA-3' (antisense); Arg-1, 5'-TGGGTGACTCCCTGCATATCT-3' (sense), 5'-TTCCATCACCTGCCAATCC-3'; IL-1RA, 5'-TAGTGTGTTCTTGGGCATCC-

3' (sense), 5'-CGCTTGTCTTCTTCTTTGTTCT-3' (antisense); and TGF-β, 5'-ATTCTGGCGTTACCTTGG-3' (sense), 5'-AGCCCTGTATTCCGTCTCT-3' (antisense).<sup>5,6</sup>

### Flow Cytometric Analysis

The retinas of OIR mice treated with PBS or PDTC were digested in preheated papain solution (Worthington Biochemical Corp., Lakewood, NJ, USA) for 30 minutes. The obtained cell digestion suspension was filtered, centrifuged, and suspended with MACS buffer (BD Biosciences, San Jose, CA, USA). After incubation with anti-mouse



**FIGURE 4.** Intravitreal injection of PDTC reduced the quantity of macrophages in the retina of OIR mice. (A) RNV and macrophage recruitment in flat-mounted retinas of OIR mice were detected by immunofluorescence staining. Retinas from OIR mice were stained with FITC-lectin and PE-F4/80 antibody. We found a reduction in the area of RNV at P18, accompanied by decreased macrophage infiltration (PBS groups, n = 6 eyes; PDTC groups, n = 7 eyes). The rightmost pictures designate locally enlarged images for detail. (B) Data are shown as mean  $\pm$  SEM analyzed by Student's *t*-test. (C) Immunofluorescence staining of FITC-lectin and PE-F4/80 in OIR mice treated with PBS (n = 3) or PDTC (n = 3). (D) Immunofluorescence staining of FITC-lectin plus iNOS, FITC-lectin plus Arg-1, F4/80 plus iNOS, F4/80 plus Arg-1 in OIR mice treated with PBS (n = 3) or PDTC (n = 3). The *arrowheads* indicate the positive regions for each labeling staining.

CD11b magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) at 4°C for 20 minutes, these cells were screened with pre-humidified mass spectrometry column (BD Biosciences). Collected CD11b<sup>+</sup> cells were incubated with FITC-conjugated anti-mouse F4/80 (eBioscience), PE-conjugated anti-mouse CD11c (eBioscience), Alexa Fluor 647-conjugated CD206 (Bio-Rad AbD Serotec, Ltd., Oxford, UK), and the matching control isotype IgG (MCA421; Bio-Rad AbD Serotec) at 4°C for 30 minutes. After washing and resuspension, the cells were analyzed by flow cytometry (BD Biosciences). F4/80<sup>+</sup>/CD11c<sup>+</sup>/CD206<sup>-</sup> (F4/80<sup>+</sup> and CD11c<sup>+</sup>) cells were identified as M1 polarized macrophages, and F4/80<sup>+</sup>/CD11c<sup>-</sup>/CD206<sup>+</sup> (F4/80<sup>+</sup> and CD206<sup>+</sup>) cells were identified as M2 polarized macrophages as previously described.<sup>6,20,21</sup> Data analysis was performed by FlowJo software (BD Life Sciences, Franklin Lakes, NJ, USA).

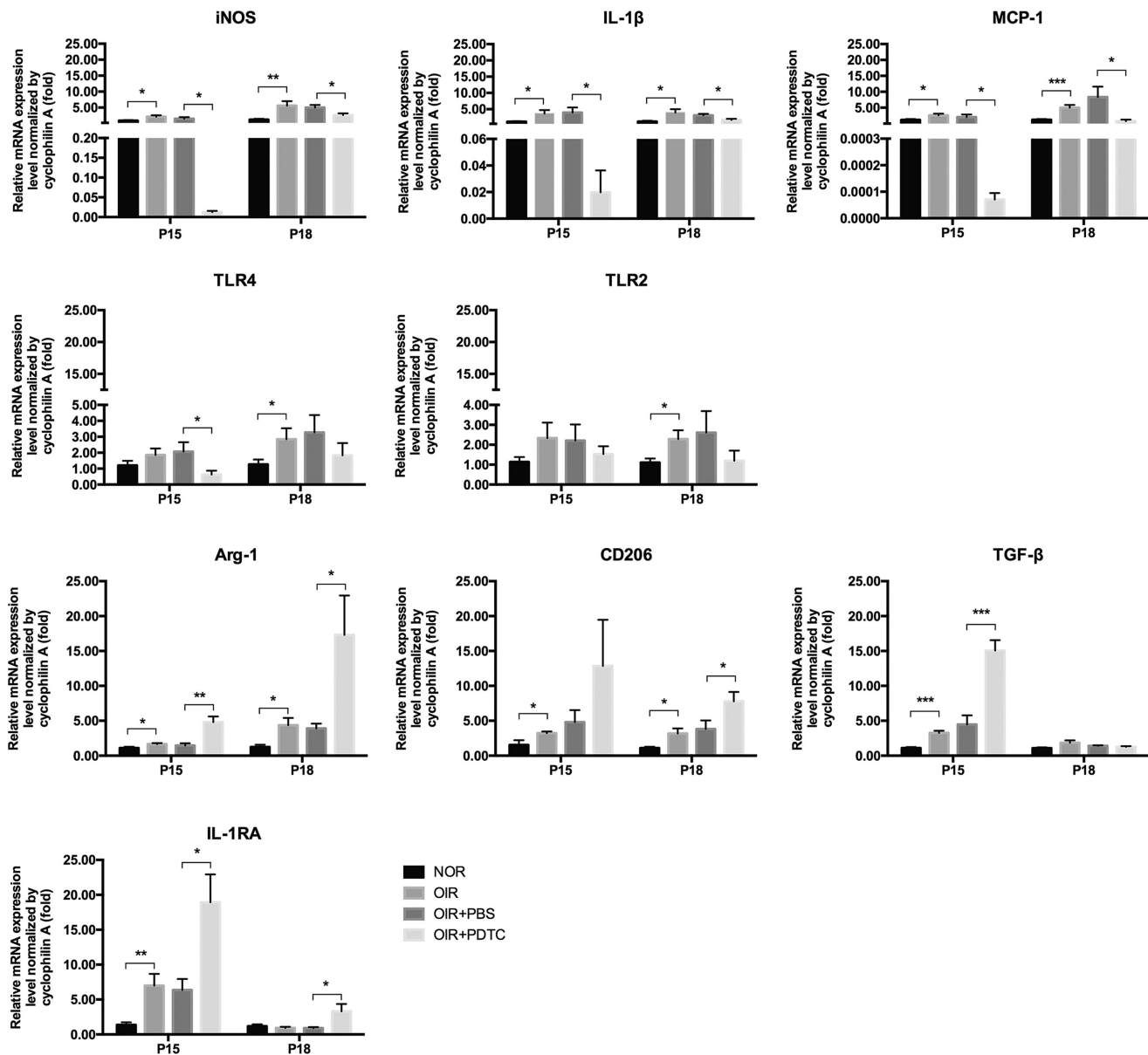
### Statistical Analysis

Mean value  $\pm$  SEM was used to present all data. The differences between two groups were analyzed by the two-tailed Student's *t*-test. The Student–Newman–Keuls method was applied to analyze multiple comparisons. When *P* < 0.05, the data were considered statistically significant. All statistical analysis was carried out using SAS 9.0 software (SAS Institute, Inc., Cary, NC, USA).

### RESULTS

#### Protein Expression Levels of p-I $\kappa$ B $\alpha$ , I $\kappa$ B $\alpha$ , p-p65, and p65 Were Elevated in the Retinas of OIR Mice

Immunofluorescence staining was performed on retinal cross-sections of OIR and normal mice at P18. Increased

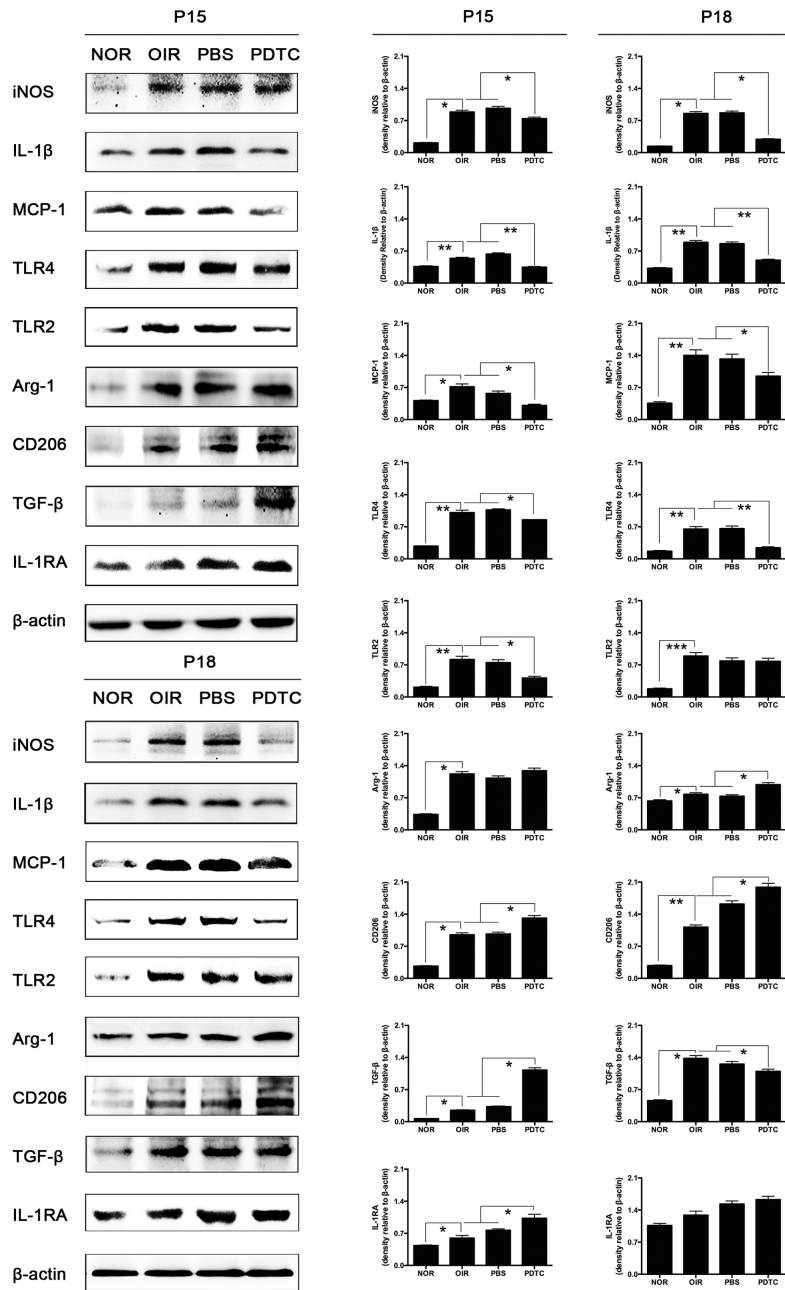


**FIGURE 5.** Messenger RNA expression levels of macrophage polarization-associated genes in OIR mice were detected by RT-PCR. To investigate the role of NF- $\kappa$ B signaling on macrophage polarization, mRNA expression levels of M1 polarized macrophage-associated genes (iNOS, IL-1 $\beta$ , MCP-1, TLR4, and TLR2) and M2 polarized macrophage-associated genes (Arg-1, CD206, TGF- $\beta$ , and IL-1RA) were compared at P15 and P18 ( $n = 5-8$  mice per group). Housekeeping gene cyclophilin A was used to normalize the relative expression of target genes. The relative quantification of different groups was calculated by the  $2^{-\Delta\Delta CT}$  method. Statistical analysis was performed with Student's  $t$ -test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

expression of p-I $\kappa$ B $\alpha$  and p-p65 was observed in OIR mice retinas (Fig. 1A). The merged images demonstrated co-localization of p-I $\kappa$ B $\alpha$  (I $\kappa$ B $\alpha$ , p-p65, p65) and lectin. Western blotting was used to determine the expression levels of p-I $\kappa$ B $\alpha$ , I $\kappa$ B $\alpha$ , p-p65, and p65 in the retinas of OIR mice. At P15, the ratios of p-I $\kappa$ B $\alpha$  to I $\kappa$ B $\alpha$  and p-p65 to p65 were significantly increased ( $P < 0.001$  and  $P = 0.001$  respectively) in the retinas of OIR mice compared with age-matched controls (Figs. 1B–1D).

### PDTC Inhibited Activation of NF- $\kappa$ B Signaling in the Retinas of OIR Mice

OIR mice received different concentrations of PDTC (0 $\mu$ M, 1 $\mu$ M, 10 $\mu$ M, or 100 $\mu$ M) at P12 and were subsequently sacrificed at P15. Western blot assays of isolated retinas demonstrated that PDTC significantly inhibited the phosphorylation of NF- $\kappa$ B signaling (p-I $\kappa$ B $\alpha$  and p-p65) at 10  $\mu$ M and 100  $\mu$ M concentrations compared with PBS-injected eyes ( $P < 0.001$ ) (Fig. 2).



**FIGURE 6.** Protein expression levels of macrophage polarization-associated genes in OIR mice were detected by western blotting. OIR mice received intravitreal injections of either 1  $\mu$ L of PBS or 1  $\mu$ L of PDTC (10  $\mu$ M) at P12, and eyes were harvested at P15 and P18 ( $n = 3$  mice for each group). Retinas were removed and total protein was isolated. Protein expression levels of macrophage polarization-associated genes in OIR and normal mice were detected by western blotting assays. Three independent experiments were performed. The data are shown as mean  $\pm$  SEM and were analyzed by the Student–Newman–Keuls method. \* $P < 0.05$ , \*\* $P < 0.01$ .

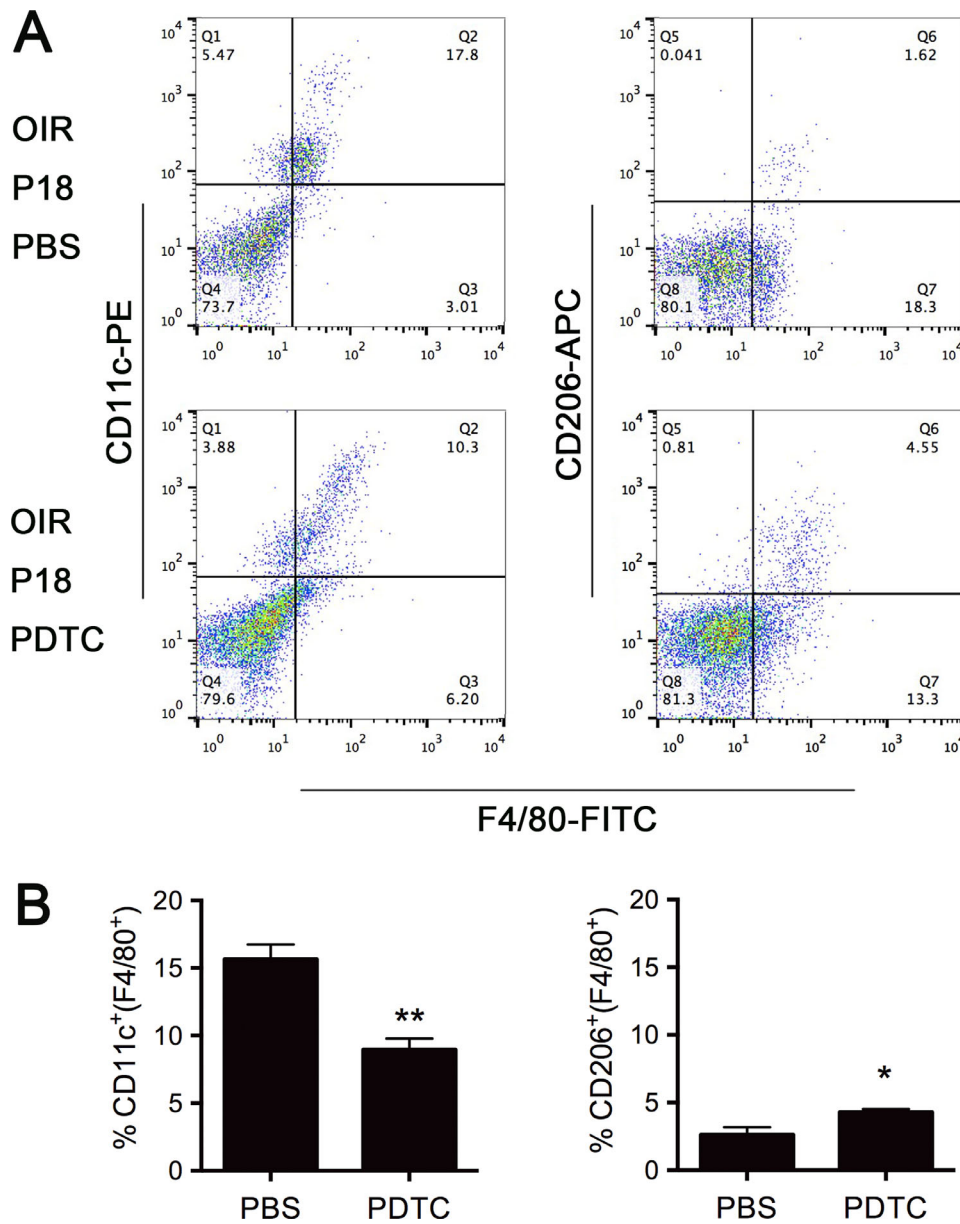
### Intravitreal Injection of PDTC Suppressed RNV in OIR Mice

In OIR mice, eyes injected with PDTC resulted in a significant reduction in RNV compared to eyes injected with PBS ( $P < 0.001$ ) (Fig. 3A). There was no significant difference in the effect at the different concentrations of PDTC tested in this study ( $P > 0.05$ ) (Figs. 3B–3D). These findings demonstrate that NF-κB signaling plays a supportive role in the development of RNV and that inhibiting the activation of NF-κB signaling by PDTC reduces RNV in OIR mice.

### Intravitreal Injection of PDTC Reduced Macrophage Recruitment in Retinas of OIR Mice

In order to investigate the role of PDTC in RNV, we quantified RNV and macrophages in retinas from OIR mice treated with either PDTC or PBS. A reduction in neovascularization was observed in the PDTC-treated group compared to the PBS group. Using F4/80 immunofluorescence staining, PDTC was also noted to significantly inhibit oxygen-induced recruitment of retinal macrophages (Figs. 4A, 4B). In addition, the inhibition of macrophage recruitment and RNV by





**FIGURE 7.** Flow cytometry analysis of M1 polarized and M2 polarized macrophage infiltration in the retinas of PBS- and PDTC-treated OIR mice. OIR mice received intravitreal injections of PBS or PDTC at P12, and retinas were harvested at P18 (n = 3 mice for each group). M1 polarized macrophages (F4/80<sup>+</sup> and CD11c<sup>+</sup>) and M2 polarized macrophage (F4/80<sup>+</sup> and CD206<sup>+</sup>) infiltration was detected by flow cytometry. Data are shown as mean  $\pm$  SEM from three independent experiments and were analyzed by Student's *t*-test. \**P* < 0.05, \*\**P* < 0.01.

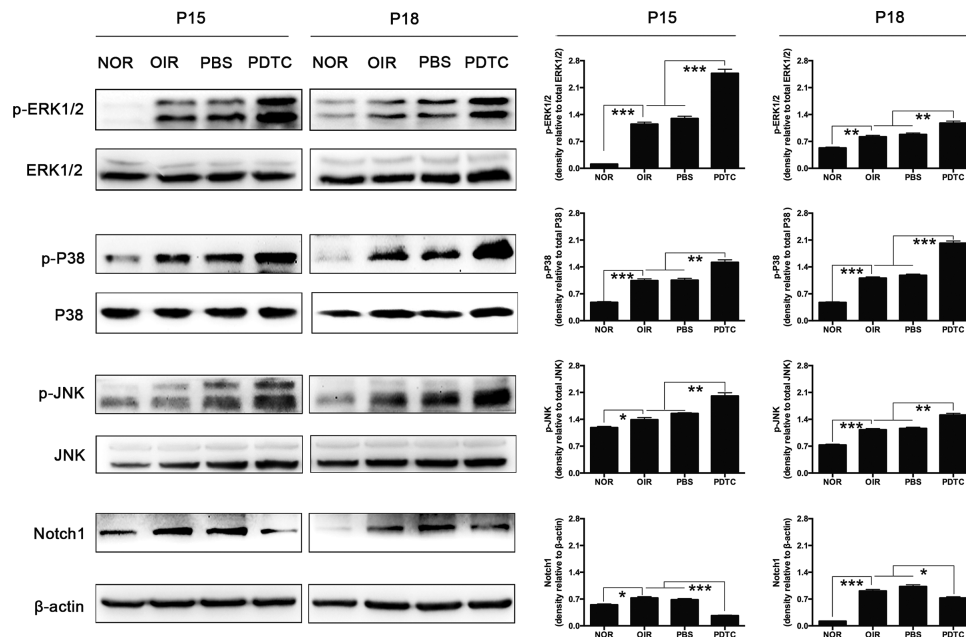
PDTC was also confirmed by F4/80 and lectin immunofluorescence analysis in retinal cross-sections (Fig. 4C).

**M1 Polarized Macrophage and Associated Cytokines Expression Decreased and M2 Polarized Macrophage and Associated Cytokines Expression Increased in Retinas of OIR Mice Treated with PDTC**

The immunofluorescence staining of iNOS and Arg-1 was used to indicate M1 and M2 polarized macrophages, respectively, as previously reported.<sup>7</sup> In retinal cross-sections of OIR mice treated with PDTC, iNOS expression was low but Arg-1 expression was markedly high (Fig. 4D).

qRT-PCR and western blotting were performed to determine macrophage polarization-associated gene expression. Inflammation-associated genes (iNOS, IL-1 $\beta$ , MCP-1, TLR4, and TLR2), which are most likely M1 cytokines, increased in the retinas of P15 and P18 OIR mice. In OIR mice that received intravitreal injection of PDTC, mRNA expression of these genes was significantly reduced. Genes relevant to M2 macrophages (Arg-1, CD206, TGF- $\beta$ , and IL-1RA) were increased in the retinas of P15 and P18 OIR mice and even more in PDTC-treated OIR mice (Fig. 5). The protein expression of genes relevant to M1 macrophages was reduced, and the protein expression of genes relevant to M2 macrophages was increased in retinas of OIR mice treated with PDTC (Fig. 6). Flow cytometric analysis was adopted to detect the type of macrophage polarization. The results showed





**FIGURE 8.** Western blotting analysis of ERK/p-ERK, P38/p-P38, JNK/p-JNK, and Notch1 protein expression in C57BL/6 mice. C57BL/6 mice received intravitreal injections of either PDTC or PBS ( $n = 3$  mice for each group). Western blotting was used to detect expression levels of p-ERK1/2 plus total ERK1/2, p-P38 plus P38, p-JNK plus JNK, and Notch1 at P15 and P18. The ratios of p-ERK1/2 and ERK1/2, p-P38 and P38, p-JNK and JNK, and Notch1 and  $\beta$ -actin are shown respectively. Three independent experiments were performed. Data are shown as mean  $\pm$  SEM and were analyzed by the Student–Newman–Keuls method. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

that compared with the PBS control group, M1 polarized macrophages (F4/80<sup>+</sup> and CD11c<sup>+</sup>) significantly decreased while M2 polarized macrophages (F4/80<sup>+</sup> and CD206<sup>+</sup>) significantly increased in the PDTC-treated group (Fig. 7). These results suggest the potential role of NF- $\kappa$ B signaling in promoting macrophage polarization. After inhibition of NF- $\kappa$ B signaling activation, M1 polarized macrophages were mitigated and M2 polarized macrophages were enhanced in retinas of OIR mice.

### Mitigation of M1 Polarization Signaling but Enhancement of M2 Polarization Signaling in Macrophages by PDTC

Having observed that PDTC increased M2 macrophage polarization and reduced M1 macrophage polarization, we subsequently explored the key pathways of NF- $\kappa$ B signaling regulation in hypoxia-induced macrophage polarization. Previous studies indicated that activation of the mitogen-activated protein kinase (MAPK) pathway was related to activation of M2 macrophages, whereas activation of the Notch1 pathway was related to activation of M1 macrophages.<sup>6,22,23</sup> In our study, we found hypoxia-induced MAPK (extracellular signal-regulated kinase [ERK], P38, Jun N-terminal kinase [JNK]) phosphorylation and Notch1 accumulation. However, following intravitreal injection of PDTC, we found that MAPK phosphorylation increased, while Notch1 was reduced (Fig. 8). In summary, these data suggest that PDTC mediates macrophage polarization by regulating MAPK activation and Notch1 inhibition.

## DISCUSSION

RNV-associated ocular diseases result in visual impairment and are a major cause of blindness. The complete

pathophysiology of RNV remains unknown. Previous studies have demonstrated that NF- $\kappa$ B, a multidirectional and multifunctional nuclear transcription factor, was associated with neovascularization. NF- $\kappa$ B binds to many cytokines, cell adhesion factors, immunomodulators, and other gene promoters or enhancers to promote the transcriptional expression of these genes and participates in cell proliferation, tumorigenesis, the immune response, inflammation, and other physiological and pathological processes.<sup>24</sup> We detected the phosphorylation levels of I $\kappa$ B and p65 in OIR mice and found a significant increase at P15, a critical time point in the hypoxic phase of neovascularization.<sup>25,26</sup> This may imply an initiating role for NF- $\kappa$ B signaling in the progression of RNV. We subsequently studied the morphological relationship between NF- $\kappa$ B signaling and neovascularization. Immunofluorescent double-labeling staining showed that p-I $\kappa$ B, I $\kappa$ B, p-p65, and p65 were enriched in the retina of OIR mice, co-localizing with isolectin, an endothelial cell marker, suggesting that endothelial cells might be one of the main sources of NF- $\kappa$ B signaling proteins.<sup>6</sup> In order to further examine the role of NF- $\kappa$ B signaling in RNV, we employed a strategy of inhibiting activation of this signaling by PDTC and analyzed the effect of intravitreal PDTC on RNV in OIR mice. The area of RNV was decreased in OIR mice with PDTC, emphasizing the proangiogenic role of NF- $\kappa$ B signaling in RNV and suggesting it might be a new target for the treatment of RNV.

It is well established that macrophages are involved in phagocytosis, chemotactic directional movement, and secretion of a large number of cytokines. Prior studies have confirmed that macrophages are key cells in both acute and chronic inflammation and play an important role in the formation of neovascularization.<sup>27–30</sup> In this study, the number of recruited macrophages in retina was significantly reduced after PDTC intervention, which

means that PDTC may inhibit the formation of RNV by inhibiting macrophage recruitment. Depending on the microenvironment, macrophages are polarized into two phenotypes: classical M1 macrophages and alternative M2 macrophages.<sup>31</sup> Pro-inflammatory M1 macrophages, characterized by production of IL-1 $\beta$ , MCP-1, iNOS, TLR2, and TLR4, are associated with tissue destruction.<sup>32</sup> M2 macrophages produce CD206, IL-1RA, Arg-1, and TGF- $\beta$  and play a role in tissue homeostasis, tissue healing, and the resolution of inflammation.<sup>33–35</sup> Research has shown that the shift of macrophages from the M1 phenotype to the M2 phenotype can attenuate experimental inflammatory colitis.<sup>36,37</sup> In the mouse model of herpes simplex virus-induced inflammation, downregulating the M1/M2 ratio of macrophages has been shown to reduce Behçet's disease-like symptoms.<sup>38,39</sup> Other studies have demonstrated that M2 macrophages facilitate normalization of retinal vasculature and reduce pathological neovascularization.<sup>40</sup> Cao et al. postulated that M2 macrophages had protective effects on the aging retina and choroid.<sup>41,42</sup> Some studies have suggested that after laser treatment, both M1 and M2 macrophages gene transcripts are upregulated and M1 macrophages may be involved in the early stage of choroidal neovascularization (CNV), whereas M2 macrophages play an important role in the development and remodeling of CNV.<sup>36</sup> Previous studies have demonstrated that NF- $\kappa$ B plays a role in the polarization of macrophages and is the prototypic signaling cascade that drives classical (M1) activation of macrophages.<sup>43,44</sup> Therefore, we speculated that inhibiting the activation of NF- $\kappa$ B signaling by PDTC could regulate the formation of RNV by regulating macrophages polarization.

In order to verify the relationship between NF- $\kappa$ B and macrophages in the formation of RNV, we chose OIR mice at P15 and P18 and age-matched controls to detect the expression of macrophage polarization-associated genes. Inflammation-associated genes (IL-1 $\beta$ , MCP-1, iNOS, TLR4, and TLR2), most probably M1 cytokines, and genes relevant to M2 macrophages (CD206, Arg-1, IL-1RA, and TGF- $\beta$ ) were increased at P15 and P18 in OIR mice. We found that in OIR mice that received intravitreal PDTC, the expression of genes relevant to M1 macrophages was reduced, and the expression of genes relevant to M2 macrophages was increased. Flow cytometry results also showed that, compared with the PBS control group, M1 polarized macrophages significantly decreased but M2 polarized macrophages significantly increased in the PDTC group. These results suggest the potential role of NF- $\kappa$ B signaling in promoting macrophage polarization. After inhibition of NF- $\kappa$ B signaling activation, M1 polarized macrophages were mitigated and M2 polarized macrophages were enhanced in retinas of OIR mice. The MAPK family includes three parallel signal-transduction modules: ERK1/2, p38, and JNK. Research has shown that MAPK signaling is involved in the promotion of M2 macrophage polarization.<sup>22</sup> In addition, Singla et al.<sup>23</sup> demonstrated that Notch1 signaling played a key role in the differentiation of M1 macrophages, thereby enhancing the inflammatory response. Inhibiting the downstream signaling of Notch1 increases M2 polarized macrophage and enhances the anti-inflammatory regulation. In order to further explore the role of NF- $\kappa$ B signaling in macrophage polarization, we detected the expression level and phosphorylation state of these kinases in OIR mice. Phosphorylation levels of MAPK signaling increased, whereas expression of M1 polarization-related

Notch1 decreased in OIR mice treated with intravitreal PDTC.

In summary, using a variety of experimental methods including gene, protein, and morphological analysis, this study demonstrates that inhibiting NF- $\kappa$ B signaling activation by PDTC reduces RNV by decreasing macrophage infiltration and promoting a polarization shift in macrophages. Furthermore, NF- $\kappa$ B signaling mediates macrophage polarization by regulating MAPK phosphorylation, including an increase in p38MAPK, JNK, ERK, and Notch1. These findings contribute to our understanding of the pathological mechanism of RNV and support NF- $\kappa$ B as a potential therapeutic target for the prevention of RNV-associated ocular diseases.

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