Molecular Vision 2014; 20:616-628 <http://www.molvis.org/molvis/v20/616>
Received 3 September 2013 | Accepted 30 April 2014 | Published 2 May 2014

Retinal neuronal MCP-1 induced by AGEs stimulates TNF-α expression in rat microglia via p38, ERK, and NF-κB pathways

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Purpose: Retinal microglia can be activated by retinal neuronal monocyte chemoattractant protein-1 (MCP-1) and play a pivotal role in early retinal degeneration. The current study investigates the pathways via which retinal neuronal MCP-1 stimulates tumor necrosis factor-α (TNF-α) expression in rat microglia.
Methods: Primary rat retinal neurons and microglia were separated and cocultured in a Transwell apparatus. The levels of TNF-α mRNA and soluble TNF-α produced by the microglia in response to advanced glycation end product (AGE)-induced retinal neuronal MCP-1 were measured with real-time PCR and enzyme-linked immunosorbent assay (ELISA). The ability of neuronal MCP-1 to stimulate microglia activation was examined by preexposing the retinal neurons to AGEs and an MCP-1 antibody or by pretreating microglia with AGEs and siRNA specific for CC-chemokine receptor 2 (CCR2) knockdowns. Additionally, we investigated the effects of microglial activation on neuronal MCP-1-induced nuclear factor-κB (NF-κB) activation and phosphorylation of mitogen-activated protein kinases (MAPKs).
Results: Stimulation with AGEs significantly increased the expression of TNF-α mRNA and soluble TNF-α in the microglial cells. Retinal neurons that had been pretreated with AGEs and an MCP-1 antibody or microglia that were CCR2 knockdowns displayed greatly reduced TNF-α secretion. Using signaling pathway-specific inhibitors, we showed that blocking the p38, extracellular signal-regulated kinase (ERK), and NF-κB signaling pathways significantly reduced the expression of TNF-α by retinal neuronal MCP-1-stimulated microglia.
Conclusions: This study indicates that TNF-α was released from the activated microglia induced by retinal neuronal MCP-1 via the p38, ERK, and NF-κB pathways, but not c-Jun N-terminal kinase (JNK), which may be an important finding in diabetic retinopathy pathogenesis.

Diabetes mellitus (DM) has been a leading public health problem in China for the past decade and imposes a heavy economic burden on Chinese patients [1,2]. With the increasing prevalence of DM in the community, diabetic retinopathy (DR) has become a serious public health issue [3,4]. Recent studies have shown that the increased influx of macrophages, leucocytes, and proinflammatory cytokines, as well as microglial activation in the retina or vitreous, is involved in DR-related damage [5-8]. Furthermore, all evidence demonstrates that local inflammation may represent the central pathway leading to DR.

Microglia are resident immunocompetent and phagocytic cells and comprise 10%–20% of all cells in the central nervous system (CNS). It is widely recognized that microglial activation represents a major histopathological change in DR [6]. Activated microglia not only act as scavengers but also serve as rapid sensors of neuronal damage and are responsible for tissue repair and neural regeneration [9-11]. Immunomodulatory molecules released by activated microglia regulate the influx of inflammatory cells to the damaged area, causing vascular breakdown and releasing cytotoxins that kill retinal neurons. These released molecules include inflammatory proteins such as cytokines and chemokines, reactive oxygen and nitrogen species, and complement factors. An increase in the release of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), from microglia has the potential to induce apoptosis, fibroblast proliferation, nuclear factor-kappaB (NF-κB) activation, and cell adhesion molecule activation [12-14].

Nevertheless, factors that mediate microglial activation and the origin of the chemostimulants in the retina remain unknown. In recent years, in vivo and in vitro evidence has increasingly shown that microglial activity is prominent after neuronal damage, suggesting that neurons play an important role in activating microglia [15-18]. In response to injury, neurons release chemokines that act on microglial cell receptors to induce migration and activation [19,20].

Studies have shown that the chemokine monocyte chemo-attractant protein-1 (MCP-1) that binds CC-chemokine receptor 2 (CCR2) is upregulated in models of endotoxin-induced uveitis, retinal neovascularization, and retinal degeneration in mice [21-25]. We previously showed for the first time that a marked increase in the expression of MCP-1

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was distributed mainly around retinal vessel walls, as well as in the cell bodies and synapses of the retinal ganglion cells (RGCs) in a rodent DR model after streptozotocin (STZ) injection, and this expression was consistently upregulated at 3 and 5 months [26]. We have also shown previously that stimulation with advanced glycation end products (AGEs) significantly increases the expression of MCP-1 in retinal neurons in vitro, which in turn increases microglial migration and activation [26]. In DR, retinal neuronal MCP-1 has been shown to activate or attract microglia; however, the signaling pathways contributing to the pathologic changes have not been elucidated, to the best of our knowledge.

Thus, in this study, retinal neurons and microglia were separated and cultured in a Transwell apparatus in which they shared the same medium but could not form direct cell-cell interactions. Retinal neurons were stimulated with AGEs to mimic the diabetic microenvironment. We also examined the secretion of TNF-α from microglia activated by retinal neuronal MCP-1. We subsequently investigated the signaling pathways by which retinal neuronal MCP-1 activates or attracts microglia, by evaluating the inhibition of NF-κB translocation and deactivation of mitogen-activated protein kinase (MAPK) signaling.

METHODS

Primary retinal microglia and neuronal culture: Microglial cells and retinal neural cells were cultured from 3-day-old Sprague-Dawley (SD) rats. All protocols involving animals were conducted in accordance with the Animal Experimental Ethical Review Committee of Capital Medical University and the Association for Research in Vision and Ophthalmology (ARVO) on the Use of Animals in Ophthalmology and Vision Research. The method of cell culture has been described in detail previously [26].

Transwell culture and treatment: The Transwell culture and treatment method has been described in detail previously [26]. Briefly, freshly isolated microglia were resuspended and plated at 2.5×10^5 cells per well on polycarbonate Transwell inserts (4.67 cm^2) with a 0.4-μm pore size (Corning, NY). After 4 h, these inserts were placed on top of six-well plates containing the 7-day-old primary cultures of retinal neurons plated at a density of 5×10^6 per well. Next, the experiments were processed in four groups with different treatments. In Group 1, the cells were cultured in the culture medium alone for 24 h and used as the control. In Group 2, the cells were treated with AGEs (750 μg/ml; Catalog 2221–10; BioVision, Milpitas, CA) in the culture medium for 24 h. In Group 3, to determine the role of MCP-1 produced by the retinal neurons, primary cultured retinal neurons were preincubated with AGEs (750 μg/ml) and an MCP-1 blocking peptide (15 μg/ml) for 8 h, followed by coculture with the previously described isolated microglia in the Transwell apparatus for another 24 h. In Group 4, the primary cultured retinal neurons were preexposed to AGEs for 24 h. The previously described isolated microglia and CCR2 siRNA were preincubated for 24 h. Then, they were cocultured in the Transwell apparatus for another 24 h.

To determine the pathways by which retinal neuronal MCP-1 stimulates TNF-α expression in rat microglia, the primary cultured retinal neurons were preexposed to AGEs for 24 h. The previously described isolated microglia and inhibitors, namely, SB203580 (1, 10, 20 μm), U0126 (1, 10, 20 μm), SP610025 (1, 10, 20 μm), or PDTC (1, 10, 20 nm), were preincubated for 1 h. Then, they were cocultured in the Transwell apparatus for another 24 h.

To exclude the effects of MCP-1 and/or TNF-α from the microglia exposed to AGEs in the Transwell apparatus, the experiments were processed in four groups with different treatments. In Group 1, the primary cultured retinal neurons were cultured in the culture medium alone for 48 h and used as the control. In Group 2, the primary cultured retinal neurons were treated with AGEs (750 μg/ml) in the culture medium for 24 h. In Group 3, the primary cultured retinal neurons were pretreated with AGE (750 μg/ml) in the culture medium for 24 h, then washed with PBS (1X; 137 mM NaCl, 2.7 mM KCl, 4.3 mM NaH_2PO_4, 1.47 mM KH_2PO_4, pH 7.4) for 3 times and removed AGE, followed by co-culture in the culture medium alone for 24 h. In Group 4, the primary cultured retinal neurons were pretreated with AGEs (750 μg/ml) in the culture medium for 48 h.

Inhibition of MCP-1/CCR2 system: The method for preparing and transfecting siRNA has been described in detail previously [26]. Briefly, CCR2 siRNAs were purchased from Ambion (Austin, TX) and complexed with Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) in six-well plates according to the manufacturer's instructions. Two microliters Lipofectamine 2000 was diluted in 50 μl Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (DMEM/F12; Sigma, St Louis, MO) and combined with 0.01 to 0.20 μg siRNA after a 15-min incubation at room temperature. The transfection was continued for 24 h at room temperature. The knockdown of CCR2 in microglia was determined with western blot analysis.

Cell immunofluorescence: For immunocytochemistry, the primary separated microglia were seeded onto the 12-mm glass coverslips (BD PharMingen, San Diego, CA), which were put into the upper chamber of the retinal neuron-microglia Transwell culture system beforehand. After

stimulation, the cells were rinsed twice with PBS and fixed for 15 min in 4% paraformaldehyde (PFA) at room temperature. Microglial cells were directly incubated with goat anti-CD11b (1:100, Serotec, Oxford, UK) and rabbit anti-TNF-α (1:50, eBioscience, San Diego, CA) antibodies overnight at 4 °C without permeabilization. Subsequently, the cells were incubated with fluorescein isothiocyanate (FITC)- or PE-conjugated secondary antibodies (1:50, Chemicon, Temecula, CA) in PBS for 1 h at room temperature. Finally, the cells were counter-stained with 4, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA), mounted in glycerol and examined by confocal microscopy (Olympus Fluoview1000, Tokyo, Japan). The specificity of the antibodies was confirmed by replacing each primary antibody with a nonspecific protein of the same isotype. Quantitative analysis of the number of positive microglial cells was performed by counting eight microscopic fields in the control cultures and the treated cultures.

Western blot analysis: The method used for western blot analysis has been described in detail previously [26,27]. Total proteins were extracted from the microglial cells with Protein Extraction Reagent (Pierce, Rockford, IL) according to the manufacturer's instructions. Equal amounts of each protein extract (50 µg) were incubated at 4 °C overnight with primary antibodies, namely anti-phospho-extracellular signal–regulated kinase (ERK1/2) (1:2,000), anti-ERK1/2 (1:1,000), anti-phospho-p38 (1:1,000), anti-p38 (1:1,000), anti-phospho-c-Jun N-terminal kinase (JNK; 1:1,000), anti-JNK (1:2,000; all were from Cell Signaling Technology, Beverly, MA), anti-p65 (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA), and anti-p50 (1:1,000, Santa Cruz Biotechnology). After washing, the membranes were incubated with a peroxidase-conjugated secondary antibody and visualized with a chemiluminescence detection system (Immobilon P, Millipore, Denmark). Images were obtained using a densitometer (Bio-Rad, Hercules, CA) and analyzed quantitatively with Multi-Analyst Macintosh Software. The band densities were normalized relative to the level of β-actin in each sample, which was detected as an internal control.

Real-time PCR: Total RNA was isolated from microglial cells using Trizol Reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed to cDNA using a PrimeScript RT reagent kit (TaKaRa, Dalian, China). The mRNA levels of the target genes were quantified with SYBR Green-based real-time PCR analysis (Bio-Rad). PCR amplification was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad) and the following cycling conditions: Thermocycling was initiated at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 58–61 °C for 30 s depending on the specific set of primers, and extension at 72 °C for 30 s. Data were analyzed using the comparative threshold cycle (Ct) method, and the results were expressed as the fold difference normalized to GAPDH. The primer sequences used were as follows: TNF-α (sense 5′-CCC TCA CAC TCA GAT CAT CTT CT-3′ and antisense 5′-GCT ACG ACG TGG GCT ACA G-3′); GAPDH (sense 5′-AGG TCG GTG TGA ACG GAT TTG-3′ and antisense 5′-TGT AGA CCA TGT AGT TGA GGT CA-3′).

ELISA: The concentrations of TNF-α released in the Transwell culture were tested with enzyme-linked immunosorbent assay (ELISA) kits (Pierce). Briefly, the samples described were incubated in 96-well plates coated with TNF-α antiserum for 2 h. The samples were treated with enzyme working reagent for 30 min and with TMB One-Step substrate reagent for 30 min in the dark, and the reaction plates were read within 30 min in an ELISA plate reader (Molecular Devices, Eugene, OR) at 450 nm, with 620 nm as the reference. The detection limit was 1 pg/ml.

Statistical analysis: All experiments were performed at least three times. Quantitative data are presented as the mean±standard error of the mean (SEM) and were analyzed with one-way ANOVA (ANOVA) or the Student *t* test. A p value of <0.05 was considered statistically significant.

RESULTS

CCR2 knockdown in primary retinal microglia: To determine the effects of CCR2 siRNA in the primary retinal microglia on CCR2 expression, western blot analysis was used to measure the amount of CCR2 protein. We downregulated CCR2, with a maximum knockdown of approximately 75% at 24 h (Figure 1).

Retinal neurons activated microglia following AGE exposure: The expression of TNF-α mRNA in the primary retinal microglia was analyzed with real-time PCR (Figure 2A,C), and the concentration of soluble TNF-α in the culture medium was measured with an ELISA kit (Figure 2B,D). A cultured retinal microglia model was developed to characterize the microglial response to recombinant rat MCP-1 (Peprotech, Rocky Hill, NJ) treatment. As shown in Figure 2A, TNF-α mRNA were set to 100%, when the cells were cultured with culture medium alone for 24 h and used as a control. With the use of this model, incubation with exogenous MCP-1 significantly stimulated TNF-α production from microglia (Figure 2A,B). In addition, CCR2 knockdown led to downregulation of TNF-α release from microglia induced by exogenous MCP-1 in this model (Figure 2B).

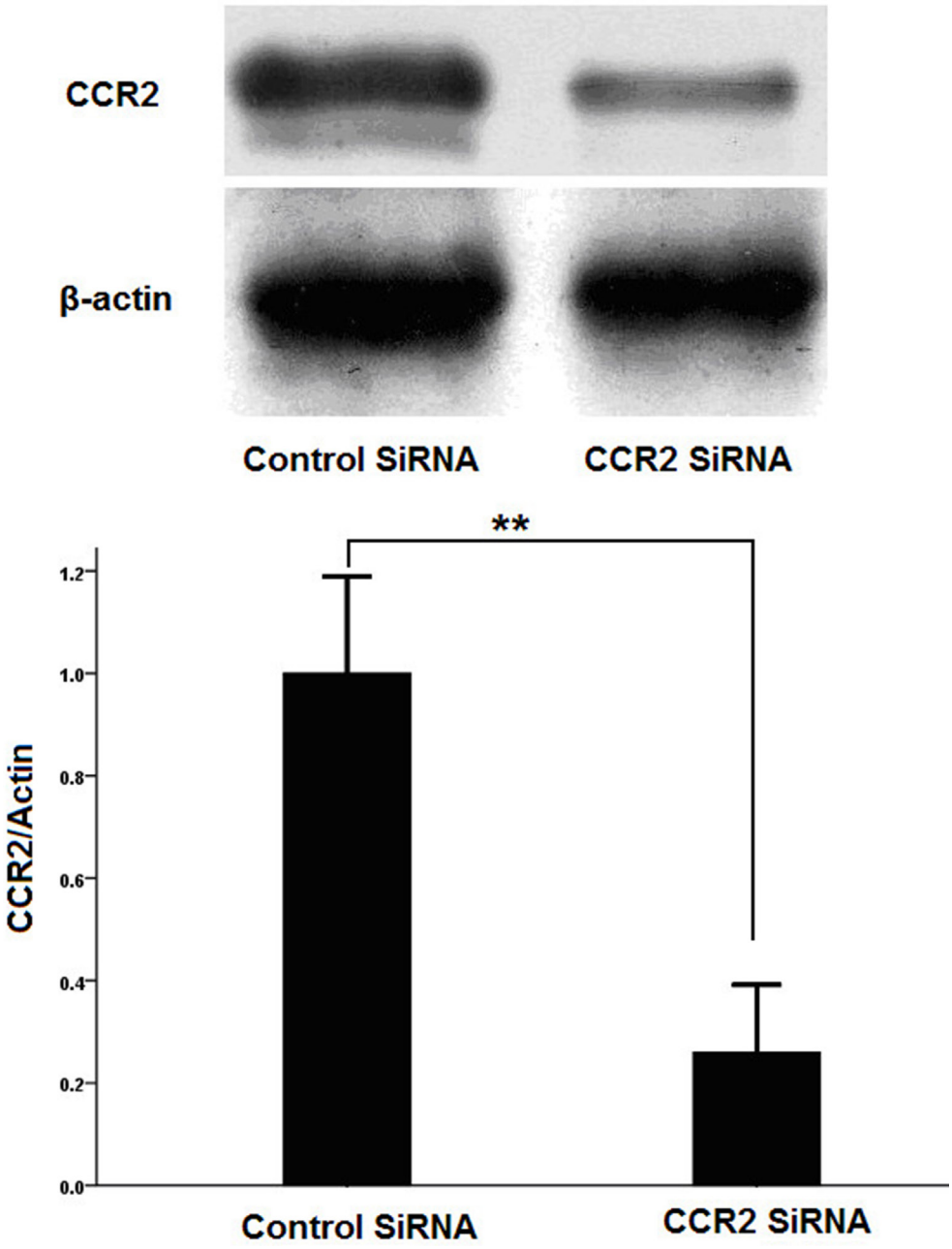

Figure 1. Effects of CC-chemokine receptor 2 (CCR2) siRNA on CCR2 expression in transfected cells. Primary cultured retinal microglia were treated with specific siRNA to knock down the expression of CCR2. Representative CCR2 knockdown was identified with western blot analysis with maximum downregulation of approximately 75%. Results are statistically significant (**p<0.01 Student *t* test).

A Transwell culture system was designed in which retinal neurons and microglial cells shared the same medium but had no direct cell–cell interaction. In this system, interactions between neurons and microglia were mediated only by the release of soluble factors. As shown in Figure 2C, TNF-α mRNA were set to 100%, when the cells were cultured with culture medium alone in the retinal neuron–microglia Transwell culture system for 24 h and used as a control. When cultured with AGEs, the retinal neurons had a markedly increased effect on retinal microglial activation in the Transwell culture system as evidenced by 585.68% expression of TNF-α mRNA relative to the control cells (Figure 2C). TNF-α mRNA expression was reduced to 152.31% relative to the control cells when the MCP-1 blocking peptide was used, while CCR2 siRNA-transfected cells showed 183.14% expression of TNF-α relative to the control cells (Figure 2C). In addition, the incubation of control rat immunoglobulin G (IgG; Abcam, Cambridge, UK) or control siRNA did not decrease TNF-α production.

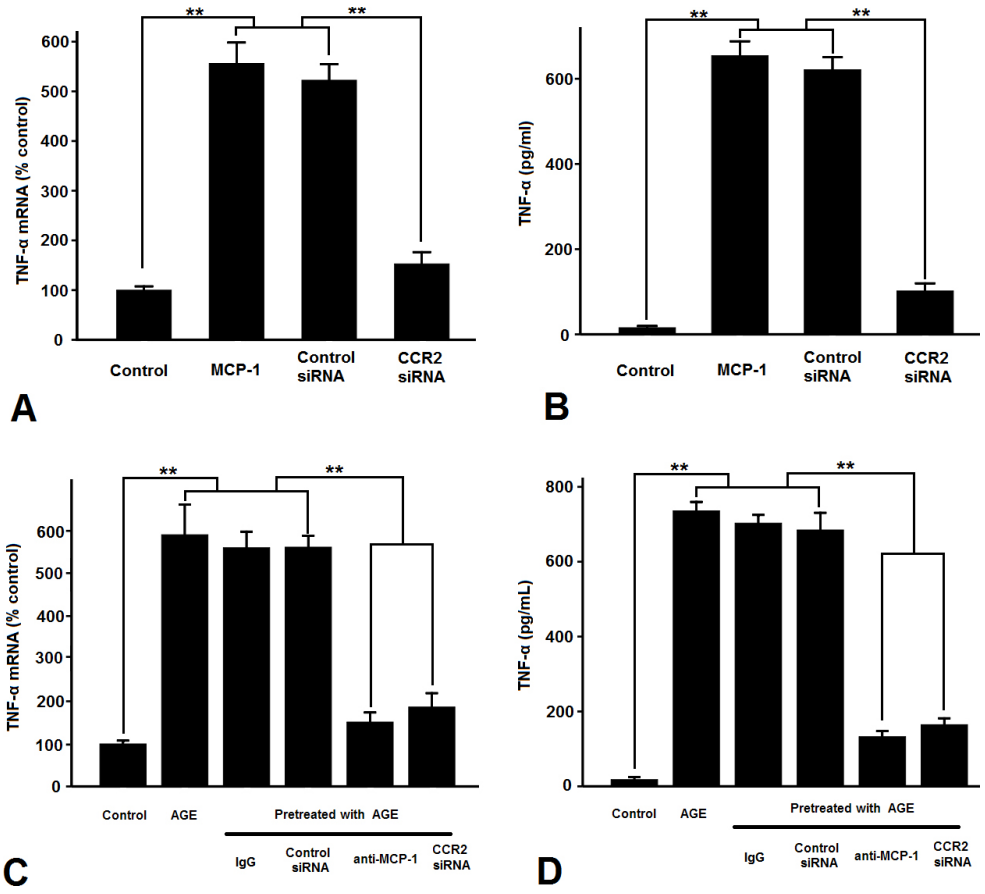

Figure 2. Effects of retinal neuronal monocyte chemoattractant protein-1 (MCP-1) on activated microglial cells. **A**: Tumor necrosis factor-α (TNF-α) mRNA from microglia was significantly stimulated by exogenous MCP-1 and CC-chemokine receptor 2 (CCR2) knockdown, which led to downregulation of TNF-α mRNA from microglia. **B**: The concentrations of soluble TNF-α were significantly stimulated by exogenous MCP-1. In addition, CCR2 knockdown led to downregulation of TNF-α release from microglia induced by exogenous MCP-1. **C**: The expression of TNF-α mRNA was significantly increased by neuronal MCP-1 in the retinal neuron–microglia Transwell culture system. In addition, TNF-α mRNA expression was reduced when the MCP-1 blocking peptide and CCR2 siRNA were used. However, the incubation of control immunoglobulin G (IgG) or control siRNA did not reduce the TNF-α production. **D**: Enzyme-linked immunosorbent assay (ELISA) was used to measure the soluble TNF-α concentration in the retinal neuron–microglia Transwell culture system. When cultured with advanced glycation end products (AGEs), the retinal neurons had a markedly increased effect on retinal microglial activation in the Transwell culture system. In addition, the concentration of soluble TNF-α was decreased when the MCP-1 blocking peptide and CCR2 siRNA were used. However, the incubation of control IgG or control siRNA did not reduce TNF-α production. Data shown are the mean±SD (**p<0.01).

Soluble TNF-α was detected in the unstimulated medium (9.12±2.58 pg/ml); however, exposure to AGEs led to upregulation of TNF-α release (729.63±33.76 pg/ml), and exposure to AGEs accompanied by MCP-1 blocking peptide (136.89±22.45 pg/ml) and CCR2 knockdown (177.56±28.13 pg/ml) led to downregulation of TNF-α release in the retinal neuron–microglia Transwell culture system (Figure 2D). However, the incubation of control rat IgG or control siRNA did not decrease TNF-α production.

Retinal neuronal MCP-1 induced by AGESs stimulates TNF-α expression in rat microglia by phosphorylation of p38: First, a p38 inhibitor, SB203580, significantly decreased the expression of TNF-α mRNA (Figure 3A) and inhibited soluble TNF-α release (Figure 3B) in a dose-dependent manner in the retinal neuron–microglia Transwell culture system. The levels of phosphorylated p38 MAPK from the microglial cells increased when stimulated by AGEs in the Transwell culture system (Figure 3C). MCP-1 is the most potent microglial chemoattractant and acts by binding to CCR2 receptors on microglial cell surfaces [28]. Anti-MCP-1 in the retinal neuron cells led to downregulation in the levels of phospho-p38, lending support to the fact that retinal neuronal MCP-1 induced by AGEs increases phospho-p38 levels in the microglial cells. The p38 inhibitor downregulated the number of CD11b and TNF-α double-stained cells (Figure 3D).

Retinal neuronal MCP-1 induced by AGEs stimulates TNF-α expression in rat microglia by phosphorylation of ERK: An ERK inhibitor, U0126, significantly decreased the expression of TNF-α mRNA (Figure 4A) and inhibited soluble TNF-α release (Figure 4B) in a dose-dependent manner. Phospho-ERK levels from the microglial cells induced by AGEs (750 μg/ml) and mediated by MCP-1 increased in the retinal neuron–microglia Transwell culture system (Figure 4C). However, anti-MCP-1 led to downregulation of the levels

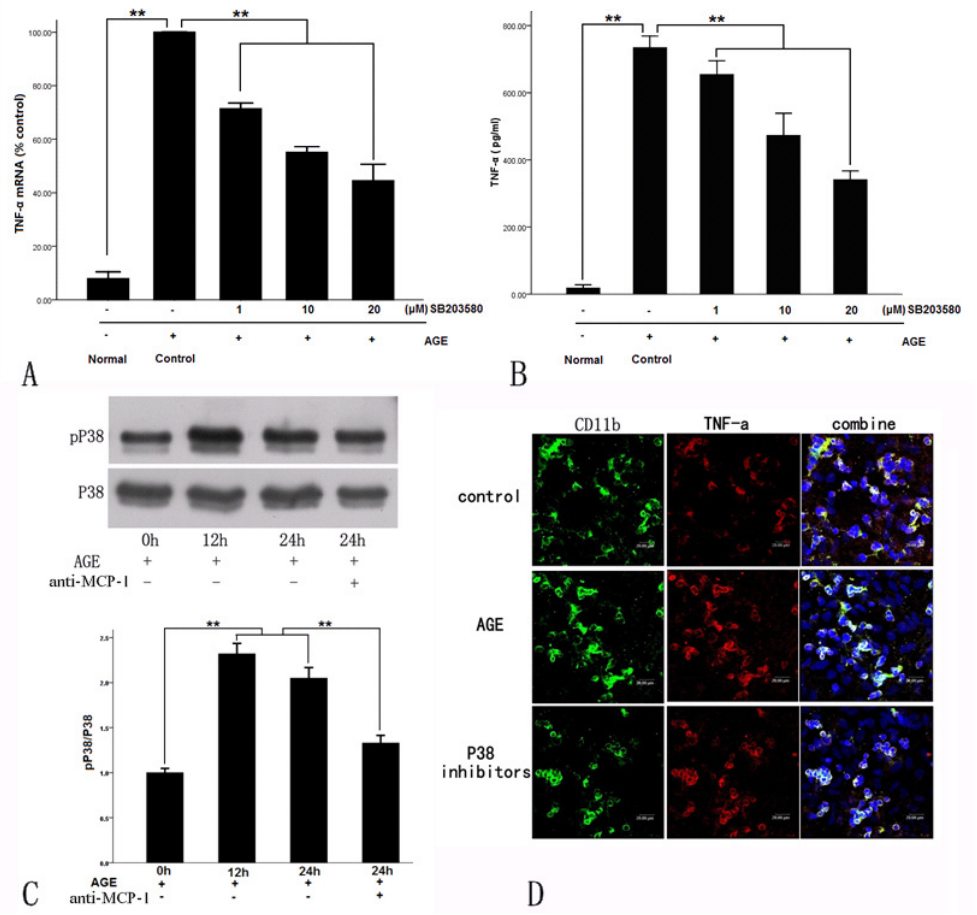

Figure 3. Tumor necrosis factor-α (TNF- α) was released from the activated microglia induced by retinal neuronal monocyte chemoattractant protein-1 (MCP-1) via the phosphorylation of p38. **A**: Real-time PCR was used to measure TNF-α mRNA expression. Dose-dependent inhibition of the expression of TNF-α mRNA was induced by retinal neuronal MCP-1 in the retinal neuron–microglia Transwell culture system by p38 inhibitors. **B**: Enzyme-linked immunosorbent assay (ELISA) was used to measure the soluble TNF-α concentration. Dose-dependent inhibition of the expression of soluble TNF-α was induced by p38 inhibitors. **C**: Phospho-p38 levels were detected with western blotting. Phosphorylated p38 MAPK levels from the microglial cells increased due to retinal neuronal MCP-1; however, anti-MCP-1 preincubated with primary cultured retinal neurons led to downregulation of the levels of phosphorylated p38 (**p<0.01). **D**: Purified microglia were stained with fluorescein isothiocyanate (FITC)-CD11b (green) and the expression of TNF-α labeled with PE (red). The number of CD11b and TNF-α double-stained cells (activated microglia) decreased markedly after advanced glycation end product (AGE) treatment with p38 inhibitors (control: 12.21±5.32 cells/microscopic visual field; AGE: 28.23±3.62 cells/microscopic visual field; p38 inhibitors: 18.36±6.17 cells/microscopic visual field; p=0.013).

of phospho-ERK, lending additional support to the fact that retinal neuronal MCP-1 induced by AGEs increases phospho-ERK levels in the microglial cells. The ERK inhibitor downregulated the number of CD11b and TNF-α double-stained cells (Figure 4D).

TNF-α expression induced by retinal neuronal MCP-1 in rat microglia is not mediated by phosphorylation of JNK: As shown in Figure 5, TNF-α mRNA when the cells were treated with AGE (750 μg/ml) in the retinal neuron–microglia Transwell culture system for 24 h was set to 100% and was used as a control. When cultured with the JNK inhibitor SP610025, the expression of TNF-α mRNA did not significantly decrease relative to the control cells (p>0.05), and it was not a dose-dependent relationship (Figure 5A). In addition, the JNK inhibitor did not significantly inhibit soluble TNF-α release (p>0.05; Figure 5B). Phospho-JNK levels from the microglial cells remained unchanged over the entire experimental period in the retinal neuron–microglia Transwell culture system, and anti-MCP-1 did not lead to downregulation of the levels of phospho-JNK (Figure 5C).

Retinal neuronal MCP-1 induced by AGEs stimulates TNF-α expression in rat microglia by NF-κB: Treatment with PDTC (an NF-κB inhibitor) significantly decreased the expression of TNF-α mRNA (Figure 6A) and inhibited soluble TNF-α release (Figure 6B) in a dose-dependent manner. The levels of the p50 and p65 subunits of NF-κB from the microglial nuclear fractions induced by AGEs increased in the retinal neuron–microglia Transwell culture system (Figure 6C). However, anti-MCP-1 led to attenuation of the nuclear translocation of NF-κB induced by retinal neuronal MCP-1 (Figure 6C). The NF-κB inhibitor downregulated the number of CD11b and TNF-α double-stained cells (Figure 6D).

Retinal neuronal MCP-1 induced by AGEs stimulates TNF-α expression in rat microglia via p38, ERK1/2, and NF-κB

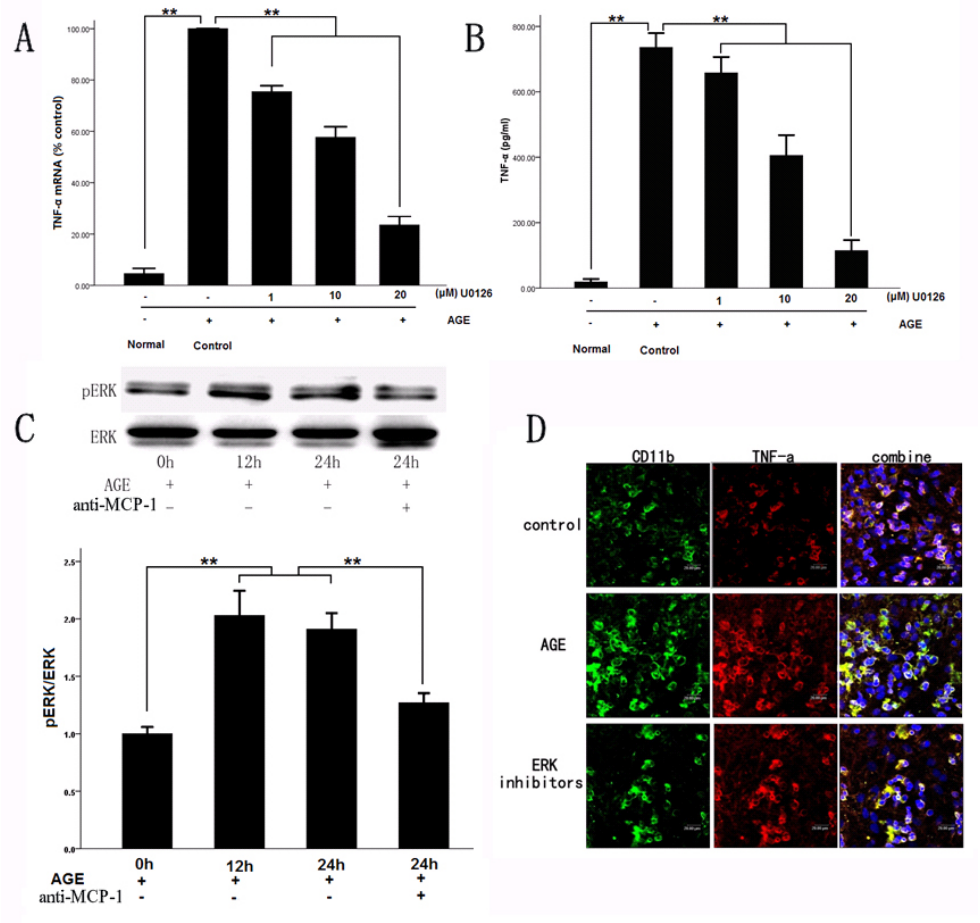

Figure 4. Tumor necrosis factor-α (TNF-α) was released from the activated microglia induced by retinal neuronal monocyte chemoattractant protein-1 (MCP-1) via the phosphorylation of extracellular signal-regulated kinase (ERK). **A**: Real-time PCR was used to measure TNF-α mRNA expression. Dose-dependent inhibition of the expression of TNF-α mRNA was induced by retinal neuronal MCP-1 in the retinal neuron–microglia Transwell culture system by ERK inhibitors. **B**: Enzyme-linked immunosorbent assay (ELISA) was used to measure the soluble TNF-α concentration. Dose-dependent inhibition of the expression of soluble TNF-α was induced by ERK inhibitors. **C**: Phospho-ERK levels were detected with western blotting. Phospho-ERK levels from the microglial cells increased due to retinal neuronal MCP-1; however, anti-MCP-1 led to downregulation of the levels of phospho-ERK (**p<0.01). **D**: Purified microglia were stained with fluorescein isothiocyanate (FITC)-CD11b (green), and the expression of TNF-α was labeled with PE (red). CD11b and TNF-α double-stained cells were detectable in the control medium. The number of double-stained cells (activated microglia) increased markedly after the advanced glycation end product (AGE) treatment. In contrast, the number of CD11b and TNF-α double-stained cells (activated microglia) decreased markedly after AGE treatment with ERK inhibitors (control: 14.58±4.54 cells/microscopic visual field; AGE: 38.84±6.11 cells/microscopic visual field; ERK inhibitors: 26.25±3.66 cells/microscopic visual field; p=0.021).

pathways, but not JNK: As shown in Figure 7A, retinal neuronal MCP-1 mRNA was set to 100%, when the primary cultured retinal neurons were cultured with culture medium alone for 48 h and used as a control. When cultured with AGEs for 24 h or 48 h, the retinal neurons had a markedly increased about 5.8 times or 7.6 times expression of MCP-1 mRNA relative to the control cells (Figure 7A), and had an increased effect on releasing soluble MCP-1 (Figure 7B). The expression of MCP-1 mRNA and soluble MCP-1 in the pretreated AGE 24 h and removed AGE groups were compared to the control cells.

To exclude the effects of MCP-1 and/or TNF-α from the microglia exposed to AGEs in the Transwell apparatus, the primary cultured retinal neurons were pretreated with AGEs (750 μg/ml) in the culture medium for 24 h, then washed with PBS three times and removed AGEs, followed by coculture with the previously described isolated microglia in the Transwell apparatus for another 24 h (Figure 7C,D,E). As shown in Figure 7C, TNF-α mRNA was set to 100%, when the primary cultured retinal neurons and the primary cultured retinal microglia were cultured with culture medium alone in the retinal neuron–microglia Transwell culture system for 24 h and used as a control. When the retinal neurons were pretreated with AGEs (750 μg/ml) and removed AGEs, the retinal neurons had a markedly increased effect on retinal microglial activation in the Transwell culture system as evidenced by 533.16% expression of TNF-α mRNA relative to the control cells (Figure 7C). TNF-α mRNA expression was reduced to 153.1% relative to the control cells when CCR2 siRNA-transfected retinal microglia (Figure 7C). Soluble TNF-α was detected in the unstimulated medium (18.12±9.56 pg/ml); however, exposure to AGEs led to upregulation of

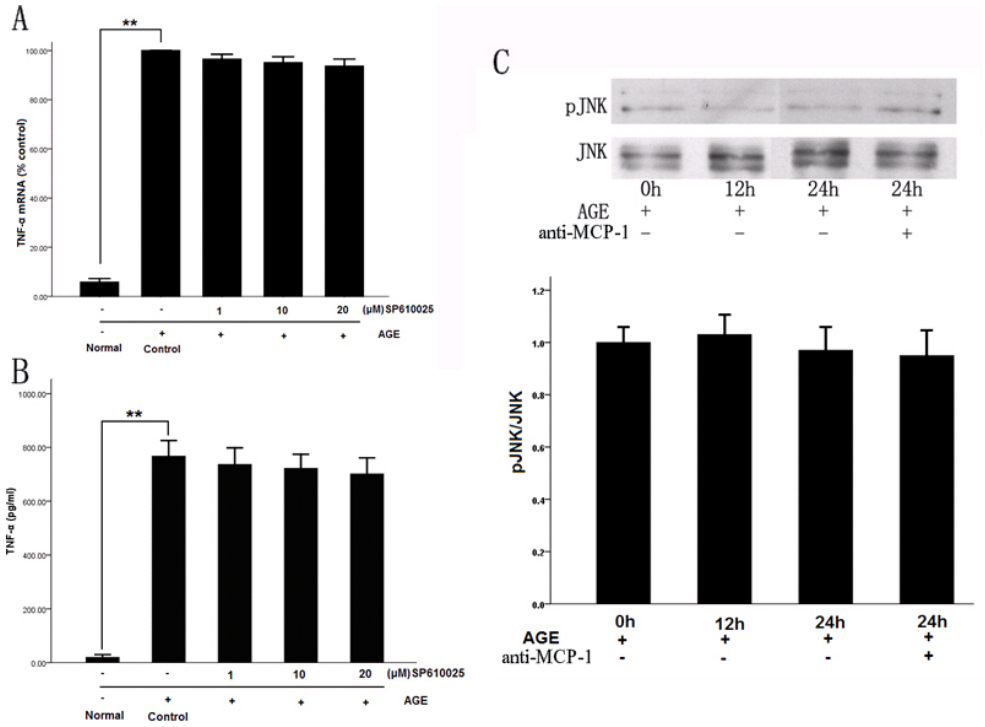

Figure 5. Phospho-c-Jun N-terminal kinase (JNK) does not cause tumor necrosis factor-α (TNF-α) release in activated retinal microglial cells induced by retinal neuronal monocyte chemoattractant protein-1 (MCP-1). **A**: Real-time PCR was used to measure TNF-α mRNA expression. JNK inhibitors did not significantly decrease the expression of TNF-α mRNA induced by retinal neuronal MCP-1 in the retinal neuron–microglia Transwell culture system. **B**: Enzyme-linked immunosorbent assay (ELISA) was used to measure the soluble TNF-α concentration. JNK inhibitors did not significantly decrease the concentration of soluble TNF-α. **C**: Phospho-JNK levels were detected with western blotting. Phospho-JNK levels from the microglial cells did not increase due to retinal neuronal MCP-1, and anti-MCP-1 did not downregulate the levels of phospho-JNK (**p<0.01).

TNF-α release (646.53±61.19 pg/ml), and exposure to AGEs accompanied by CCR2 knockdown (145.41±10.97 pg/ml) led to downregulation of TNF-α release in the retinal neuron–microglia Transwell culture system (Figure 7D).

As shown in Figure 7E, the levels of phospho-p38, phospho-ERK, and the p50 and p65 subunits of NF-κB from the microglial cells increased in the retinal neuron–microglia Transwell culture system. However, the levels of phospho-p38, phospho-ERK, and the p50 and p65 subunits of NF-κB from the microglial cells decreased accompanied by CCR2 knockdown to retinal microglia in the Transwell culture system. Phospho-JNK levels from the microglial cells remained unchanged over the entire experimental period in the retinal neuron–microglia Transwell culture system, and CCR2 knockdown did not lead to downregulation of the levels of phospho-JNK.

DISCUSSION

Currently, it is known that retinal inflammation is consistent with several molecular and physiologic abnormalities of DR and represents a relatively early event, even before vascular dysfunction [29]. In addition, many of the anti-inflammatory therapies (aspirin or other salicylates) have been found to significantly inhibit development of DR in animal models [6,30,31]. However, the Early Treatment Diabetic Retinopathy Research study, a prospective clinical trial in humans, showed that aspirin failed to inhibit retinopathy [32]. The contradictory conclusions might indicate that the mechanisms of inflammation in the retina in diabetes are complex and need further research.

Although microglia are the primary source of chemokines and microglial activation has been recognized as a key mechanism leading to inflammation, the mechanisms by which diabetes activates microglia to release inflammatory proteins such as cytokines and chemokines remain elusive. There is evidence that inflammatory changes might lead to degeneration of retinal neurons and secrete chemokine such as MCP-1 [33,34]. In addition, several studies have demonstrated that microglia express CCR2, a receptor of MCP-1, suggesting that the MCP-1/CCR2 pathway is potentially involved in the recruitment of microglial precursors from the periphery to lesions and microglia activation [35].

In the present study, to determine that retinal neuronal MCP-1 induced by AGEs stimulates TNF-α expression in rat microglia, a Transwell culture system was designed in which interactions between neurons and microglia were mediated only by their release of soluble factors. Neutralizing neuronal MCP-1 with a specific antibody or downregulating the

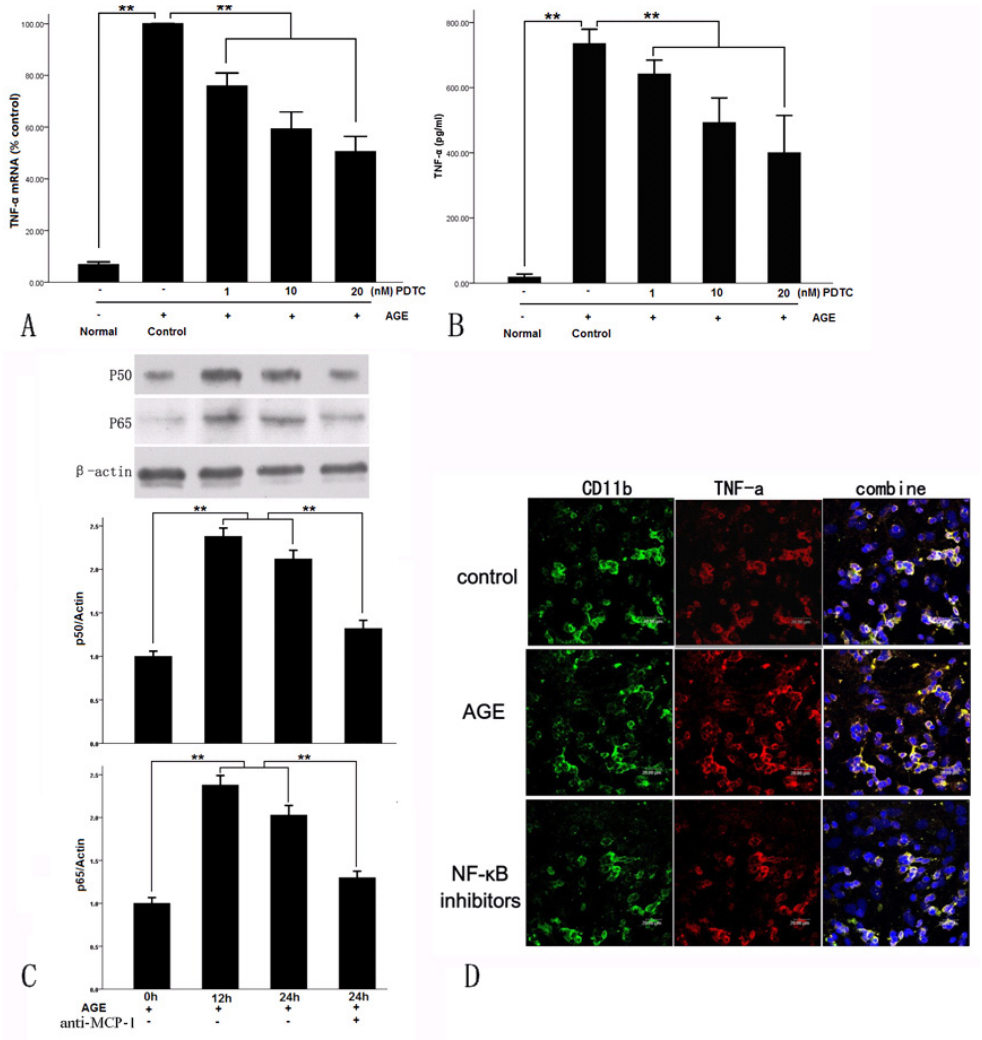

Figure 6. Tumor necrosis factor-α (TNF-α) was released from the activated microglia induced by retinal neuronal monocyte chemoattractant protein-1 (MCP-1) via the nuclear factor-κB pathways. **A**: Real-time PCR was used to measure TNF-α mRNA expression. Dose-dependent inhibition of the expression of TNF-α mRNA was induced by retinal neuronal MCP-1 in the retinal neuron–microglia Transwell culture system by nuclear factor-κB (NF-κB) inhibitors. **B**: Enzyme-linked immunosorbent assay (ELISA) was used to measure the soluble TNF-α concentration. Dose-dependent inhibition of the expression of soluble TNF-α was induced by NF-κB inhibitors. **C**: Western blot analysis of NF-κB p50 and p65 subunits in nuclear fractions of microglial cells treated with advanced glycation end products (AGEs; 750 μg/ml) in the presence or absence of anti-MCP-1. The levels of the p50 and p65 subunits of NF-κB from the microglial nuclear fractions increased by retinal neuronal MCP-1; however, anti-MCP-1 led to downregulation (**p<0.01). **D**: Purified microglia were stained with fluorescein isothiocyanate (FITC)-CD11b (green), and the expression of TNF-α was labeled with PE (red). The number of CD11b and TNF-α double-stained cells (activated microglia) decreased markedly after AGE treatment with NF-κB inhibitors (control: 11.33±3.56 cells/microscopic visual field; AGE: 26.36±4.52 cells/microscopic visual field; NF-κB inhibitors: 17.89±2.87 cells/microscopic visual field; p=0.015).

MCP-1/CCR2 pathway with CCR2 knockdown can greatly inhibit the effect of retinal neurons on microglia activation. In addition, to exclude the effects of MCP-1 and/or TNF-α from the microglia exposed to AGE in the Transwell apparatus, the primary cultured retinal neurons were pretreated with AGEs (750 μg/mL) in the culture medium for 24 h, then washed with PBS three times and removed AGEs, followed by coculture with the isolated microglia in the Transwell apparatus for another 24 h (Figure 7C,D,E). Based on our current in vitro studies, we demonstrate that retinal neuronal MCP-1 activates microglia by the p38, ERK, and NF-κB pathways, but not JNK.

The inflammatory process involves multiple disorders in signaling networks, and deregulation of MAPK systems is involved in the inflammatory process [36,37]. MAPKs, which comprise a family of protein-serine/threonine kinases, link extracellular signals from activated receptors located in the plasma membrane to various cellular compartments, notably the nucleus, leading to different cellular responses such as cell proliferation, survival, death, and differentiation. The phosphorylation of molecules involved in the MAPK system ultimately signals the expression of proinflammatory mediators, especially cytokines. A previous study showed that MAPK signaling pathway molecules are involved in the production of the proinflammatory cytokines interleukin-1β

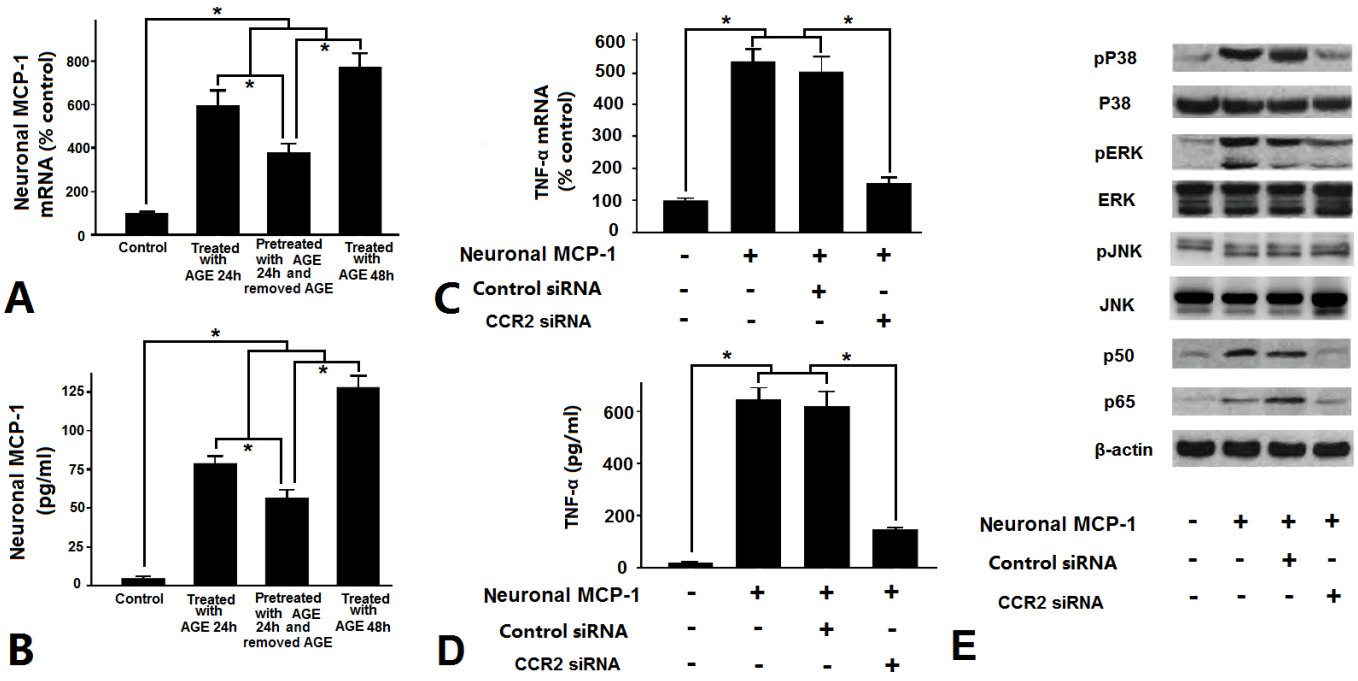

Figure 7. Retinal neuronal monocyte chemoattractant protein-1 (MCP-1) induced by advanced glycation end products (AGEs) stimulates tumor necrosis factor-α (TNF-α) expression in rat microglia via the p38, extracellular signal-regulated kinase (ERK), and nuclear factor-κB (NF-κB) pathways, but not the c-Jun N-terminal kinase (JNK) pathway. **A**: Real-time PCR was used to measure MCP-1 mRNA expression. **B**: Enzyme-linked immunosorbent assay (ELISA) was used to measure the soluble MCP-1 concentration. **C**, **D**, **E**: To exclude the effects of MCP-1 and/or TNF-α from the microglia exposed to AGEs in the Transwell apparatus, the primary cultured retinal neurons were pretreated with AGEs (750 μg/ml) in the culture medium for 24 h, then washed with PBS three times and removed AGEs, followed by coculture with the previously described isolated microglia in the Transwell apparatus for another 24 h. **C**: Real-time PCR was used to measure TNF-α mRNA expression. **D**: ELISA was used to measure the soluble TNF-α concentration (*p<0.05). **E**: The levels of phospho-p38, phospho-extracellular signal-regulated kinase (ERK), and the p50 and p65 subunits of NF-κB from the microglial cells increased. However, the levels of phospho-p38, phospho-ERK, and the p50 and p65 subunits of NF-κB from the microglial cells decreased accompanied by CC-chemokine receptor 2 (CCR2) knockdown to retinal microglia in the Transwell culture system with western blotting. Phospho-c-Jun N-terminal kinase (JNK) levels from the microglial cells remained unchanged over the entire experimental period in the retinal neuron–microglia Transwell culture system, and CCR2 knockdown did not lead to downregulation of the phospho-JNK levels.

and TNF-α by microglia [36]. The MAPK systems, such as ERK, JNK, and p38, are mainly involved in the inflammatory process; however, ERK5/BMK1 is responsible for regulating cell proliferation and differentiation and plays a critical role in cardiovascular development and vascular integrity.

P38 MAPK, one of the families of intracellular signaling molecules that transduce extracellular responses, plays an important role in regulating the lipopolysaccharides (LPS)-induced activation of microglia [38-40]. Consistent with previous studies, we found that p38 MAPK is involved in activating retinal microglia and that SB203580 significantly suppressed the expression of proinflammatory mediators such as TNF-α via transcriptional and post-transcriptional mechanisms [41]. CD11b is a β-integrin marker of microglia, and the increased CD11b expression corresponds to microglial activation [42]. In our study, a p38 inhibitor, SB203580, reduced CD11b-positive staining of primary retinal microglia, which provided evidence for the suppressive effect on microglial activation in vitro (Figure 3D). It has therefore been suggested that inhibiting p38 MAPK would abrogate TNF-α, thus providing an anti-inflammatory therapeutic potential.

ERK1 (p44 MAPK) and ERK2 (p42 MAPK) are proteins with 84% sequence homology in their central components and typically share many features. ERK MAPKs are mainly activated by growth factors that bind to membrane receptor tyrosine kinases (RTKs) and cytokines that bind to receptors linked to tyrosine kinases of the JAK family (Janus kinases). Once activated, ERK proteins can be phosphorylated and can activate retinal microglia [43]. Consistent with the previous studies, ERK MAPKs were found to be involved in activating retinal microglia [13,41], and U0126 significantly decreased the expression of TNF-α mRNA and soluble TNF-α release in a dose-dependent manner.

Although JNK and p38 mediate transcriptional and post-transcriptional responses to proinflammatory stimuli, the phosphorylation of both molecules ultimately signals different components. For example, p38 is involved in regulating NF-κB, whereas JNK is responsible for the phosphorylation of c-Jun, a component of AP-1 [37]. Wang et al. showed that AGEs mediated the expression and secretion of TNF-α in rat retinal microglia via the ERK, p38, and JNK pathways [13]. However, our results show that retinal neuronal MCP-1 induced by AGEs activates microglia by the p38 and ERK pathways, but not the JNK pathway, which are not in agreement with the study by Wang et al. [13]. This discrepancy may be attributed to the use of a different JNK inhibitor. Curcumin, the JNK inhibitor used by Wang et al., has a broad selectivity for various targets such as reactive oxygen species (ROS) and NF-κB pathways [44].

We postulate that the NF-κB pathway is also involved in retinal neuronal MCP-1 induced activation of microglia. The NF-κB signaling pathway is the major signaling mechanism involved in inflammation and transcriptional regulation of inflammatory mediators [45,46]. Our study has shown that treatment with PDTC (an NF-κB inhibitor) significantly decreased the expression of TNF-α mRNA and soluble TNF-α release in a dose-dependent manner. In addition, NF-κB consists of five subunits, with p50 and p65 the most abundant. Our study demonstrated that anti-MCP-1 led to attenuation of the levels of the p50 and p65 subunits from the microglial nuclear fractions induced by retinal neuronal MCP-1 (Figure 6C). Consistent with previous studies, we conclude that the NF-κB pathway is an important signaling mechanism in the neuronal MCP-1/TNF-α pathway for retinal microglia activation [13]. In summary, the current study provides new insights into understanding the inflammatory mechanisms involved in DR and shows that retinal neuronal MCP-1 induced by AGEs stimulates the secretion of TNF-α from activated microglia via the p38, ERK, and NF-κB pathways, but not JNK, which may be an important finding in DM pathogenesis.

ACKNOWLEDGMENTS

Supported by research grants from National Natural Science Foundation for the Young Scholars of Beijing Shijitan Hospital, Capital Medical University (No. 2013QB01).

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Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 2 May 2014. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.