Differential Involvement of Phosphoinositide 3-Kinase/Akt in Human RPE MCP-1 and IL-8 Expression

Zong-Mei Bian, Susan G. Elner, Ayako Yoshida, and Victor M. Elner

Purpose. To investigate the role of the phosphatidylinositol 3-kinase (PI3K) pathway and the signal mediator AP-1 in monocyte chemotactic protein (MCP)-1 and interleukin (IL)-8 gene expression in human retinal pigment epithelial (hRPE) cells.

Methods. hRPE cells were stimulated with IL-1β and TNF-α and by coculturing with monocytes in the presence or absence of a series of kinase inhibitors. The induction of MCP-1 and IL-8 protein and mRNA was determined by ELISA and RT-PCR, respectively. Western blot analysis, kinase assays, and electrophoretic mobility shift assays were used to detect the activation of signaling mediators and transcription factors.

Results. Concomitant with the induction of chemokine expression by the stimuli, there was phosphorylation of PI3K and its downstream targets—namely, Akt, GSK, and FKHR. Ly294002, a specific inhibitor of PI3K, resulted in time- and dose-dependent blockade of MCP-1 mRNA expression and protein production. The IC_{50} for inhibition of MCP-1 secretion induced by IL-1β, TNF-α, and hRPE-monocyte binding was 16, 12, and less than 3 μM, respectively. In contrast, Ly294002 did not inhibit the IL-8 expression induced by any of the stimuli. Ly294002 as well as U0126, SB202190, and SP600125, the selective inhibitors of MEK, p38, and JNK, respectively, strongly inhibited induced c-fos expression, whereas Ly294002 did not inhibit induction of MEK, p38, or JNK. Blockade of PI3K/Akt abolished IL-1β–induced nuclear translocation of AP-1, whereas the induction of IκB degradation was unchanged.

Conclusions. The Ly294002-sensitive PI3K/Akt pathway regulates MCP-1, but not IL-8 expression in hRPE cells independent of MAPK and IκB. PI3K-dependent induction of hRPE c-fos and AP-1 nuclear translocation may be a target for therapies aimed at modulating MCP-1 in retinal diseases. (*Invest Ophthalmol Vis Sci.* 2004;45:1887–1896) DOI:10.1167/iovs.03-0608

Leukocyte activation and infiltration into choroidal and retinal tissue play critical roles in infectious and noninfectious retinal diseases, including proliferative vitreoretinopathy (PVR), age-related macular degeneration (ARMD), and overtly inflammatory ocular diseases such as uveitis.[1,2] The retinal pigment epithelium (RPE) is a key component of the blood–retina barrier and is believed to be the major participant in these pathologic processes. In addition to responding to ambient proinflammatory cytokines IL-1β and TNF-α,[3–6] we have recently shown that human RPE (hRPE) cells respond to monocytes binding to their cell surfaces by secreting IL-8 and MCP-1, the principal chemokines secreted by RPE cells.[6,7] MCP-1 belongs to the C-C chemokine family and functions as a chemoattractant and an activator for lymphocytes and monocytes, causing monocyte/macrophage infiltration into tissues.[8] Interleukin (IL)-8, a member of the C-X-C chemokine family, is a potent activator and chemoattractant of neutrophils.[9] Transcription factors NF-κB and AP-1 binding motifs are found in MCP-1 and IL-8 gene promoters. Thus, it is not surprising that these two chemokines are often coinduced in different cells by several stimuli.[9,10] MCP-1–directed monocyte extravasation and cell–cell contact between monocytes/macrophages and hRPE cells are also important sequential events in retinal diseases, such as PVR, ARMD, and uveitis. Thus, hRPE MCP-1 induced by IL-1β, TNF-α, and monocyte/hRPE cell contact may initiate and perpetuate ocular inflammation by recruiting and activating monocytes and lymphocytes in diseased retinal tissue.

IL-1β and TNF-α have high potential to activate a range of protein kinases, but the postreceptor signaling pathways may vary, depending on cell type and the nature of the stimulus.[11,12] The signaling pathways mediating IL-1β- and TNF-α–induced hRPE MCP-1 and IL-8 expression have been reported in our previous studies.[13] We have demonstrated that extracellular signal-regulated kinase ERK1/2 and p38 mitogen-activated protein kinase (MAPK) pathways, and NF-κB-induced kinase (NIK) pathway are the major signaling pathways. Our recent studies have shown that direct monocyte/hRPE contact, but not monocyte-conditioned medium (CM), induces hRPE MCP-1 and IL-8 production and that coactivation of ERK1/2, p38, and NIK pathways are essential for the rapid induction of these hRPE chemokines.[7,14]

In addition to MAPK and NIK pathways, the phosphatidylinositol-3-OH-kinase (PI3K)/Akt pathway and the transcription factor AP-1 have been shown to be involved in MCP-1[15–17] and IL-8[18–20] expression in cell types other than RPE cells, whereas PI3K also actives the NIK pathway.[21] Thus, we investigated the role of the PI3K pathway and the activation of AP-1 in induction of hRPE MCP-1 and IL-8 by IL-1, TNF, and monocyte contact.

Materials and Methods

Cell Isolation and Culture

HRPE cells were isolated within 24 hours of death from donor eyes obtained from the Midwest Eye Bank, as previously described.[5] The donor eyes were obtained in accordance with the provisions of the Declaration of Helsinki for research involving human tissue. In brief, the sensory retinal tissue was separated gently from the hRPE monolayer, and the hRPE cells removed from Bruch's membrane with papain (5 U/mL). Human RPE cells were cultured in DMEM containing 15% fetal bovine serum, penicillin G (100 U/mL), streptomycin sulfate (100 μg/mL), and amphotericin B (0.25 μg/mL) in culture plates (Falcon Primaria; BD Biosciences, Bedford, MA) to inhibit fibroblast growth. Cells grown in culture were further subcultured into 6- to 12-well plates, grown to confluence, and used for experiments. Most of the data described in this study were from hRPE cultures at passages 4 and 5. We did not find observable differences in the morphologic or biochemical properties of interest in cultures from passages 2 to 7. The

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hRPE monolayers exhibited typical hexagonal arrays with uniform immunohistochemical staining for fibronectin, laminin, and type IV collagen in the chicken-wire distribution characteristic of these epithelial cells. The identity of hRPE cells in the culture was also confirmed by apical immunohistochemical staining of Na^+-K^+-ATPase. The cultured hRPE cells, therefore, had morphologic and biochemical similarities to the native hRPE, but significant structural and functional differences inherent in the cultured cells are undoubtedly present when compared with native hRPE cells.

Enzyme-Linked Immunosorbent Assay

The levels of antigenic MCP-1 and IL-8 in the CM overlying cultured hRPE cells were quantitated by modification of a double-ligand ELISA method, as previously described.[22] The monoclonal anti-IL-8 antibody (Ab), monoclonal anti-MCP-1 Ab, biotinylated anti-IL-8 Ab, and biotinylated anti-MCP-1 Ab were purchased from R&D Systems (Minneapolis, MN). The IL-8 detection monoclonal Ab used for ELISA is directed against the mature form of IL-8 and may also be used to neutralize the activity of mature human IL-8 (R&D Systems; Technical Correspondent, Steven K. Orstad, personal communication, July 2003). We confirmed this independently by comparing results of our ELISA to those of functional IL-8 inhibition in the same hRPE CM using specific, another neutralizing anti-human IL-8 Ab.[6] Standards included 0.5 log dilutions of rMCP-1 (R&D Systems) and rIL-8 (R&D Systems) from 5 pg to 100 ng/well.

Western Blot Analysis

For preparation of whole-cell extracts, the hRPE cells were lysed with lysing buffer containing 50 mM HEPES (pH 7.4), 1% Triton X-100, 0.15 M sodium chloride, 10% glycerol, 1.5 mM magnesium chloride, 1 mM EDTA, and a mixture of protease inhibitors. The total cell lysate was obtained by sonication and centrifugation. Protein concentrations were determined with a bicinchoninic acid kit for protein determination (Sigma-Aldrich, St. Louis, MO). Western blot analyses of the hRPE cellular extracts were performed with a kit (Phospho-Akt Pathway Sampler Kit; Cell Signaling, Beverly, MA). Briefly, samples containing 20 to 50 μg of protein were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then were electrotransferred to nitrocellulose membranes. For signal protein detection, samples were blocked with a solution of Tris-buffered saline containing 5% dry milk and 0.1% Tween-20 (TBST) at room temperature for 1 hour, incubated at 4°C overnight with appropriate rabbit polyclonal Ab, and washed three times in TBST. Next, the membranes were incubated with horseradish peroxidase-conjugated polyclonal anti-rabbit secondary Ab for 1 hour at room temperature and washed three additional times with TBST. The membranes were then visualized using an enhanced chemiluminescence (ECL) assay (NEN Life Science Products, Inc., Boston, MA).

Kinase Assay

The activity of Akt kinase and MAPKs were analyzed by using assay kits for Akt and MAPK (Cell Signaling). Kinase assays were performed according to the manufacturer's protocols. Briefly, hRPE cells were serum-deprived for 24 hours and then stimulated with IL-1β, TNF-α, or monocytes in the presence or absence of inhibitors. After stimulation, the media were removed and the treated cells were rinsed with ice-cold PBS. For monocyte cocultures, the hRPE cells were washed with Ca^{2+}- and Mg^{2+}-free PBS containing 5 mM EDTA and checked under a microscope to ensure that all monocytes had been removed. Protein concentrations from cell lysates were equalized before the kinase assays. The cell lysates were mixed overnight with immobilized anti-Akt Ab, anti-phospho p38 or p42/44 monoclonal Ab, or anti-c-jun fusion protein beads. After immunoprecipitation of Akt, p38, ERKs, and JNK/SAPK with the corresponding Ab, the immunocomplexes were incubated with adenosine triphosphate (ATP) and corresponding substrates glycogen synthase kinase-3 (GSK-3) fusion protein (Akt), ATF-2 (p38), Elk-1 (ERKs), and c-jun fusion protein (JNK) at 30°C for 30 minutes. The reactions were terminated with adding SDS sample buffer. The samples were separated on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose membranes. The membranes were blocked with blocking buffer and then incubated with anti-phospho-GSK-3α/β, -ATF-2, -Elk-1, or -c-jun Ab. Finally, the membranes were incubated with horseradish peroxidase–conjugated anti-rabbit IgG Ab and visualized with ECL kits (New England Biolabs, Beverly, MA). Quantitation of immunoreactive bands was determined by using commercial software (ImageQuant; Molecular Dynamics, Sunnyvale, CA).

Electrophoretic Mobility Shift Assay

An electrophoretic mobility shift assay (EMSA) kit (Dig Gel Shift Kit; Roche Molecular Biochemicals, Mannheim, Germany) was used in the gel shift assays for detecting sequence-specific DNA-binding proteins. The nuclear extracts were prepared as previously described.[22] The probes were end labeled with digoxigenin-11-ddUTP (DIG). The labeled DNA fragments containing the AP-1 (5′-CGCTTGATGAGTCAGCCGGAA-3′ and 5′-TTCCGGCTGACTCATCAAGCG-3′) or NF-κB (5′-GCAAATCGTGGAATTTCCTCTGA-3′ and 5′-GTCAGAGGAAATTCCACGATTTG-3′) binding sites were incubated with hRPE nuclear extracts (1 μg) at 37°C for 15 minutes. The reactions contained poly[d(I-C)] for AP-1 or poly[d(A-C)] for NF-κB in the presence or absence of a 125-fold excess of unlabeled oligonucleotide for specific competition. The mixtures were then transferred to 8% polyacrylamide gel for electrophoresis. After electrophoretic separation, the oligonucleotide-protein complexes were electroblotted onto positively charged nylon membranes (Roche Diagnostics, Indianapolis, IN). The DIG-labeled DNA fragments were visualized by an enzyme immunoassay using the anti-DIG Ab.

Semiquantitative Reverse Transcription–Polymerase Chain Reaction

Human RPE cDNA synthesis was performed according to the protocol for a reverse transcription system (Invitrogen, Carlsbad, CA). Briefly, total cellular RNA was isolated from nearly confluent cultures of hRPE cells (TRIzol extraction reagent; Invitrogen) according to the manufacturer's procedure. Five micrograms of RNA was added to the reaction mixture with M-MLV reverse transcriptase (100 U/μL) and 1 μL random primers in a total volume of 20 μL. PCR for each product was performed with 1, 0.1, and 0.03 μL of the cDNA solution for IL-8 or 1, 0.2, and 0.1 μL for MCP-1, respectively, and three different cycles (15, 25, and 35). The PCR reactions were accepted as semiquantitative when individual amplifications were performed in the midlinear portion of the response curve. Specific cDNA was amplified using 28 (1 μL cDNA), 35 (0.1 μL cDNA), 25 (0.2 μL cDNA), 30 (0.2 μL cDNA), and 20 (1 μL cDNA) cycles for IL-8, MCP-1, c-jun, c-fos, and β-actin, respectively. The following conditions were used in PCR reaction for IL-8, MCP-1, and β-actin: denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute, and extension at 72°C for 2 minutes. The conditions for c-jun and c-fos were denaturation at 95°C for 45 seconds, annealing at 65°C for 45 seconds, and extension at 72°C for 1 minute. The reactions were initiated by adding 0.15 μL of *Taq* DNA polymerase (5 U/μL) to a final volume of 20 μL. The human synthetic oligonucleotide primers were: IL-8, 5′-AAGCTGGCCGTGGTCCTCTTG-3′ (sense) and AGCCCTCTTCAAAAACTTCTC-3′ (anti-sense); MCP-1, 5′-GCTCATAGCAGCCACCTTCATTC-3′ (sense) and GTCTTCGGAGTTTGGGTTTGC-3′ (anti-sense); c-jun, 5′-GCATGAGGAACCGCATCGCTGCCTCCAAG-3′ (sense) and GACCAAGTCCTTCCCACTCGTGCACACTG-3′ (anti-sense); and c-fos, 5′-GAGAATCCGAAGGGAAAGGAATAAGATG-3′ (sense) and GTGAAGACGAAGGAAGACGTGTAAGCAG-3′ (anti-sense). To ensure that an equal amount of hRPE template was used in each amplification reaction, human β-actin sense (5′-GTGGGGCGCCCCAGGCACCA-3′) and anti-sense (5′-GCTCGGCCGTGGTGGTGAAGC-3′) primers were used in parallel. Each PCR product was analyzed by electrophoresis on a 2% agarose gel and stained with ethidium bromide. The intensity of the ethidium bromide luminescence was measured by an image sensor with a computer-controlled display.

Northern Blot Analysis

The total cellular RNA was isolated as described earlier. The RNA was separated by electrophoresis using 1% formaldehyde-agarose gels and transferred overnight to the positively charged nylon membranes by capillary blotting. The blots were hybridized overnight at 50°C with DIG-labeled oligonucleotide probes (25 ng/mL). Bound probes were detected with an anti-DIG Fab fragment conjugated to alkaline phosphatase with the chemiluminescent substrate CSPD (disodium 3-(4-methoxyspiro {1,2-dioxetane-3, 2′-(5′-chloro) tricyclo [3.3.1.1.^{3,7}] decan}-4-yl)phenyl phosphate). Specific chemokine mRNA was quantified by laser densitometry. Equivalent amounts of total RNA load per gel lane were assessed by monitoring 18s and 28s ribosomal RNA.

Statistical Analysis

Data in the text and figure legends are expressed as the mean ± SEM. Differences were calculated by ANOVA or were taken to be significant at $P < 0.05$.

Results

Activation of PI3K/Akt by IL-1β, TNF-α, and Monocyte Coculture

Activation of PI3K induces expression of MCP-1 and IL-8 in a variety of cell types.[15–18] Therefore, the activation of the PI3K/Akt pathway by IL-1β, TNF-α, and monocyte coculture was examined in this study. The serine-threonine kinase Akt is one of the major downstream targets of PI3K. To assess activation of Akt by IL-1β and TNF-α, the hRPE cells were incubated with IL-1β (0.2 and 2 ng/mL) or TNF-α (2 and 20 ng/mL) at 37°C for 7, 15, 30, and 60 minutes. For monocyte cocultures, fresh-isolated human monocytes (1×10^5 cells/cm^2) were overlaid onto hRPE cells for 15, 30, and 60 minutes. The hRPE cell lysates were prepared for Western blot analysis and kinase assays.

To determine phosphorylation of Akt, the samples were immunoblotted with antibody against the active (phosphorylated) form of Akt. To ensure that equal amounts of samples were used, phosphorylation-independent anti-Akt or anti-actin Ab was used in parallel assays. IL-1β (0.2 ng/mL), TNF-α (2 ng/mL), and monocytes/hRPE coculture each induced Akt phosphorylation (Figs. 1A, 1C). Activation of Akt was further examined by detecting phosphorylation of its substrates GSK and forkhead transcription factor (FKHR). Phosphorylation of GSKβ and FKHR was determined using phospho-specific anti-GSKβ and -FKHR Abs. IL-1β (0.2 ng/mL), TNF-α (2 ng/mL), and monocytes/hRPE cocultured for 30 minutes all markedly induced phosphorylation of GSKβ and FKHR (Figs. 1B, 1C), suggesting that IL-1β, TNF-α, and monocyte coculturing all activate Akt and its downstream targets. The Akt enzyme activity induced by IL-1β, TNF-α, and monocytes was time and dose dependent. When compared with the 30-minute untreated cells, the Akt enzyme activities after simulation for 7, 15, 30, and 60 minutes were increased by 2.6-, 2.8-, 3.2-, and 6.5-fold with IL-1β, and 2.1-, 2.5-, 3.3-, and 1.3-fold with TNF-α (Fig. 2A). The activation reached a maximum at 60 minutes for IL-1β and at 30 minutes for TNF-α. As the IL-1β and TNF-α doses increased from 0.2 to 2 ng/mL and from 2 to 20 ng/mL, respectively, the Akt enzyme activity was also enhanced. Similarly, the Akt enzyme activities induced by monocyte coculture for 15, 30, and 60 minutes were 4.7-, 2.7-, and 1.5-fold higher than the 30-minute untreated cells (Fig. 2B). The Akt enzyme activity induced by monocyte coculture reached a maximum by 15 minutes, whereas the increases in Akt activity by all three stimuli were sustained for at least 60 minutes (Fig. 2). As expected, when the hRPE cells were treated with

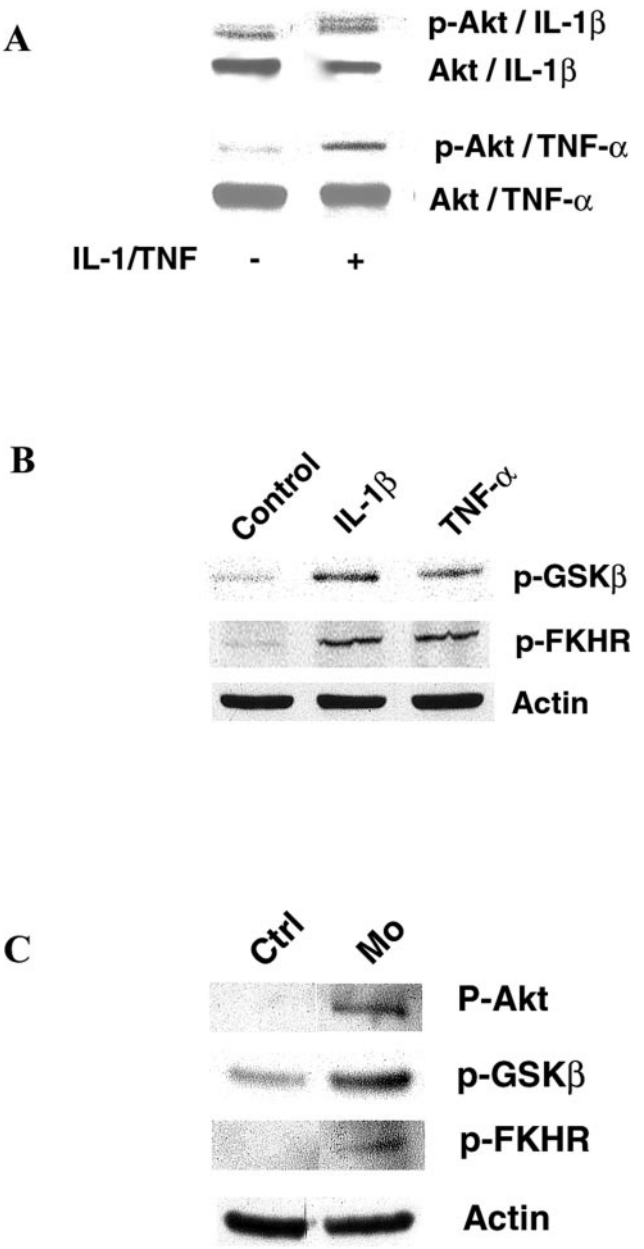

Figure 1. IL-1β-, TNF-α-, and monocyte (Mo)/hRPE coculture-induced phosphorylation of Akt, GSKβ, and FKHR in hRPE cells. Western blot analysis of whole-cell lysates from hRPE cells that were unstimulated (control, Ctrl), exposed to IL-1β (0.2 ng/mL), or exposed to TNF-α (2 ng/mL) for 30 minutes were detected by anti-phosphorylation–specific or phosphorylation-independent Akt Ab (**A**), or anti-phosphorylation–specific GSKβ and FKHR Ab (**B**). The hRPE cell lysates after monocyte/hRPE coculture were analyzed similarly. Anti-actin Ab was the control probe (**C**).

Ly294002, the specific inhibitor of PI3K,[23] the monocyte-induced hRPE Akt enzyme activity was abolished (Fig. 2B).

Activation of PI3K/Akt in hRPE MCP-1 Expression

We then assessed the involvement of the PI3K/Akt pathway in hRPE IL-8 and MCP-1 synthesis and secretion known to be induced by IL-1β, TNF-α, or monocytes.[3,4,6,7] Ly294002 was used to monitor PI3K-mediated signaling. First, nearly confluent hRPE cells were preincubated with or without Ly294002 for 1 hour. Then hRPE cells were challenged with IL-1β (0.2

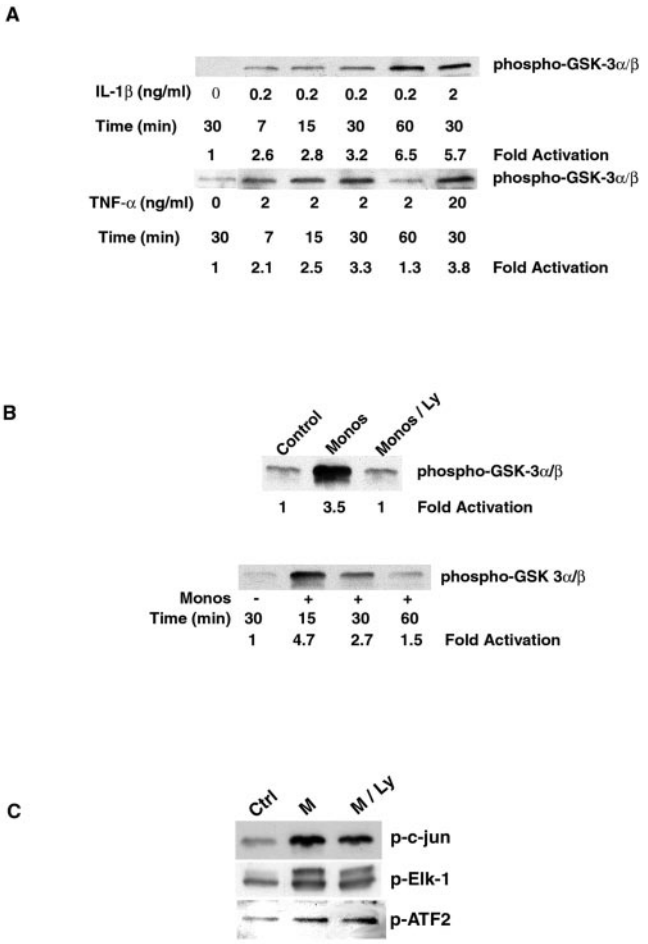

FIGURE 2. IL-1β-, TNF-α-, and monocyte (Monos; M)/hRPE coculture-induced activation of hRPE Akt and the effects of Ly294002. Human RPE cells were treated with IL-1β (0, 0.2, or 2 ng/mL) and TNF-α (0, 2, or 20 ng/mL) for 7, 15, 30, or 60 minutes (**A**) or preincubated with Ly294002 (Ly; 0, 100 μM) for 1 hour before monocytes were overlaid onto hRPE cells for 15, 30, or 60 minutes in the absence or presence of the Ly294002 (**B**, **C**). Unstimulated hRPE cells were used as control (Ctrl). Induced activation of hRPE Akt as detected by Ab binding to phosphorylated GSK (phospho-GSK-3α/β) (**A**, **B**) and induced activation of hRPE p38, ERK1/2, or JNK/SAPK as detected by Ab binding to phosphorylated ATF-2 (p-ATF2), Elk-1 (p-Elk-1), or c-jun (p-c-jun), respectively (**C**). Multiples of change in expression (fold activation), indicated under the bands (**A**, **B**), was quantitated by densitometry.

ng/mL), TNF-α (2 ng /mL), or monocyte coculture for 6 hours (mRNA assay) or 24 hours (protein assay). The CM were collected for MCP-1 and IL-8 ELISA, and the hRPE cells were harvested for MCP-1 and IL-8 mRNA detection. The PI3K inhibitor Ly294002 markedly reduced hRPE MCP-1 protein production in a dose-dependent manner (Figs. 3A, 3B). Ly294002 (100 μM) strongly inhibited ($P < 0.001$) hRPE MCP-1 secretion induced by IL-1β or TNF-α, whereas Ly294002 at 50 to 100 μM completely abrogated MCP-1 induced by hRPE-monocyte coculture, suggesting that PI3K activation is required for the MCP-1 production. The monocytes/hRPE coculture-induced MCP-1 production also was more sensitive to Ly294002 inhibition, since the calculated IC_{50} values for Ly294002 inhibition of MCP-1 secretion stimulated by IL-1β, TNF-α, and monocyte coculture were 16, 12, and less than 3 μM, respectively. In contrast to the marked inhibition of MCP-1 induction, Ly294002 did not affect IL-8 production (Table 1). Consistent with selective inhibition of MCP-1 protein production, Ly294002 inhibited only hRPE MCP-1 steady state mRNA induced by each of the stimuli (Fig. 4). These results indicate that the Ly294002-sensitive PI3K pathway appears to be selective for induction of hRPE MCP-1, but not for hRPE IL-8. The inhibition of MCP-1 mRNA to near the control levels by Ly294002 strongly suggests that the regulation by PI3K/Akt is mainly at the transcriptional level.

Induction of AP-1, but Not NF-κB by the PI3K/Akt Pathway

AP-1 and NF-κB are the major transcription factors for MCP-1 and IL-8 gene expression.[9,24] We first determined whether blocking PI3K by Ly294002 could alter AP-1 activation. AP-1 consists of a heterodimer of c-Jun and c-Fos proteins. To analyze c-jun and c-fos mRNA levels, serum-starved hRPE cells were overlaid with monocytes in the presence or absence of Ly294002 (100 μM) for 0.5, 1, 2, 4, and 6 hours, or hRPE cells were treated with IL-1β (0.2 ng/mL) and TNF-α (2 ng/mL) for 2 hours. At the end of the incubation, the media were removed, and the total RNA was isolated from the hRPE cells for RT-PCR analysis. The conditions for RT-PCR were predetermined to ensure semiquantitative estimation (data not shown). The housekeeping gene β-actin mRNA was used as an internal control. In the untreated hRPE cells, low levels of constitutive expression of c-jun and c-fos mRNA were observed (Fig. 5). Coculture with monocytes led to increases in both c-jun and c-fos hRPE mRNA (Fig. 5A). Similar enhanced expression of c-jun and c-fos hRPE mRNA was observed with IL-1β or TNF-α treatment (Fig. 5B). The monocyte coculture-stimulated c-jun and c-fos mRNA expression was transient. As shown in Figure 5A, the induced c-jun and c-fos hRPE mRNA expression was time dependent with peaks at 0.5 hour after the onset of stimulation. The time course for c-fos induction differed from that of c-jun. During 6 hours of cocultures with monocytes, the induced c-jun mRNA had only slight degradation, whereas c-fos mRNA returned to the constitutive level of expression by 4 hours (Fig 5A). Moreover, inhibition of PI3K by Ly294002 (Fig. 5C) reduced c-fos, but not c-jun mRNA expression, suggesting that PI3K pathway is selectively involved in c-fos, but not c-jun, gene induction. To further demonstrate the PI3K-mediated activation of AP-1 and NF-κB, whole-cell and nuclear extracts were made from hRPE cells under the same experimental conditions. The extracts were analyzed by Western blot to determine IκB degradation and by gel mobility shift assays to determine AP-1 and NF-κB binding to specific DNA probes. As seen in Figure 6D, IL-1β markedly increased AP-1 binding in hRPE nuclear extracts but this increase was reduced to basal levels by Ly294002.

In contrast to the inhibition of hRPE AP-1 activation by LY294002, the induced nuclear translocation of NF-κB was not as strongly inhibited (Fig. 6D). As shown in our previous studies,[13] the IκB degradation, a key step for NF-κB activation was induced by IL-1β (Fig. 6C), and this degradation was effectively blocked by parthenolide, an inhibitor of IκB degradation. In this study, the IκB degradation induced by IL-1β was not changed by Ly294002 (Fig. 6C), suggesting that activation of this NF-κB pathway induced by IL-1β is independent of the activation of PI3K. These results indicate that the PI3K pathway that mediates MCP-1 gene expression is unlikely to have crosstalk with the NIK pathway (Figs. 6C, 6D), as that described for other cell lines.[25]

Activation of c-Fos by PI3K/Akt Does Not Require MAPK Pathways

It has been documented that MAPK pathways may lead to activation of AP-1[26,27] and MAPK can be activated through PI3K.[28] Therefore, we first investigated how inhibition of PI3K would affect activation of the ERK, p38, and JNK, MAPK

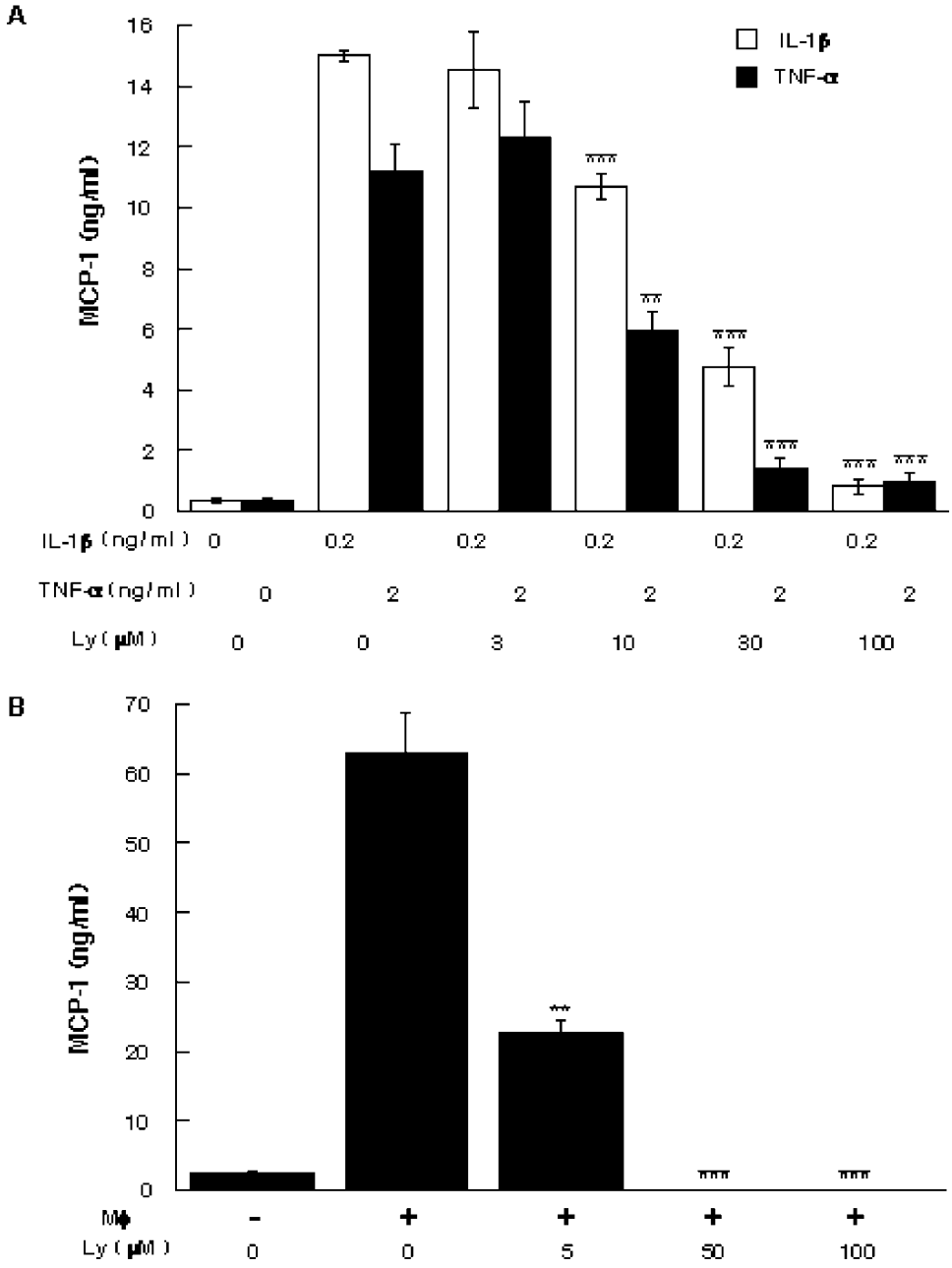

FIGURE 3. Effects of PI3K/Akt inhibition on IL-1β-, TNF-α-, and monocyte (Mϕ)/hRPE coculture-induced hRPE MCP-1. Human RPE cells were pretreated for 1 hour with Ly294002 (Ly; 0, 3, 10, 30, and 100 μM) and then stimulated with IL-1β (0.2 ng/mL) or TNF-α (2 ng/mL) for 24 hours in the absence or presence of Ly294002 (**A**). Human RPE cells were pretreated for 1 hour with Ly294002 (Ly; 0, 5, 50, and 100 μM), before stimulation with monocytes overlaid onto hRPE cells for 24 hours in the absence or presence of Ly294002 (**B**). Constitutive and stimulated MCP-1 secretion, measured by ELISA ($n = 3$), were compared with and without Ly294002 ($^{**}P < 0.01$ and $^{***}P < 0.001$).

pathways in hRPE cells. Phosphorylation of Elk1, c-jun, and ATF2 by ERK, JNK, and p38, respectively, were assessed after stimulation at 37°C for 30 minutes by monocytes in the absence or presence of the Ly294002. As a result, Ly294002 had no effect on the induced ERK, p38, and JNK enzyme activities as demonstrated in Figure 2C. Next, to determine the role of the MAPK pathway in the activation of c-fos (AP-1), SB202190, U0126, and SP600125—specific inhibitors for p38, ERK, and JNK/SAPK, respectively—were used to block these kinase activities during stimulation by IL-1β (0.2 ng/mL) at 37°C for 30 minutes. Similar to Ly294002, SB202190, U0126, and SP600125 each suppressed IL-1β–induced AP-1 expression as shown by reduced levels of c-fos (Figs. 6A, 6B). These results suggested converged downstream activation of c-fos (AP-1) by multiple signaling pathways at the gene level. Activation of AP-1 (c-fos) by PI3K is independent of the ERK, p38, or JNK MAPK pathways.

TABLE 1. Effect of Ly 294002 on IL-1β, TNF-α, and Monocyte-Induced IL-8 Secretion by hRPE Cells

	IL-8 (ng/mL)
RPE alone	<0.01
Coculture of RPE and monos	46.71 ± 4.01
Coculture of RPE and monos/Ly	44.21 ± 0.91
RPE alone	0.05 ± 0.00
IL-1β (0.2 ng/mL)	23.93 ± 1.63
IL-1β (0.2 ng/mL)/Ly	21.69 ± 0.63
TNF-α (2 ng/mL)	10.46 ± 0.61
TNF-α (2 ng/mL)/Ly	12.34 ± 0.73

Human RPE cells were not pretreated (control) or were pretreated with Ly294002 (100 μM) for 1 hour. Monocytes were then overlayed onto hRPE cells or IL-1β or TNF-α were added to hRPE cultures. IL-8 was measured in conditioned media collected after 24 hours of hRPE cocultured with monocytes, IL-1β, or TNF-α without or with the inhibitor present.

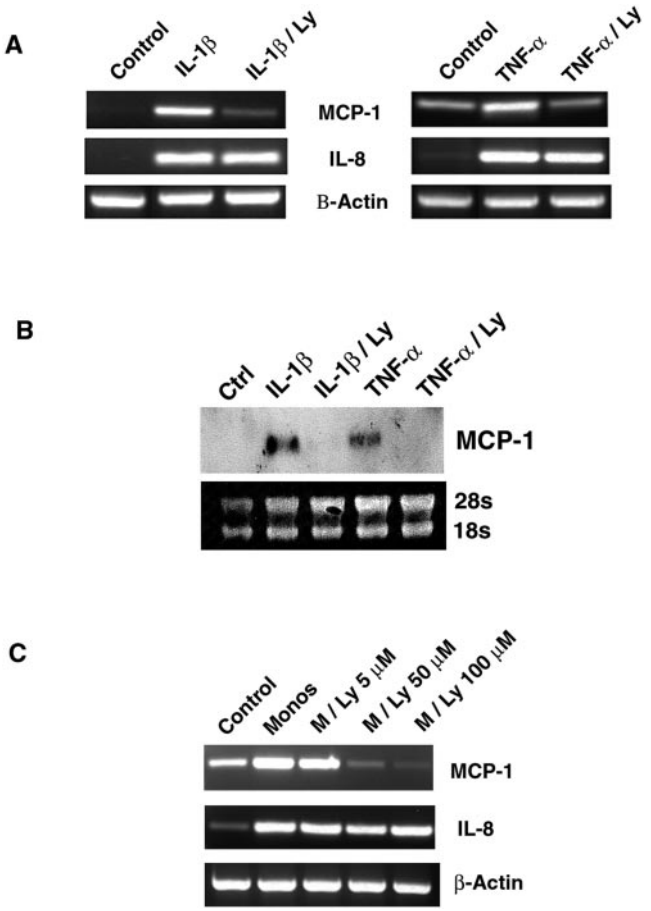

FIGURE 4. Effects of Ly294002 on IL-1β-, TNF-α-, or monocyte (monos; M)/hRPE coculture-induced steady state hRPE MCP-1 and IL-8 mRNA. Unstimulated hRPE cells were used as control (Ctrl). hRPE cells were pretreated with Ly294002 (Ly; 0 or 100 μM) for 1 hour and then stimulated with IL-1β (0.2 ng/mL) or TNF-α (2 ng/mL) in the absence or presence of the inhibitor for 6 hours (**A**, **B**) or pretreated with Ly294002 (Ly; 0, 5, 50, or 100 μM) before exposure to monocytes for 6 hours in the absence or presence of Ly294002 (**C**). Semiquantitative RT-PCR (**A**, **C**) and Northern blot (**B**) with 18s and 28s RNA controls.

DISCUSSION

PI3K comprises a family of lipid signaling enzymes that promote phosphorylation of phosphoinositides (PtdIns). Among them, PtdIns-3 and -4-P_2 and PtdIns-3, -4, and -5-P_3 are the second messengers that bind to the pleckstrin homology (PH) domain of Akt (protein kinase B, PKB) and protein kinase 1 (PDK1). IL-1, TNF, and cell-to-cell contact have been known to activate the PI3K pathway.[15,18,29,30] Activation of PI3K mediates TNF[15] and platelet-derived growth factor (PDGF)-[16] induced MCP-1 expression and PDGF-, IL-1-,[18] and hepatocyte growth factor (HGF)-[19] induced IL-8 expression in other cell types. In this study, we demonstrated the important role of the PI3K/Akt pathway in inducing expression of hRPE MCP-1. However, hRPE IL-8 expression induced by the same stimuli was totally insensitive to Ly294002. This observation was in clear contrast to the concomitant inhibition of both MCP-1 and IL-8 expression by blocking NIK and MAPK pathways.[13,14] Although the selective stimulation of either IL-8 or MCP-1 by a given stimulus has been reported in various cell types, selective induction of MCP-1, but not IL-8, by a particular signaling pathway is rare. The selective involvement of PI3K in MCP-1, but not IL-8, expression as reported herein is quite similar to one of our previous observations showing that glycated human serum albumin–induced MCP-1, but not IL-8, was susceptible to inhibition by AE490, a selective inhibitor of jak2.[22] Ly294002-sensitive PI3K is thus a second signaling pathway observed in hRPE cells that selectively stimulates MCP-1. Of note, selective alteration of MCP-1, but not IL-8, expression has been reported in other cell types in at least two cases. One report showed that MCP-1, but not IL-8, may be downregulated by IL-13 through the PI3K pathway in the human colonic epithelial cell,[31] whereas another report demonstrated that alprazolam selectively suppresses MCP-1, but not IL-8, mRNA expression.[32] These two recent observations, together with our results, suggest that stimuli may specifically trigger downstream effectors regulating MCP-1 gene expression.

As shown in this and our previous studies,[13,14] hRPE cell IL-8 and MCP-1 are induced in parallel through multiple signaling pathways by a variety of stimuli (Fig. 7). The joint stimulation of IL-8 and MCP-1 mRNA expression is consistent with the finding that both IL-8 and MCP-1 promoters contain the common sites for the promiscuous transcription factors NF-κB and AP-1, both of which may be activated by IL-1 and TNF. NF-κB functions as a dimer, composed of a family of subunits including NF-κB1 (p50), NF-κB2 (p52), c-REL, REL-A (p65), and REL-B.[20] The transcription factor AP-1 is a homo- or heterodimer formed by c-JUN, JUN-D, JUN-B, ATF-2, c-FOS, FRA-1, FRA-2, and other members of this family.[20]

The molecular mechanism underlying the selective induction of MCP-1, but not IL-8, gene expression by the Ly294002-

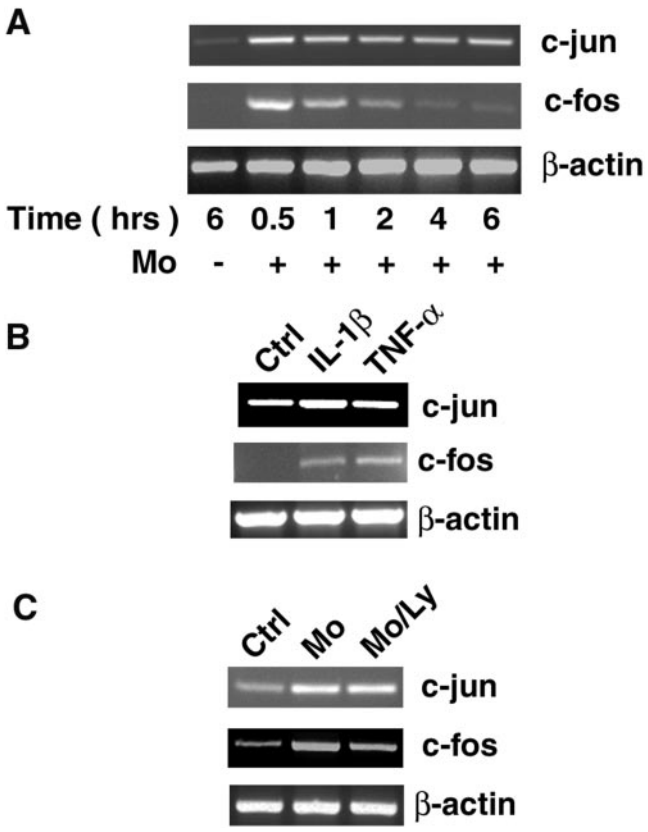

FIGURE 5. Semiquantitative RT-PCR of hRPE c-jun and c-fos mRNA induced by monocyte (Mo)/hRPE coculture (**A**, **C**), IL-1β (0.2 ng/mL) (**B**), or TNF-α (2 ng/mL) (**B**). Unstimulated hRPE cells were used as the control (Ctrl). Human RPE cells were pretreated for 1 hour with Ly294002 (Ly; 0, 100 μM) at select wells and challenged by IL-1β, TNF-α, or monocyte/hRPE coculture for 0.5, 1, 2, 4, or 6 hours in the absence or presence of the inhibitor.

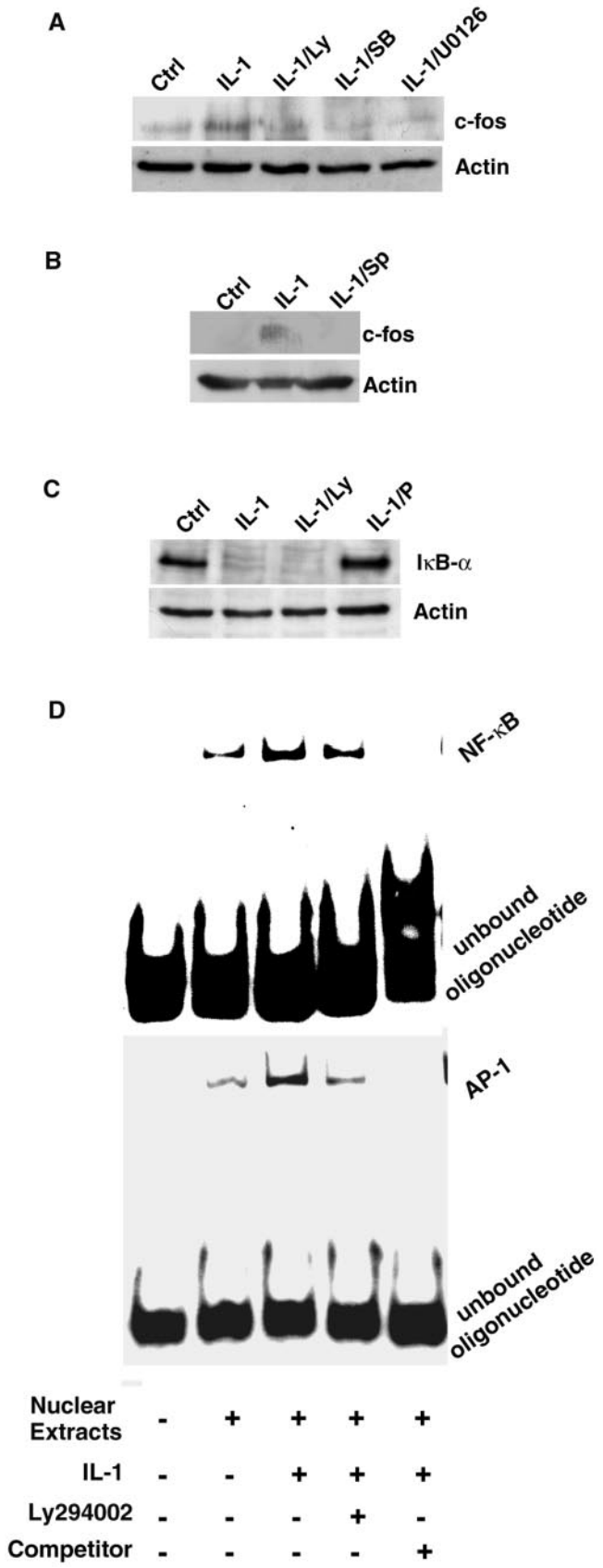

FIGURE 6. Effects of Ly294002 and MAPK inhibitors on IL-1β–induced hRPE c-fos gene expression, IκB-α degradation, and NF-κB and AP-1 binding. Unstimulated hRPE cells were used as the control (Ctrl). HRPE cells were pretreated for 1 hour without or with inhibitors: Ly294002 (Ly; 100 μM), SB202190 (SB; 30 μM), U0126 (20 μM), SP600125 (Sp; 20 μM), or parthenolide (P; 50 μM) and then stimulated with IL-1β (0.2 ng/mL) for 30 minutes (Western blots, **A**– **C**) or 2 hours (EMSA, **D**) in the absence or presence of inhibitors. Western blot analysis of hRPE whole-cell lysates induced by IL-1β were probed with anti-c-fos Ab (**A**, **B**), anti-IκBα subunit Ab (**C**) as well as anti-actin Ab (**A**– **C**). For electrophoretic mobility shift assay (EMSA), hRPE nuclear extracts (1 μg) were incubated with DIG-labeled oligonucleotides of NF-κB and AP-1, resolved by gel electrophoresis, transferred, and developed with substrate to show DNA-NF-κB and DNA-AP-1 protein complexes (**D**). A 125-fold excess of the unlabeled oligonucleotides (Competitor) was added to show specific competitive inhibition.

sensitive PI3K pathway remains elusive. Molecular studies of MCP-1 and IL-8 promoters have revealed the differences between the two genes. In contrast to the existence of only a single NF-κB binding site with high affinity for p65[33] in the human IL-8 promoter, two NF-κB binding sites, A1 and A2, have been found in the human MCP-1 promoter. The A2 site, which has a high affinity for c-Rel/p65, is the most important NF-κB binding site for MCP-1 expression.[33] The different structured and properties of NF-κB sites in the promoters of these two chemokine genes imply that the selective inhibition of MCP-1 expression may result from blocking NF-κB binding to one of the two NF-κB sites through an Ly294002-sensitive PI3K pathway. Supporting this contention is a report in which alprazolam suppressed MCP-1, but not IL-8, expression by selective inhibition of cRel/p50 binding to the A2 site of MCP-1 promoter, whereas binding of p50/p65 to the IL-8/NF-κB site was unaffected.[32] Another possibility for the selective inhibition of MCP-1 expression by PIK3 inhibitors is that the PI3K pathway may activate certain transcription factors required for MCP-1. For example, the binding of activated Sp1 to the proximal GC box appears to be critical in the maintenance of basal MCP-1 transcription.[24,34] Moreover, Sp1 can be further activated by both IL-1 and TNF, both of which induce MCP-1[10,35] The PI3K pathway has been reported to be required for small tumor antigen-induced Sp1-dependent promoter transcriptional activation.[36] Thus, when activated, PI3K may differentially activate Sp1 in the MCP-1 transcription.

As in other cell types, PI3K is necessary to induce hRPE c-fos gene expression whose product, c-fos, complexes with c-jun to form AP-1.[37] AP-1 appears to be more important than NF-κB for MCP-1 expression in human endothelial cells,[38] whereas the reverse appears to be true of IL-8 in tumor cells.[39] Moreover, IL-1 activation of PI3K results in much stronger induction of AP-1 than of NF-κB.[29] Because we found that IL-1-induced AP-1 binding to hRPE DNA was almost completely inhibited by LY294002, PI3K-dependent AP-1 activation appears to be at least as important as NF-κB binding for hRPE MCP-1 induction. We found that Ly294002 did not inhibit hRPE IL-8 induction. However, not all members of the PI3K family, most notably PI3K-C2α, are resistant to inhibition by Ly294002.[40] Thus, induction of IL-8 may be through an Ly294002-insensitive PI3K.

Leukocytic infiltration of the choroid and retina often occurs in diseases such as PVR, ARMD, and proliferative diabetic retinopathy (PDR), all of which may finally result in severe visual loss. Inflammation appears to be important mechanistically in the pathogenesis of these diseases. For example, RPE, glial, and fibroblast cellular proliferation leading to the formation of periretinal membranes follows the initial inflammatory stage of PVR.[41] In ARMD, macrophages, and lymphocytes as well as reactive, migrating, or proliferating hRPE cells that are often found adjacent to the newly formed vessels in subretinal space.[2,42–45] These ARMD lesions appear to underlie the subretinal plasma leakage and subretinal neovascularization seen

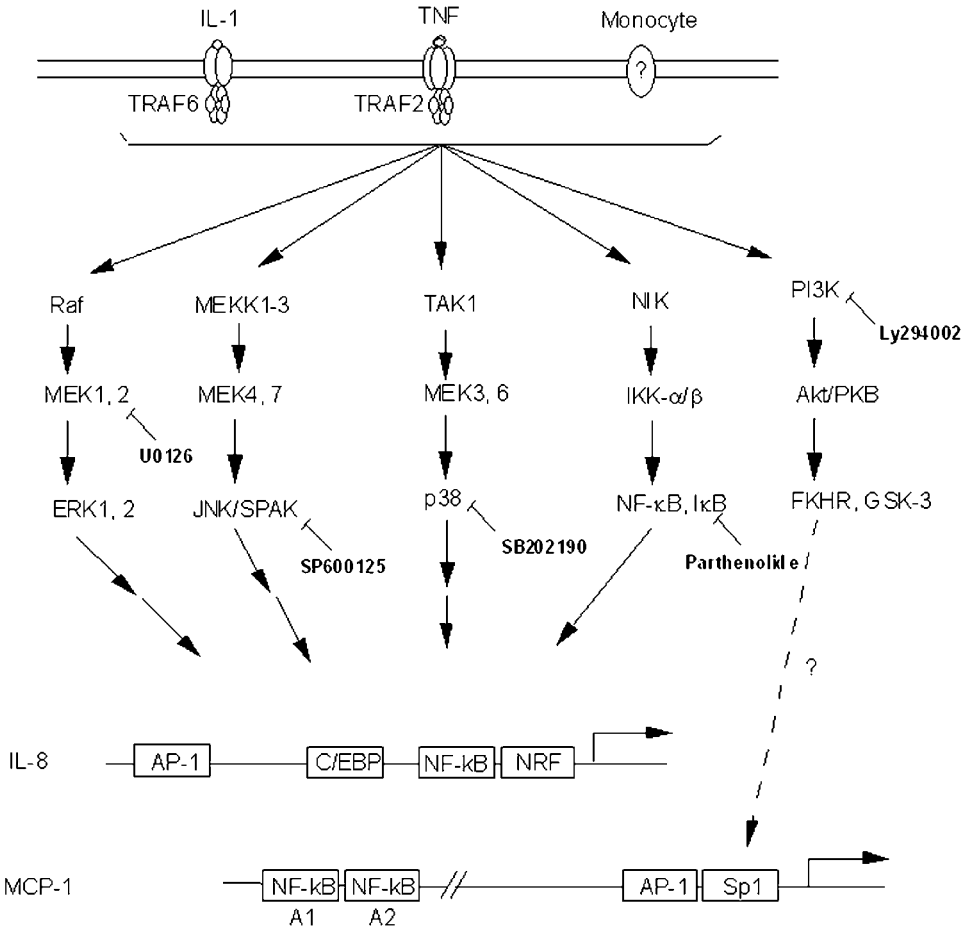

FIGURE 7. Schematic of proposed signal pathways for hRPE MCP-1 and IL-8 gene expression. IL-1, TNF-α, and monocyte binding all induce the p38, ERK1/2, JNK, NIK, and PI3K pathways. In unstimulated cells, the negative regulatory element (NRF) prevents IL-8 transcription.[21,33]

clinically.[42] Mononuclear phagocytes and lymphocytes have also been identified in surgically removed human PDR fibrovascular membranes.[46] Therefore, hRPE cells and mononuclear phagocytes and their products may be responsible for initiation and perpetuation of these retinal diseases. Our studies have shown that the proinflammatory cytokines IL-1 and TNF, as well as monocyte binding, induce hRPE cells to secrete MCP-1 and IL-8. These hRPE chemokines may directly participate in retinal neovascularization and periretinal proliferation. Recently, in an in vitro model, hRPE MCP-1 and IL-8 were shown to be involved in vascular tube formation.[47] IL-8 has been known to induce angiogenesis and is present in the vitreous of patients with retinal neovascularization.[48,49] The importance of MCP-1 in neovascularization of the posterior segment of the eye has been suggested by several studies. For instance, high levels of MCP-1 in vitreous have been detected in PVR (72%) and in PDR (76%).[50] Another study has shown that proliferation of RPE cells is associated with upregulation of MCP-1 expression.[51] In addition, vitreous treatment (a model for studying PVR) upregulates MCP-1 gene expression in ARPE-19 cells.[52] Likewise, in patients with ARMD, MCP-1 has been found in association with keratin-positive cells (RPE cells) and CD68-positive cells (macrophages/RPE) in surgical excised choroidal neovascular membranes (Murata T, et al. *IOVS* 2001; 42:ARVO Abstract 1215). Furthermore, intravitreous injection of anti-MCP-1 significantly reduces ischemia-induced retinal neovascularization.[53] Actually, on the one hand MCP-1 mediates the recruitment of monocyte infiltration, producing the indirect effect on neovascularization.[52] On the other hand, MCP-1 induces endothelial cell migration directly, as first reported by Weber et al. in 1999.[54] Further study has demonstrated that MCP-1 induces endothelial cell migration in vitro, and endothelial cells arising from aortic rings in the absence of an inflammatory response produced in vivo angiogenesis in a matrigel plug assay,[55] and was an inducer in corneal neovascularization.[56]

The important role of the PI3K pathway in hRPE MCP-1, but not IL-8, induction, illuminates targets for the selective regulation of these hRPE chemokines. Cultured hRPE cells have been widely used as a valuable tool to delineate potential roles for RPE cells in normal and diseased conditions. Because cultured cells are significantly different from native cells, both functionally and structurally, confirmation of our results in situ and/or in vivo may be necessary.

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