

Proinflammatory cytokines decrease the expression of genes critical for RPE function

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Purpose: Proinflammatory cytokines interferon gamma (IFN- γ), tumor necrosis factor alpha (TNF- α), and interleukin-1 beta (IL-1 β) secreted by infiltrating lymphocytes or macrophages may play a role in triggering RPE dysfunction associated with age-related macular degeneration (AMD). Binding of these proinflammatory cytokines to their specific receptors residing on the RPE cell surface can activate signaling pathways that, in turn, may dysregulate cellular gene expression. The purpose of the present study was to investigate whether IFN- γ , TNF- α , and IL-1 β have an adverse effect on the expression of genes essential for RPE function, employing the RPE cell line ARPE-19 as a model system.

Methods: ARPE-19 cells were cultured for 3–4 months until they exhibited epithelial morphology and expressed mRNAs for visual cycle genes. The differentiated cells were treated with IFN- γ , TNF- α , and/or IL-1 β , and gene expression was analyzed with real-time PCR analysis. Western immunoblotting was employed for the detection of proteins.

Results: Proinflammatory cytokines (IFN- γ + TNF- α + IL-1 β) greatly increased the expression of chemokines and cytokines in cultured ARPE-19 cells that exhibited RPE characteristics. However, this response was accompanied by markedly decreased expression of genes important for RPE function, such as *CDH1*, *RPE65*, *RDH5*, *RDH10*, *TYR*, and *MERTK*. This was associated with decreased expression of the genes *MITF*, *TRPM1*, and *TRPM3*, as well as microRNAs miR-204 and miR-211, which are known to regulate RPE-specific gene expression. The decreased expression of the epithelial marker gene *CDH1* was associated with increased expression of mesenchymal marker genes (*CDH2*, *VIM*, and *CCND1*) and epithelial–mesenchymal transition (EMT) promoting transcription factor genes (*ZEB1* and *SNAI1*).

Conclusions: RPE cells exposed to proinflammatory cytokines IFN- γ , TNF- α , and IL-1 β showed decreased expression of key genes involved in the visual cycle, epithelial morphology, and phagocytosis. This adverse effect of proinflammatory cytokines, which could be secreted by infiltrating lymphocytes or macrophages, on the expression of genes indispensable for RPE function may contribute to the RPE dysfunction implicated in AMD pathology.

The RPE is an integral part of the human visual system and consists of a polarized monolayer of epithelial cells located in the eye between the retinal photoreceptors and the choroid [1,2]. The RPE forms the blood–retinal barrier, provides nutrients to the photoreceptor cells, and phagocytizes rod outer segment discs undergoing circadian shedding. The RPE plays an integral role in the visual cycle by regenerating 11-*cis*-retinal, the chromophore of the visual pigment rhodopsin. A normally functioning RPE is indispensable for vision, and dysfunction of the RPE may be associated with the pathogenesis of age-related macular degeneration (AMD). Inflammation is thought to be a major contributor to AMD pathogenesis, and inflammatory mediators secreted by lymphocytes and macrophages infiltrating the posterior compartment of the eye in this context could trigger RPE dysfunction [3-6]. The response of RPE cells

to proinflammatory cytokines interferon gamma (IFN- γ), tumor necrosis factor alpha (TNF- α), and interleukin-1 beta (IL-1 β) has been widely investigated. These cytokines can bind to their specific receptors present on RPE cells and highly increase the expression of several cytokines and chemokines [7-14]. This inflammatory response is associated with increased generation of reactive oxygen species (ROS) in the RPE cell [15]. The response of RPE cells to oxidant injury is also thought to play a role in the pathogenesis of AMD [16-20]. The activation of signaling pathways by IFN- γ , TNF- α , and IL-1 β and the subsequent increase in ROS generation could potentially trigger RPE dysfunction by perturbing the expression of genes critical for the normal function of the RPE.

RPE cells are known to express a large number of genes to support their specialized function. *CDH1* (Gene ID: 999; OMIM: 192090), which encodes cadherin-1 protein (CDH1, E-cadherin), is a gene that supports the epithelial function [21]. The *RPE65* (Gene ID: 6121; OMIM: 180069) gene encodes the RPE-specific protein 65 kDa (RPE65), which is an essential visual cycle enzyme required for the conversion

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of all-*trans*-retinol to 11-*cis*-retinol [22,23]. The *RDH5* (Gene ID: 5959; OMIM: 601617) gene encodes 11-*cis*-retinol dehydrogenase while the *RDH10* (Gene ID: 157506; OMIM: 607599) gene encodes a retinol dehydrogenase that catalyzes the conversion of all-*trans*-retinol to all-*trans*-retinal [24,25]. The *RLBPI* (Gene ID: 6017; OMIM: 180090) gene encodes the 11-*cis*-retinal binding protein retinaldehyde-binding protein 1 (RLBPI), which is also known as cellular retinaldehyde-binding protein (CRALBP) [26]. *TYR* (Gene ID: 7299; OMIM: 606933) encodes tyrosinase, the most important enzyme involved in the generation of melanin pigment from tyrosine [27]. The phagocytosis function of RPE cells is controlled by tyrosine-protein kinase MER encoded by the *MERTK* (Gene ID: 10461; OMIM: 604705) gene [28]. Microphthalmia-associated transcription factor (MITF) encoded by the *MITF* (Gene ID: 4286; OMIM: 156845) gene is a known regulator of RPE differentiation [29]. MITF keeps RPE cells at a differentiated stage by highly upregulating the expression of the RPE characteristic microRNAs miR-204 and miR-211 [30,31]. This transcription factor is also known to promote melanogenesis by inducing *TYR* and to increase the expression of the *transient receptor potential cation channel, subfamily M, member 1 (TRPM1)*; Gene ID: 4308; OMIM: 603576) and *transient receptor potential cation channel, subfamily M, member 3 (TRPM3)*; Gene ID: 80036; OMIM: 608961) genes [30]. We hypothesized that the interaction of IFN- γ , TNF- α , and IL-1 β with RPE cells may adversely affect the expression of one or more genes required for the normal functioning of the RPE. Therefore, we investigated the effect of these inflammatory cytokines on the expression of several genes involved in RPE function. We employed ARPE-19, a widely used adult human RPE-derived cell culture system, for this purpose [32]. Here, we show that IFN- γ , TNF- α , and IL-1 β decrease the expression of key genes involved in the visual cycle, epithelial morphology, melanogenesis, and phagocytosis in ARPE-19 cells, thus indicating that these proinflammatory cytokines could promote retinal pigment epithelial dysfunction.

METHODS

STR analysis: Authentication of ARPE-19 cells [32] used in this study has been reported earlier from our laboratory [33]. Briefly, this validation of the established cell line using short tandem repeat analysis and amelogenin gender locus determination was performed by ATCC Cell Line Authentication Service, Promega, Madison, WI. Seventeen short tandem repeat (STR) loci plus the amelogenin gender determining locus were amplified using PowerPlex 18D kit. The cell line samples along with appropriate positive and negative controls

were processed using an ABI Prism 3500xl Genetic Analyzer. Data were analyzed using GeneMapper ID-X v1.2 software (Applied Biosystems (Foster City, CA). The batch of the cells we had was found to be a perfect match for the ATCC human RPE cell line CRL-2302 (ARPE-19). The STR analysis result is shown in Appendix 1. These cells were used within 3 additional passages.

Cell culture: ARPE-19 cells were grown on tissue culture plates in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose, L-glutamine, and 1 mM sodium pyruvate supplemented with 1% fetal bovine serum, and penicillin and streptomycin (Thermo Fisher Scientific, Grand Island, NY). The cells were maintained at 37 °C in a humidified environment of 5% CO₂, with media exchange performed twice a week. The ARPE-19 cells grown for 3 to 4 months appeared differentiated as indicated by epithelial morphology, pigmentation, and expression of RPE characteristic genes [data not shown]. The differentiated cells were treated with the proinflammatory cytokines IFN- γ , IL-1 β , and/or TNF- α for either 20 h in the absence of serum or 4 days in the presence of serum. The concentrations of the cytokines used were IFN- γ , 10 u/ml; IL-1 β , 1 ng/ml; and TNF- α , 1 ng/ml. The cytokine concentration was increased tenfold in certain experiments. Human IL-1 β was purchased from R&D Systems (Minneapolis, MN) while TNF- α and IFN- γ were from Roche Applied Science (Indianapolis, IN). The effect of proinflammatory cytokines on gene expression was also tested using an RPE cell culture system established from the eyes of adult human donors (70–85 years old) by Nagineni et al. [12,34]. These cells were grown to confluence in minimum essential medium supplemented with 10% fetal bovine serum, non-essential amino acids, penicillin (100 u/ml), streptomycin (100 μ g/ml), and amphotericin B (25 ng/ml) and then exposed to IFN- γ (100 u/ml), IL-1 β (10 ng/ml), and TNF- α (10 ng/ml) for 20 h in the absence of serum.

Real-time PCR: The reagents for RNA isolation, cDNA synthesis, and real-time PCR were obtained from Life Technologies Corporation (Grand Island, NY). The total RNA fraction isolated from the ARPE-19 cells with the Ambion mirVana miRNA Isolation Kit was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit. The cDNA preparation was then used as the template for quantitative real-time PCR analysis of the gene transcripts *IL-8* (Gene ID: 3576; OMIM: 146930), *CCL2* (Gene ID: 6347; OMIM: 158105), *CCL5* (Gene ID: 6352; OMIM: 187011), *CSF2* (Gene ID: 1437; OMIM: 138960), *CXCL11* (Gene ID: 6373; OMIM: 604852), *CXCL10* (Gene ID: 3627; OMIM: 147310), *CDHI*, *RLBPI*, *RPE65*, *RDH5*, *RDH10*, *TYR*, *MERTK*, *VIM* (Gene ID: 7431; OMIM: 193060), *CCND1* (Gene ID: 595; OMIM:

168461), *CDH2* (Gene ID: 1000; OMIM: 114020), *ZEB1* (Gene ID: 6935; OMIM: 189909), *SNAIL* (Gene ID: 6615; OMIM: 604238), *MITF*, *TRPM1*, and *TRPM3*. Briefly, 96-well reaction plate containing the PCR samples was heated at 95 °C for 20 s to activate the polymerase. Amplification condition consisted of 40 cycles of 1 sec at 95 °C and 20 s at 60 °C. Each PCR reaction (20 µl) was set up using validated TaqMan probes (labeled with reporter dye FAM at the 5' end), primers specific for the gene of interest, and TaqMan Fast Advanced Master Mix. Human 18S rRNA was used as the endogenous control. An Applied Biosystems ViiA 7 Real-Time PCR System was employed for gene amplification analysis following the manufacturer's default thermal cycling conditions. The real-time PCR results were expressed as n-fold induction in gene expression calculated using the relative quantification ($\Delta\Delta CT$) method. The expression of miRNAs was also analyzed with real-time PCR using Individual TaqMan MicroRNA Assays (hsa-miR-204, hsa-miR-211, and hsa-miR-155), the TaqMan MicroRNA Reverse Transcription Kit, TaqMan Fast Advanced Master Mix, and RNU48 (endogenous control).

Western immunoblot analysis: Following treatment with proinflammatory cytokines, the cells were suspended in RIPA buffer (Sigma-Aldrich, St. Louis, MO) containing a protease/phosphatase inhibitor cocktail (Cell Signaling Technology, Danvers, MA) at 4 °C, sonicated, and then centrifuged at 12,000 ×g for 10 min. Equal amounts of the supernatants (corresponding to 20 µg protein) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and then blotted on to nitrocellulose membranes using the iBlot dry blotting system (Invitrogen, Carlsbad, CA). The blots were then probed using a rabbit anti-CDH1 antibody (1:1,000 dilution; Cell Signaling Technology, Danvers, MA) or rabbit anti-RLBP1 antibody (1:10,000 dilution; gift from Dr. John Saari, Emeritus Professor of Biochemistry/Ophthalmology, University of Washington, Seattle, WA). Mouse anti- α -tubulin (1:10,000 dilution) was used as the primary antibody to detect α -tubulin as the loading control. IRDye 800CW goat anti-rabbit immunoglobulin G (IgG; 1:15,000) and IRDye 680LT goat-anti-mouse IgG (1:15,000) were used as the secondary antibodies. Odyssey blocking buffer (PBS), mouse anti- α -tubulin antibody, and IRDye-labeled secondary antibodies were purchased from LI-COR Biosciences (Lincoln, NE). The blots were scanned using a LI-COR Odyssey Clx Infrared Imaging System for the detection of immunoreactive bands and to estimate the fluorescence intensity.

Statistical analysis: A paired Student *t* test was used for the analysis of statistical significance. The alpha value assigned

for significance was a *p* value of less than 0.05. Representative experiments are shown in the figures, and the values are shown as mean \pm standard deviation (SD).

RESULTS

The response of the RPE cells to the proinflammatory cytokines was investigated. The ARPE-19 cells maintained in culture for 4 months acquired RPE characteristics, such as epithelial morphology and visual cycle gene expression [data not shown]. The cells were treated with IFN- γ (10 u/ml), TNF- α (1 ng/ml), and IL-1 β (1 ng/ml) for 20 h in the absence of serum; cytokines were omitted from the controls. The control cells showed typical epithelial morphology characteristic of RPE cells while the treated cells exhibited an irregular shape and thickened cell junctions (Figure 1A). This was accompanied by the increased expression of several cytokines and chemokines. Real-time PCR analysis of the control and treated cells showed that the expression of transcripts for *IL8*, *CCL2*, *CCL5*, *CSF2*, *CXCL10*, and *CXCL11* was highly increased by the treatment (Figure 1B). We then analyzed the expression of several genes essential for RPE function with real-time PCR in the control and treated cells (Figure 1C). The proinflammatory cytokines considerably decreased the expression of mRNA for *CDH1*. This gene encodes the CDH1 (E-cadherin) protein that is essential for epithelial function. Interestingly, the treatment also markedly decreased the expression of genes involved in the visual cycle (*RLBP1*, *RPE65*, *RDH5*, and *RDH10*), melanin pigment synthesis (*TYR*), and phagocytosis (*MERTK*). The effect of proinflammatory cytokines on the expression of transcripts for *CDH1*, *RLBP1*, *RPE65*, and *RDH5* in ARPE-19 cells was investigated further. The cells were treated with IFN- γ (10 u/ml), TNF- α (1 ng/ml), and IL-1 β (1 ng/ml) either individually or in combination for 20 h in the absence of serum and gene expression analyzed with real-time PCR (Figure 2). TNF- α and IL-1 β when tested individually noticeably decreased the expression of the transcripts for *CDH1*, *RLBP1*, *RPE65*, and *RDH5*. IFN- γ by itself failed to decrease the expression of *CDH1* and *RLBP1* but decreased the expression of *RPE65* and *RDH5*. Combinations of two cytokines (IFN- γ + TNF- α , IFN- γ + IL-1 β , or TNF- α + IL-1 β) or all three cytokines (IFN- γ + TNF- α + IL-1 β) were highly effective in decreasing the expression of *CDH1*, *RLBP1*, *RPE65*, and *RDH5*. Thus, the interaction of proinflammatory cytokines with RPE cells may result in the decreased expression of genes necessary for RPE function.

We analyzed whether proinflammatory cytokines can decrease the expression of proteins encoded by the *CDH1* and *RLBP1* genes in ARPE-19 cells. The cells treated with a

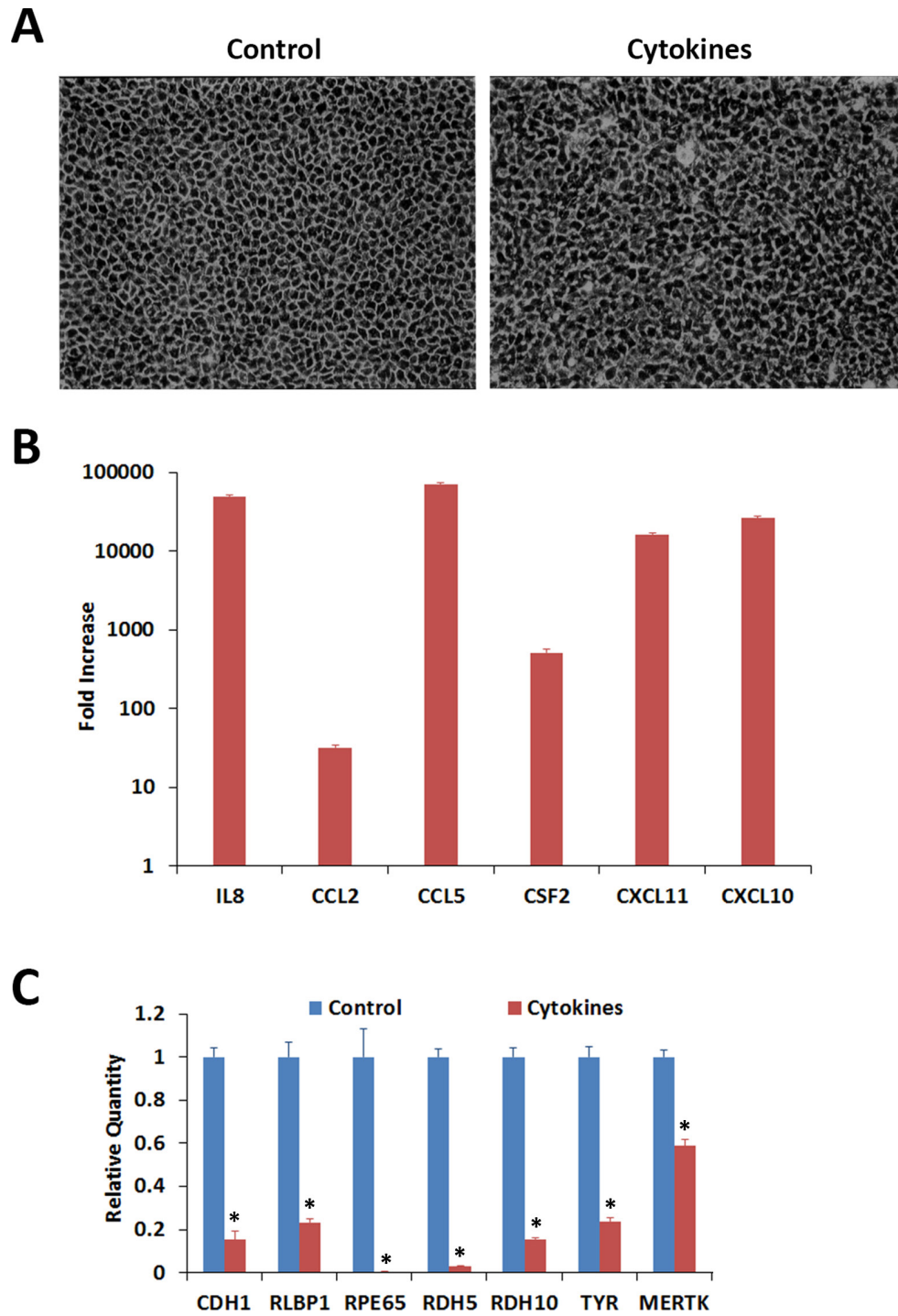


Figure 1. Effect of proinflammatory cytokines on cultured ARPE-19 cells exhibiting RPE characteristics. The cells were treated with interferon gamma (IFN- γ ; 10 u/ml), tumor necrosis factor alpha (TNF- α ; 1 ng/ml), and interleukin-1 beta (IL-1 β ; 1 ng/ml) for 20 h in the absence of serum and their gene expression analyzed with real-time PCR. **A**: Control cells exhibited typical epithelial morphology when examined with phase contrast microscopy. Cells treated with cytokines appeared distorted with thickened cell junctions. Magnification: 100X. **B**: The cells responded to the proinflammatory cytokines by highly increasing the expression of cytokines and chemokines. Gene expression in the treated and control cells was analyzed with real-time PCR. The fold increases shown are statistically significant ($p < 0.05$ compared to control, $n = 4$). **C**: The expression of the RPE characteristic genes when analyzed with real-time PCR decreased substantially in the differentiated ARPE-19 cells exposed to the proinflammatory cytokines. * $p < 0.5$ when compared to control, $n = 4$.

mixture of IFN- γ , TNF- α , and IL-1 β for 20 h under serum-free conditions, as described for the gene expression studies, were initially employed for the western blot analysis of the CDH1 and RLBP1 proteins. However, we did not observe any significant decrease in the expression of these proteins between the control and treated samples under this condition. This is not quite unexpected as proteins in a living cell are thought to turn over much more slowly compared to mRNAs. Therefore, we decided to increase the duration of the treatment of the cytokines to 4 days. However, it was necessary to use serum-containing medium in this case as the cells could not tolerate serum-free conditions for this duration. The differentiated ARPE-19 cells treated with proinflammatory cytokines for 4 days in the presence of the serum also exhibited distorted morphology similar to those treated under serum-free conditions for 20 h (data not shown). The cells were treated with a mixture of IFN- γ (10 u/ml), TNF- α

(1 ng/ml), and IL-1 β (1 ng/ml) for 4 days in the presence of serum for the western blot analysis. The results showed that the amount of CDH1 protein in the cells exposed to the cytokines was about 25% of that present in the control (Figure 3). A similar decrease was also observed when the cells were treated with ten times the indicated cytokine concentrations. Proinflammatory cytokines also decreased the expression of the RLBP1 protein in ARPE-19 cells (Figure 4). The RLBP1 protein content in the treated cells was approximately 65% of that present in the control cells.

The genes *MITF*, *TRPM1*, and *TRPM2* and microRNAs miR-204 and miR-211 are highly expressed in RPE cells, and the expression of these genes and microRNAs is necessary for maintaining RPE characteristics [30,31]. The effect of proinflammatory cytokines on the expression of these genes and microRNAs was investigated in relation to that of *RPE65* and *RLBP1*. Differentiated ARPE-19 cells were treated with

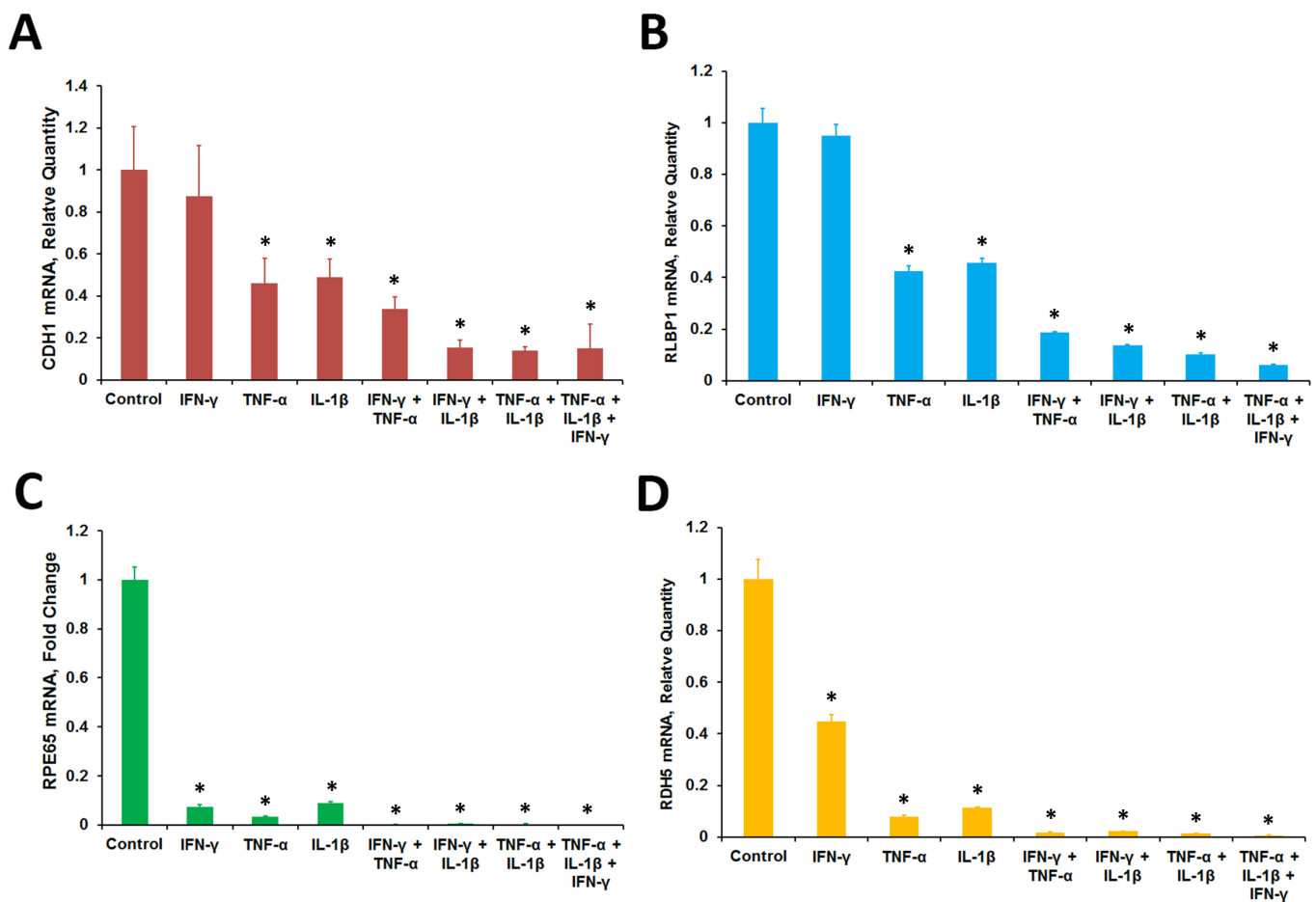


Figure 2. Proinflammatory cytokines decreased the expression of RPE characteristic genes. Differentiated ARPE-19 cells were treated with interferon gamma (IFN- γ ; 10 u/ml), tumor necrosis factor alpha (TNF- α ; 1 ng/ml), interleukin-1 beta (IL-1 β ; ng/ml) either alone or in combination for 20 h in the absence of 1% serum. The gene transcripts were analyzed with real-time PCR. A: *CDH1*. B: *RLBP1*. C: *RPE65*. D: *RDH5*. *p<0.5 when compared to control, n = 4.

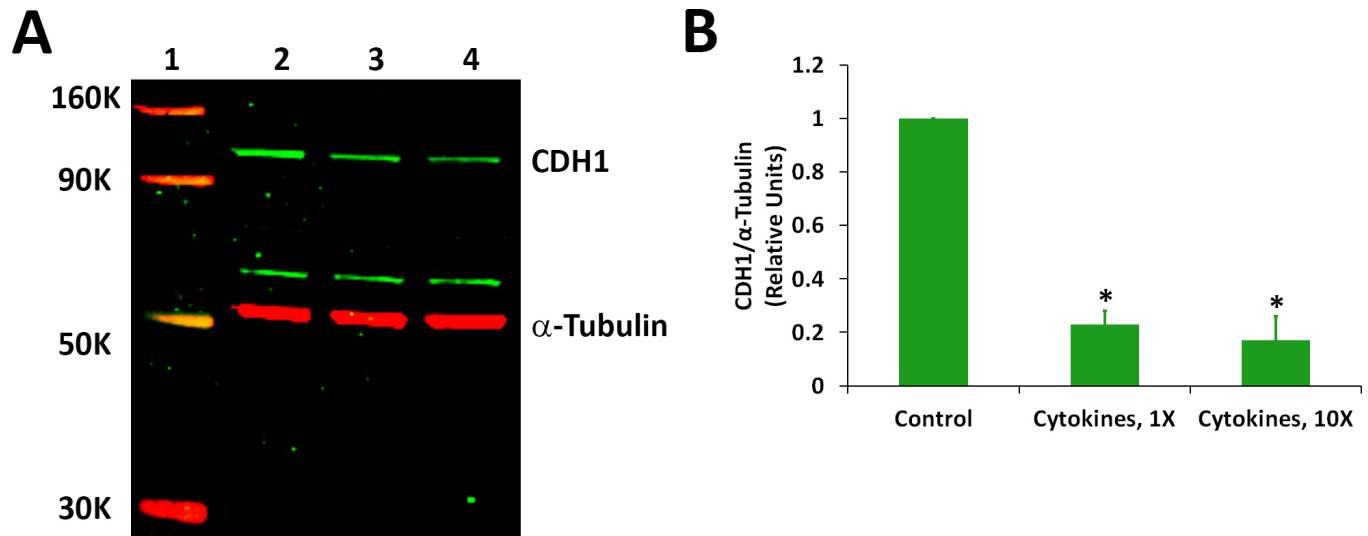


Figure 3. Proinflammatory cytokines decreased the expression of the CDH1 protein. Differentiated ARPE-19 cells were treated with the cytokines (1X = interferon gamma (IFN- γ), 10 u/ml + tumor necrosis factor alpha (TNF- α), 1 ng/ml + interleukin-1 beta (IL-1 β), 1 ng/ml) for 4 days in the presence of 1% serum. **A**: Western immunoblot analysis of CDH1 expression. Lane 1: molecular weight markers; lane 2: control; lane 3: cytokines, 1X; and lane 4: cytokines, 10X. A representative blot from three similar experiments is shown. **B**: Histogram showing CDH1 expression. The CDH1 band intensity was normalized with corresponding α -tubulin band intensity; * $p < 0.05$ when compared to control, $n = 3$.

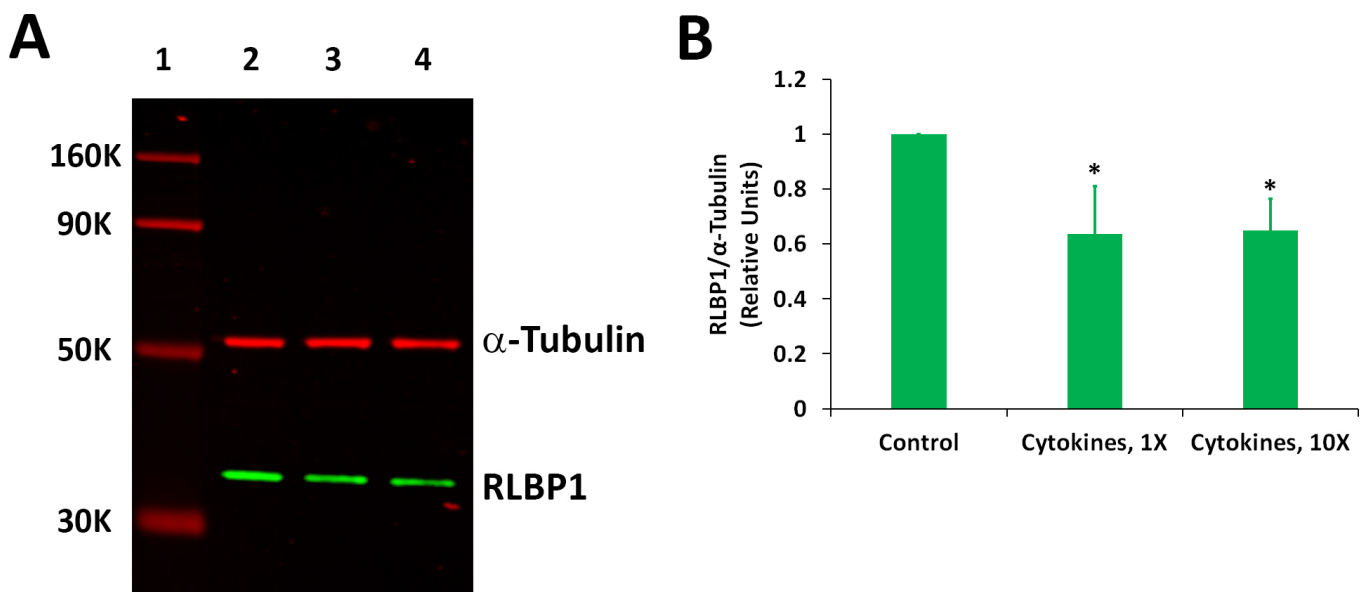


Figure 4. Proinflammatory cytokines decreased the expression of the RLBP1 protein. Differentiated ARPE-19 cells were treated with the cytokines (1X = interferon gamma (IFN- γ), 10 u/ml + tumor necrosis factor alpha (TNF- α), 1 ng/ml + interleukin-1 beta (IL-1 β), 1 ng/ml) for 4 days in the presence of 1% serum. **A**: Western immunoblot analysis of RLBP1 expression. Lane 1: molecular weight markers; lane 2: control; lane 3: cytokines, 1X; and lane 4: cytokines, 10X. A representative blot from three similar experiments is shown. **B**: Histogram showing RLBP1 expression. The RLBP1 band intensity was normalized with corresponding α -tubulin band intensity; * $p < 0.05$ when compared to control, $n = 3$.

a mixture of IFN- γ (10 u/ml), TNF- α (1 ng/ml), and IL-1 β (1 ng/ml) either for 20 h under serum-free conditions or for 4 days in the presence of 1% serum, and the gene expression was analyzed with real-time PCR (Figure 5). A decrease in the expression of the transcripts for *RPE65* and *RLBP1* was observed under the two different conditions that we employed to expose the cells to the cytokines. The expression of *MITF*, *TRPM1*, and *TRPM3* was also considerably decreased following exposure to proinflammatory cytokines under both conditions. The expression of miR-204 and miR-211 was decreased by more than 95% when the cells were exposed to the cytokines for a longer duration (4 days with serum). However, the decrease in miR-204 and miR-211 was less than 15% when the cells were treated with cytokines for a shorter time (20 h, without serum). The expression of miR-155 was considerably increased in the cells exposed to proinflammatory cytokines, and a higher increase was observed with longer exposure.

The decrease in the expression of *CDH1*, a known epithelial marker, in ARPE-19 cells on exposure to proinflammatory cytokines could indicate EMT-like changes. Therefore, we analyzed the expression of mesenchymal marker genes in cells treated with a mixture of IFN- γ (10 u/ml), TNF- α (1 ng/ml), and IL-1 β (1 ng/ml) for 20 h under serum-free conditions. The cells were also exposed to cytokine concentrations ten times higher than those indicated here. The treatment increased the expression of mesenchymal marker genes *VIM*, *CCND1*, and *CDH2* while decreasing the expression of *CDH1* (Figure 6). The expression of *ZEB1* and *SNAIL*, two transcription factors known to promote EMT, was also increased by exposing the cells to proinflammatory cytokines. Thus, a decrease in the expression of the epithelial marker gene is associated with increased expression of several mesenchymal genes.

The effect of proinflammatory cytokines on the expression of RPE characteristic genes was tested using an RPE cell culture established from adult human donor eyes by

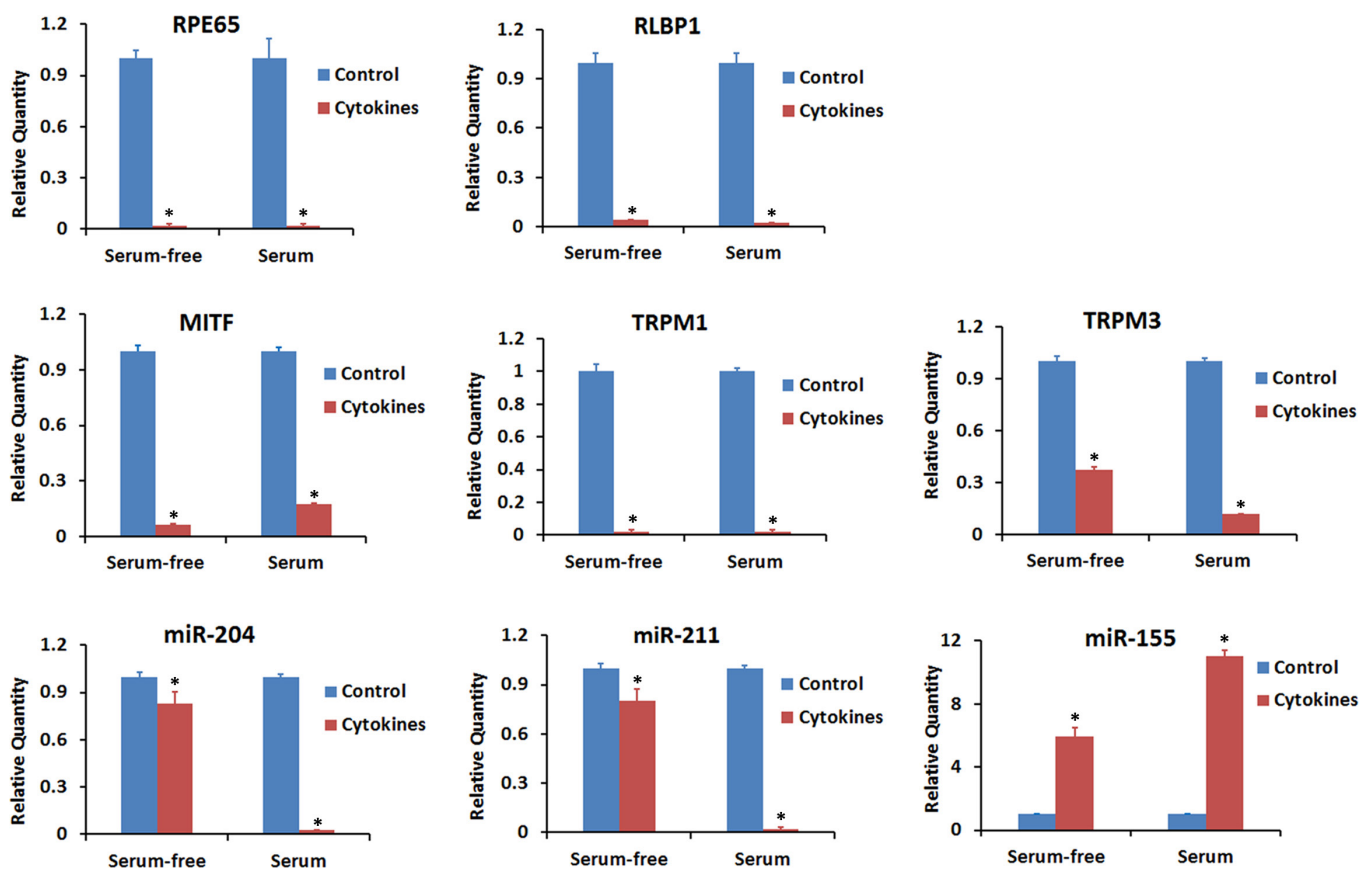


Figure 5. Proinflammatory cytokines decreased the expression of genes that control RPE function. Differentiated ARPE-19 cells were treated with the cytokines (interferon gamma (IFN- γ), 100 u/ml + tumor necrosis factor alpha (TNF- α), 10 ng/ml + interleukin-1 beta (IL-1 β), 10 ng/ml) either for 20 h under serum-free conditions or for 4 days in the presence of 1% serum. Real-time PCR was employed to analyze the expression of the indicated transcripts and miRNAs. * $p < 0.05$ when compared to respective control, $n = 3$.

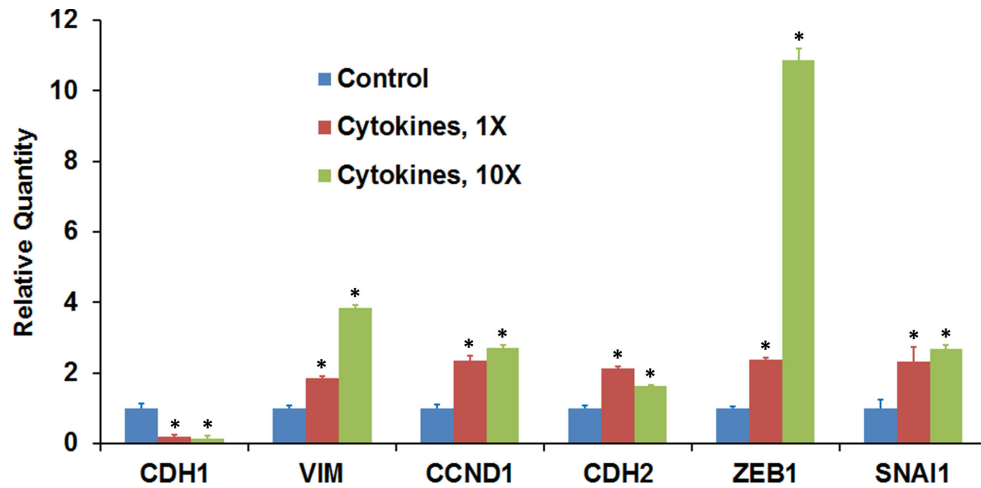


Figure 6. Proinflammatory cytokines modulate the expression of genes that regulate the EMT. Differentiated ARPE-19 cells were treated with the cytokines (1X = interferon gamma (IFN- γ), 10 u/ml + tumor necrosis factor alpha (TNF- α), 1 ng/ml + interleukin-1 beta (IL-1 β), 1 ng/ml) for 20 h in the absence of serum, and the expression transcripts for the epithelial marker gene (*CDH1*), mesenchymal

marker genes (*VIM*, *CCND1*, and *CDH2*), and transcription factor genes (*ZEB1* and *SNAI1*) was analyzed with real-time PCR. * $p < 0.5$ when compared to control, n = 4.

Nagineeni et al. [12,34]. These cells were exposed to IFN- γ (100 u/ml), IL-1 β (10 ng/ml), and TNF- α (10 ng/ml) for 20 h in the absence of serum. The expression of the transcripts for *RDH5*, *RDH10*, *MITF*, and *RPE65* was highly decreased in response to the cytokine treatment (Figure 7). Thus, these cultured RPE cells also exhibited decreased expression of RPE characteristic genes, similar to the differentiated ARPE-19 cells, in response to treatment with proinflammatory cytokines.

DISCUSSION

Inflammation is known to contribute to the pathogenesis of AMD [4,35-38]. Infiltrating lymphocytes and macrophages during ocular inflammation may facilitate RPE dysfunction

associated with AMD [5,6]. RPE cells in culture respond to the proinflammatory cytokines IFN- γ , TNF- α , and IL-1 β by increasing the expression of a large number of cytokines and chemokines [7-10]. This is associated with increased production of cytotoxic levels of reactive oxygen species in the cell [15]. In the present study, we showed that the expression of several genes important in RPE function are decreased in RPE cells exposed to IFN- γ , TNF- α , and IL-1 β . The proinflammatory cytokines decreased the expression of transcripts for visual cycle genes *RLBP1*, *RPE65*, *RDH5*, and *RDH10*; epithelial gene *CDH1*; melanogenesis gene *TYR*; and phagocytosis gene *MERTK* in treated ARPE-19 cells in culture. We also observed that the decrease in mRNA expression in the case of *CDH1* and *RLBP1* resulted in decreased expression

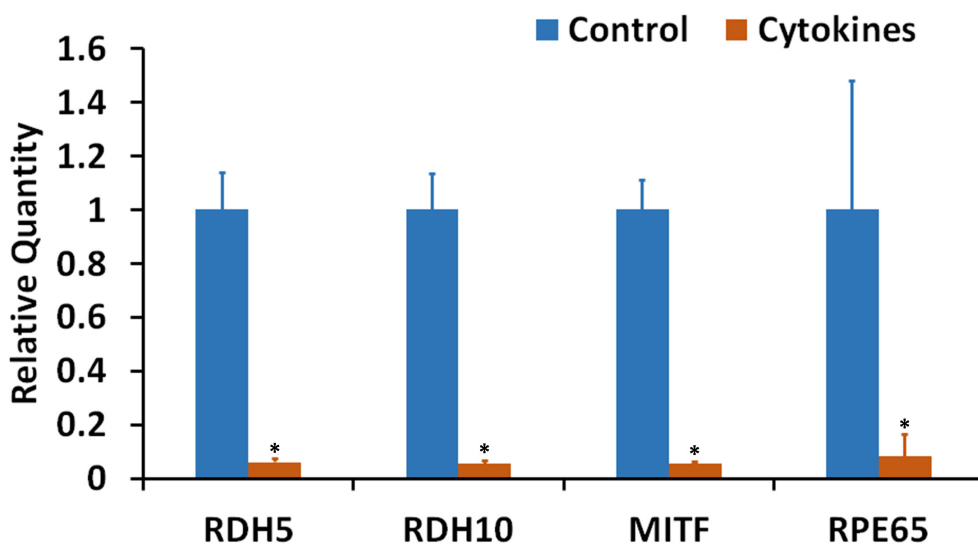


Figure 7. Proinflammatory cytokines decreased the expression of RPE characteristic genes in an RPE cell culture system established from adult human donor eyes. The cultured cells were treated with the cytokines (interferon gamma (IFN- γ), 100 u/ml + tumor necrosis factor alpha (TNF- α), 10 ng/ml + interleukin-1 beta (IL-1 β), 10 ng/ml) for 20 h under serum-free conditions. Real-time PCR analysis of the expression of mRNAs for *RDH5*, *RDH10*, *MITF*, and *RPE65* is shown. * $p < 0.05$ when compared to respective control, n = 4.

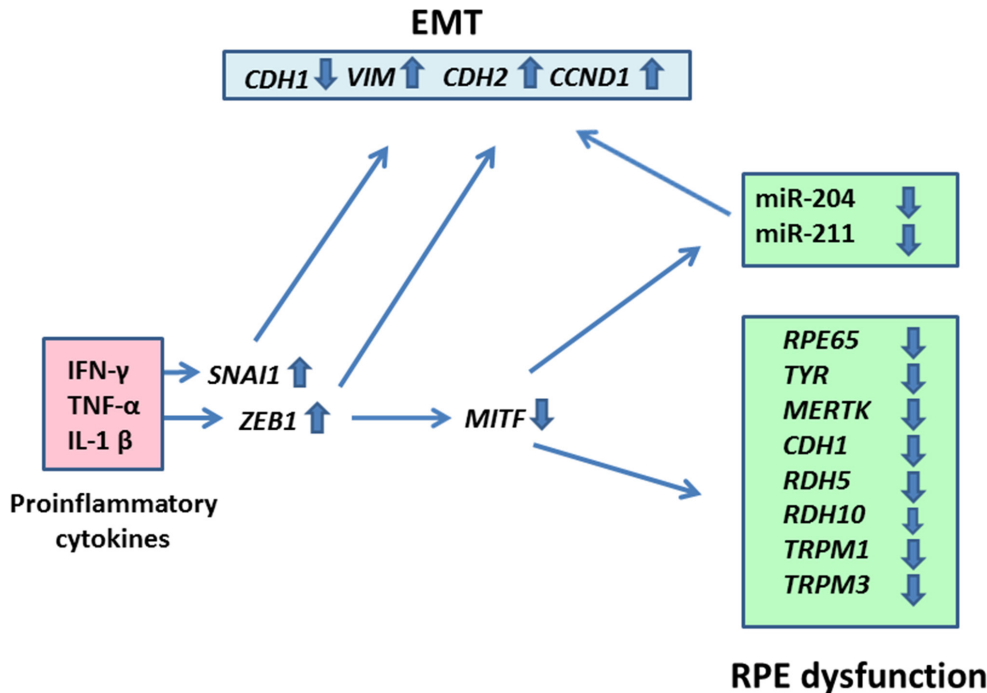


Figure 8. A schematic depiction of RPE cell dysfunction induced by IFN- γ , TNF- α , and IL-1 β . The proinflammatory cytokines increased the expression of proepithelial–mesenchymal transition (EMT) transcription factor genes *SNAI1* and *ZEB1* in the RPE cells. This could result in the downregulation of the epithelial marker gene *CDH1* and upregulation of the mesenchymal marker genes *VIM*, *CDH2*, and *CCND1*. Increased *ZEB1* expression also caused a decrease in the expression of the *MITF* gene that encodes a transcription factor that controls RPE differentiation. This, in turn, may decrease the expression of the RPE functional genes *RPE65*, *TYR*, *MERTK*, *RDH5*, *RDH10*, *TRPM1*, and *TRPM3*. The decrease in *MITF*

expression may also reduce the expression of miR-204 and miR-211, two microRNAs necessary for maintaining epithelial physiology and RPE function.

of their encoded proteins. Thus, proinflammatory cytokines have the potential to trigger RPE dysfunction by downregulating the expression of important functional genes in RPE.

We employed the adult human RPE derived ARPE-19 cell line for this study. This cell culture system developed by Dunn et al. [32] has been employed in more than 1,000 publications to study RPE function. We have previously shown that ARPE-19 cells respond to IFN- γ , TNF- α , and IL-1 β by increasing the expression of cytokines and chemokines [10]. However, ARPE-19 cells grown under normal culture conditions did not show many RPE characteristics required for the present study. Therefore, we maintained these cells in culture for the long term employing culture conditions similar to those reported by Ahmado et al. [39]. ARPE-19 cells grown under this condition for 4 months exhibited typical epithelial morphology and pigmentation, and expressed many RPE functional genes [data not shown]. These differentiated cells responded to IFN- γ , TNF- α , and IL-1 β by decreasing the expression of many RPE characteristic genes as reported in the present study. This important observation must be verified using other RPE cell culture systems. We tested the effect of proinflammatory cytokines on an RPE cell culture system established by Nagineni et al. [34] from human donor eyes. A combination of IFN- γ , TNF- α , and IL-1 β effectively decreased the expression of RPE characteristic genes *RDH5*,

RDH10, *RPE65*, and *MITF* in this cell culture system that has served as a valuable model system for studying the RPE inflammatory response [9,11-14,34,40,41]. It remains to be elucidated whether the adverse effect of inflammatory cytokines on the expression of genes important in critical RPE functions, such as the visual cycle, is applicable to in vivo situations. However, Camelo et al. [42] reported that the RPE dysfunction observed in aged or light-challenged mice is associated with a decrease in the expression of the *Rpe65* gene. Camelo et al. employed these mice model systems to study age-related retinal degeneration and found that the RPE dysfunction is triggered by the inflammatory response resulting from T lymphocyte infiltration into the retina.

We observed that proinflammatory cytokines decreased the expression of *MITF* in ARPE-19 cells. This gene encodes MITF, a basic helix–loop–helix leucine zipper transcription factor that regulates RPE development [29]. Recently, this transcription factor was reported to regulate the expression of two visual cycle genes, *RLBP1* and *RDH5*, in RPE cells [43]. Adijanto et al. [30] showed that *MITF* keeps the human fetal RPE cells in a differentiated state by upregulating the expression of miR-204 and miR-211. These two microRNAs are highly expressed in RPE cells and control RPE characteristics [31]. Fetal human RPE cells transfected with *MITF* siRNA showed decreased expression of miR-204 and miR-211

that was associated with the decreased expression of the RPE characteristic genes *RLBPI* and *RPE65* [30]. Antimers to miR-204 and miR-211 also decreased the expression of visual cycle genes [31]. *MITF* also regulated the expression of *TRPM1* and *TRPM3* along with that of miR-204 and miR-211 [30]. The present results clearly showed that the decrease in the expression of *RLBPI*, *RPE65*, and other RPE characteristic genes in ARPE-19 cells exposed to IFN- γ , TNF- α , and IL-1 β was associated with a decrease in the expression of *MITF* along with that of miR-204, miR-211, *TRPM1*, and *TRPM3*. Therefore, downregulation of *MITF* may potentially mediate the adverse effect of proinflammatory cytokines on the expression of RPE characteristic genes due to the inherent ability of *MITF* to regulate miR-204/211 expression.

Morphometric analysis of RPE from donor eyes has indicated that AMD progression is associated with the presence of RPE cells with irregular size and shape [44]. Interestingly, we observed a disruption in the typical epithelial morphology of cultured ARPE-19 cells on exposure to the proinflammatory cytokines IFN- γ , TNF- α , and IL-1 β . This disruption was associated with gene expression changes indicative of EMT. The decrease in the expression of the epithelial marker gene *CDH1* in cells treated with the proinflammatory cytokines was accompanied by increased expression of the mesenchymal marker genes *VIM*, *CDH2*, and *CCND1*. This was associated with an increase in the expression of *ZEB1* and *SNAIL*. These two transcription factor genes are known promoters of EMT [45-48]. Inhibiting the expression of miR-204 and miR-211 has been shown to induce EMT-like changes in human fetal RPE cells [30,31]. In addition, miR-204 and miR-211 are known to inhibit EMT in cancer cells [49,50]. Liu et al. [51] reported that *Zeb1* downregulated *Mitf* expression in mouse RPE cells causing EMT-like changes and the loss of RPE characteristics. We observed in the ARPE-19 cells that the increase in *ZEB1* expression is associated with decreases in the expression of *MITF*, miR-204, and miR-211. Thus, *ZEB1* may mediate the proinflammatory cytokine induced RPE dysfunction by decreasing the expression of *MITF* and miR-204/miR-211. The possibility that apoptosis plays a role in the proinflammatory cytokine-induced RPE dysfunction that we observed remains to be elucidated. A review of the literature showed that the study of apoptosis in RPE cells in response to treatment with proinflammatory cytokines is limited. No apoptosis was detected when human RPE cells in culture were exposed to TNF- α (20 ng/ml), IL-1 β (2 ng/ml), or lipopolysaccharide (1,000 ng/ml) for 24 h [52], but the effect of a combination of all these proinflammatory agents on apoptosis in human RPE cells was not tested. However, human retinal capillary endothelial cells treated with a mixture of 10 ng/ml TNF- α , 10 ng/ml

IL-1 β , and 50 units/ml IFN- γ for 48 h were reported to exhibit apoptosis in about 14% cells compared to about 7% in the case of untreated cells [53].

In summary, the present results showed that the proinflammatory cytokines IFN- γ , TNF- α , and IL-1 β decrease the expression of key genes involved in visual cycle, epithelial morphology, melanogenesis, and phagocytosis in cultured ARPE-19 RPE cells (Figure 8). Proinflammatory cytokines may elicit this effect by increasing the expression of *ZEB1* and *SNAIL*, pro-EMT transcription factor genes. The increase in *ZEB1* expression may, in turn, decrease the expression of *MITF*, a key regulator of RPE differentiation, causing a decrease in the expression of miR-204 and miR-211, two key microRNAs regulating RPE-characteristics. Thus, a decrease in the expression of critical functional genes due to targeting of RPE cells by IFN- γ , TNF- α , and IL-1 β secreted by infiltrating inflammatory cells may potentially contribute to the RPE dysfunction implicated in AMD pathology.

APPENDIX 1. STR ANALYSIS.

To access the data, click or select the words “[Appendix 1.](#)”

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