

Activation of the JAK–STAT intracellular pathway in human retinal pigment epithelial cell line ARPE-19

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Background: Retinal pigment epithelial cells constitute an important component of the blood–retinal barrier and play a pivotal role in the development of age-related macular degeneration (AMD). Understanding the underlying molecular mechanisms is a prerequisite for developing therapeutic strategies for the treatment of this disease. This study investigated cytokine-induced changes of the JAK–STAT (Janus tyrosine kinase–signal transducers and activators of transcription) signaling pathway in the human retinal pigment epithelial cell line ARPE-19 and potential implications for AMD.

Methods: Electromobility shift assay, immunofluorescence staining, and flow cytometry were used to evaluate the JAK–STAT pathway in the ARPE-19 cell line.

Results: We examined cytokine-induced expression of STATs in the ARPE-19 cell line. Strong STAT1 activation determined by electromobility shift assay and flow cytometry was demonstrated upon exposure to interferon- γ . Interferon- α upregulated STAT1, STAT2, and STAT3 in ARPE-19 cells, while interleukin-6 (IL-6) and IL-4 activated STAT3 and STAT6, respectively. Confocal microscopy identified the nuclear translocation of the STAT proteins. Flow cytometry analysis demonstrated the upregulation of major histocompatibility complex molecules on ARPE-19 cells as responses to interferon- α and interferon- γ .

Conclusion: Our data demonstrate the upregulation of members of the JAK–STAT signaling pathway in the ARPE-19 cells upon stimulation with interferon- α , interferon- γ , IL-4, and IL-6. We present a model for these signaling pathways potentially relevant for AMD, which may prove useful for screening of AMD therapeutics.

Keywords: retinal pigment epithelium, ARPE-19, age-related macular degeneration, JAK–STAT pathway

Introduction

Age-related macular degeneration (AMD) is a degenerative disease of the macula leading to irreversible visual impairment and blindness, affecting nearly 50 million people worldwide.^{1,2} Clinically and histologically, AMD can be classified into two major subtypes: dry and wet AMD. Dry AMD is characterized by macular changes consisting of abnormalities of the retinal pigment epithelium (RPE), drusen, and photoreceptor dysfunction/degeneration. The key feature of wet AMD is choroidal neovascularization (CNV), which is described as the growth of new blood vessels from the choroid into the region underlying the RPE.³

The etiology of AMD is unclear but includes hereditary components.^{4–7} Earlier stage of AMD treatment is limited to risk factor management. Major changes during AMD disease development occur in the outer retina, affecting the photoreceptors, the RPE, and the Bruch's membrane.

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Recent studies have shown that the immune system plays an important role in the development of AMD.⁸ RPE cells constitute a specialized phagocytic system similar to that of macrophages. In addition, the RPE can modulate immune response through proinflammatory cytokine production, including interleukin-1 β (IL-1 β), IL-6, and tumor necrosis factor- α .⁹ RPE cells also produce pigment epithelial growth factor, which decreases and increases IL-12 and IL-10 production, respectively.¹⁰ RPE plays a critical role in photoreceptor renewal since defects lead to photoreceptor death and retinal degeneration.^{11–14} Although it is known that dysfunctions of RPE cells are the major cause of degenerative diseases including AMD, exact molecular mechanisms of these processes remain to be elucidated.^{11,15}

The JAK–STAT (Janus tyrosine kinase–signal transducers and activators of transcription) pathway has been implicated in the control of cell progression, cell survival, and cell death. STATs are a class of transcription factors activated upon tyrosine phosphorylation. This cytoplasmic protein family functions in signaling and transcription factor regulation, participating in normal cellular responses to cytokines and growth factors.¹⁶ The JAK–STAT pathway can lead to angiogenesis by triggering angiogenic factor production, including vascular endothelial growth factor and matrix metalloproteinase.^{17–19}

Our recent research has shown abnormal STAT3 activation in choroidal neovascular membranes of AMD patients.²⁰ Therefore, we chose to focus on the expression and regulation of the JAK–STAT pathway using the ARPE-19 cell line (as in vitro model for RPE cells), a signaling cascade not extensively studied in the context of AMD pathogenesis. Our results demonstrate STAT3-specific upregulation by interferon- α (IFN- α) and IL-6. We also found a strong activation of STAT1 induced by IFN- γ . Furthermore, in AMD where multiple growth factor pathways are involved, STAT proteins, particularly STAT3 (because of its central regulatory role), represent an attractive development target for potentially effective AMD therapies.

Materials and methods

Chemicals and reagents

Human recombinant IFN- α (Roferon-A) was obtained from Roche Pharma AG (Reinach, Switzerland). Human recombinant IFN- γ , IL-6, and IL-4 were from R&D Systems (Minneapolis, MN). Antibodies against STAT1, STAT2, STAT3, and STAT6 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Primary rabbit polyclonal antibodies against phosphorylated human STAT proteins for immunofluorescence

experiments were from Cell Signaling (Danvers, MA). Recognized phosphorylated tyrosines on the respective STAT proteins can be summarized as follows: epitope Y701 on STAT1, epitope Y705 on STAT3, and epitope Y641 on STAT6. Monoclonal antibodies against human cytokeratins 8 and 18 were from Progen Biotechnik (Heidelberg, Germany). Secondary antibodies conjugated with Alexa Fluor 488 (goat anti-mouse) and Alexa Fluor 555 (goat anti-rabbit) were from Invitrogen (Carlsbad, CA). Alexa Fluor 488 mouse anti-human phospho-STAT1 (epitope Y701), anti-phospho-STAT3 (epitope Y705), anti-phospho-STAT6 (epitope Y641), fluorescein isothiocyanate (FITC)-conjugated mouse anti-human monoclonal antibodies against major histocompatibility complex (MHC) class I human leukocyte antigen-A,B,C (HLA-A,B,C), FITC-conjugated mouse anti-human HLA-DR and HLA-DQ, as well as isotype-matched IgG for flow cytometry experiments, were purchased from BD Biosciences (San Jose, CA). All cell culture reagents were obtained from Invitrogen. All cell culture plasticware were purchased from TPP (Trasadingen, Switzerland).

Cell lines

The ARPE-19, Colo 205, Daudi, HepG2, and THP-1 cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and cultivated according to the ATCC recommendations. Briefly, ARPE-19 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham F-12 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 0.1 mg/mL penicillin, and 100 U/mL of streptomycin in 5% CO₂/95% air at 37°C.

HLA modulation assay

Surface expression of MHC class I and class II was monitored by flow cytometry, using a FITC-labeled antibody for a monomorphic determinant of HLA-ABC heavy chain, HLA-DR, HLA-DQ, or control, isotype-matched reagent in cells cultured for 48 h in the presence or absence of IFN- α and IFN- γ . Mean fluorescence intensity of stained cells was measured and analyzed using a FACSCalibur analyzer and the CellQuest Pro Software (Becton-Dickinson, San Jose, CA).

Proliferation assay

Cell proliferation was evaluated using cultures of 10,000 cells/well in 24-well plates in the absence or presence of 500 U/mL of IFN- α for the indicated time period as it was previously described.^{21,22} Cells were trypsinized, and cell numbers were determined by live cell counting using a hemocytometer and 0.4% trypan blue reagent.

Electromobility shift assay

To examine the effects of cytokines on the activation of STAT proteins, electromobility shift assay (EMSA) was performed as it was previously described.²³ Briefly, cytokine treated or untreated cells (negative control) were lysed in low salt buffer (20 mM HEPES, pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.2% NP-40, 10% glycerol, 0.1 mM Na₃VO₄, 1 mM PMSF, 1 mM DTT, 2 µg/mL aprotinin, 1 µg/mL leupeptin, and 1 µg/mL pepstatin) at 4°C for 10 min. After centrifugation for 1 min at full speed, the supernatant (cytoplasmic extract) was removed, and the pelleted nuclei were incubated in a high salt buffer, same as low salt, but with 420 mM NaCl and 20% glycerol for 30 min at 4°C. Samples were cleared by centrifugation at 12000×g at 4°C. The supernatant was designated as the nuclear extract. The protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Bovine serum albumin was used as a standard. Nuclear extracts were stored at -70°C. For the EMSA, nuclear extracts were incubated for 20 min at room temperature in 20 mM HEPES, pH 7.9, 4% Ficoll, 1 mM MgCl₂, 40 mM KCl, 0.1 mM EGTA, 0.5 mM DTT, 160 mg/mL Poly(dI-dC) × Poly(dI-dC) with 1 ng of ³²P-labeled oligonucleotides. Samples were separated on a 5% nondenaturing polyacrylamide gel at 400 V for 4 h at 4°C. The following four oligonucleotides corresponding to STAT response element sequences were used in this work: O15: 5'-CTAGGAAAGGGAAA CCGAAACTGAAGC-3', binding to STAT1-STAT2-p48 complex; βCas: 5'-ATCGATTTCTAGGAATTCAATCC-3', containing the sequence of the β-casein promoter binding to STAT1, STAT5, and STAT6 homodimers; Ce: 5'-CACTTCCCAAGAACAGA-3', binding to STAT6 homodimers; and M67: 5'-GATCCATTTCCCGTAAATCAT-3', binding to STAT1, STAT1-3, STAT3, and STAT4 homo-/heterodimers. For supershift experiments, 1 µL of antibody was added to the samples as indicated in figure legends and incubated for 20 min. For oligonucleotide competition assays, a 50-fold excess of unlabeled oligonucleotide was added to the incubation mixture.

Immunofluorescence staining

ARPE-19 cells were cultured on cover glasses in 24-well tissue culture dishes. Cells were left untreated (negative control) or incubated with appropriate cytokines for 20 min at 37°C. Then cells were fixed in methanol/acetone (1:1) for 20 min on ice and were further incubated with 10% goat serum in PBS for 1 h at room temperature. Anti-phospho-STAT and anti-cytokeratin antibodies were used as primary antibodies.

Goat-anti-mouse Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 555 were used as secondary antibodies, and the preparations were examined on confocal laser scanning microscope (Zeiss LSM 510; Carl Zeiss MicroImaging GmbH, Jena, Germany). Microscopic images were collected and analyzed using proprietary image acquisition LSM software (version 3.2; Carl Zeiss MicroImaging GmbH).

Analysis of activated STAT proteins by flow cytometry

ARPE-19 cells were incubated for 20 min with appropriate cytokines or left untreated as a negative control. Cells were then fixed with 2% paraformaldehyde for 20 min and then permeabilized in 90% methanol for 30 min. Then cells were stained for 1 h with Alexa Fluor 488-conjugated anti-phospho-STAT1, anti-phospho-STAT3, or anti-phospho-STAT6 antibodies as it is indicated in figure legends and collected on FACSCalibur cytometer with CellQuest Pro software (Becton Dickinson).

Results

Effect of IFN-α and IFN-γ on HLA modulation

We first tested the effects of IFN-α and IFN-γ on HLA-expression on ARPE-19 cells by flow cytometry. We found that untreated ARPE-19 cells express high levels of MHC class I (Figure 1A, B, gray histograms). When the cells were treated with 500 U/mL of IFN-α or with 500 U/mL of IFN-γ for 48 h, we observed the upregulation of MHC class I expression (Figure 1A, B, white histograms). Moreover, 48-h treatment with 500 U/mL of IFN-γ induced the expression of MHC class II molecule HLA-DR (Figure 1C, white histogram), whereas IL-4 and IL-6 treatments did not have any effect on the expression of MHC molecules on ARPE-19 cells (data are not shown).

Inhibition of proliferation by IFN-α

The effect of IFN-α on the proliferation of ARPE-19 cells was analyzed by quantitation of cell viability using trypan blue staining. The percentage of dead cells in cultures with and without IFN-α did not exceed 10%. As it is shown in Figure 2, exogenously added human IFN-α at a concentration of 500 U/mL suppressed the proliferation of ARPE-19 cells. In cultures without IFN-α, the cell number increased, whereas in the presence of IFN-α, cell proliferation was inhibited.

Intracellular signaling

We further studied the potential effects of IFN-α, IFN-γ, IL-6, and IL-4 on the activation of STAT proteins in ARPE-19 cells.

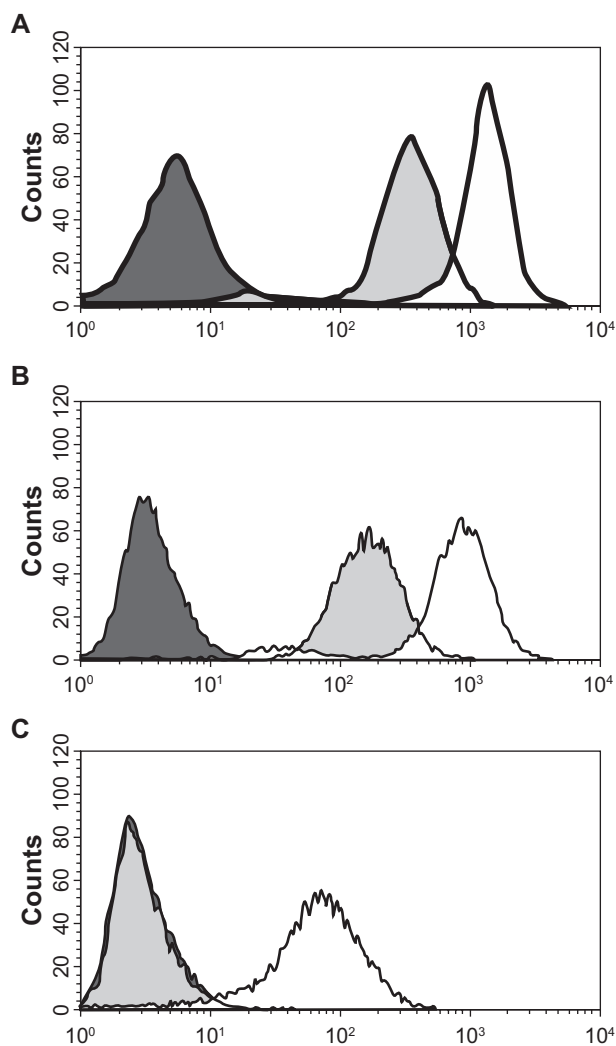


Figure 1 Interferon-induced MHC modulation in ARPE-19 cells. **A)** IFN- α -induced MHC class I upregulation. Black histogram: isotype-matched control; gray histogram: MHC class I expression in ARPE-19 cells; white histogram: cells treated for 48 h with 500 U/mL of IFN- α . **B)** IFN- γ -induced MHC class I upregulation. Black histogram: isotype-matched control; gray histogram: MHC class I expression in ARPE-19 cells; white histogram: cells treated for 48 h with 500 U/mL of IFN- γ . **C)** IFN- γ -induced MHC class II upregulation. Black histogram: isotype-matched control; gray histogram: HLA-DR expression; white histogram: cells treated for 48 h with 500 U/mL of IFN- γ . Black histogram (negative control) is fully hidden behind the gray histogram.

Toward this end, the EMSA was carried out on nuclear extracts and probed with four oligonucleotides, O15, M67, β Cas, and C ϵ , that recognize all known STAT proteins. Untreated cells were used as negative controls, and for positive controls, Daudi, HepG2, THP-1, and Colo 205 cell lines were treated with the appropriate cytokines. Representative results are shown in Figures 3–6. Stimulation with IFN- α activated a STAT1-STAT2-p48 complex that binds to the O15 probe (Figure 3). All positive nuclear extracts of stimulated cells have been verified by supershift experiments using specific anti-STAT antibodies (Figures 3, 4, and 6). The supershift experiment (Figure 3) showed that the O15 band disappears

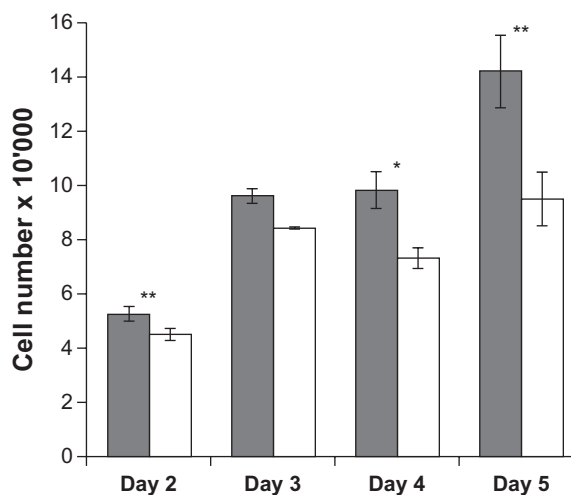


Figure 2 IFN- α -induced inhibition of proliferation in ARPE-19 cells. Proliferation of cells over a 5-day period in the presence (white bars) or absence (gray bars) of 500 U/mL of IFN- α . Values are cell number means \pm SEM of three independent sets of experiments with $n = 9$ (day 2), 3 (day 3), 6 (day 4), 8 (day 5) data points. **Notes:** * $P = 0.025$; ** $P < 0.001$ by Student's t -test comparing INF- α -treated cells with untreated controls.

after incubation with antibodies specific for STAT1 and STAT2, whereas antibodies specific for STAT3 had no effect. When tested on β Cas probe, all nuclear extracts stimulated with human IL-4 showed activated STAT6 (Figure 4). The STAT6:6 shift disappeared after addition of anti-STAT6 antibodies (Figure 4). The STAT6:6 homodimers were also visible when the nuclear extracts were tested on the C ϵ probe derived from the immunoglobulin heavy chain ϵ gene promoter (Figure 5). To confirm the specificity of the induced gel shifts, an oligonucleotide competition assay was performed. All induced complexes were competed by the excess of unlabelled oligonucleotides. One example is shown in Figure 5: the STAT6 shift bound to the C ϵ disappeared after addition of 50-fold excess of cold C ϵ probe.

The ARPE-19 nuclear extracts were also tested on M67 probe (Figure 6). As a positive control for STAT1, we used IFN- γ -stimulated Colo 205 cells (Figure 6, both panels), and as a STAT3 positive control, we used HepG2 cells stimulated with IL-6 (Figure 6, both panels). A very strong activation of STAT1:1 homodimer shift was observed after IFN- γ and IFN- α stimulation (Figure 6). Furthermore, IL-6 activated STAT3 in the ARPE-19 cells (Figure 6, the upper panel). The STAT3 shift generated by ARPE-19 cells is comparable with the STAT3 shift generated by our positive control cell line HepG2. After stimulation with IFN- α , the STAT1:STAT3 complex appeared as a band of intermediate intensity migrating slightly slower than STAT1:1 homodimers (Figure 6, on the lower panel). After

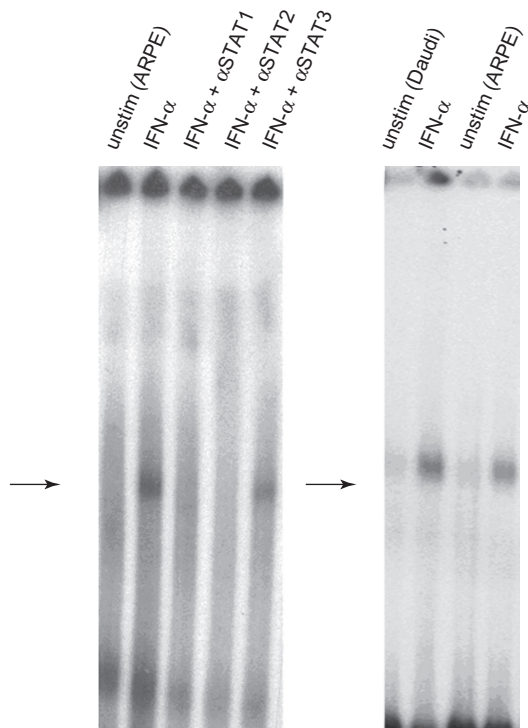


Figure 3 EMSA analysis of nuclear extracts from ARPE-19 cells using the O15 probe. ARPE-19 cells were unstimulated (negative control) or stimulated with 500 U/mL IFN- α for 20 min. Supershift experiment: IFN- α -treated cells were incubated with antisera specific for STAT1 (IFN- α + α STAT1), with antisera specific for STAT2 (IFN- α + α STAT2), or with antisera specific for STAT3 (IFN- α + α STAT3). In control experiment, the nuclear extracts of ARPE-19 cells were compared with nuclear extracts of Daudi cells (untreated and treated with 500 U/mL of IFN- α for 20 min), which were used as a positive probe for the O15 probe.

IFN- α stimulation, STAT3:3 homodimer shift was visible on overexposed films only as a faint, but reproducible band. Both STAT1:1 homodimers and STAT1:3 heterodimers could be supershifted with antibodies specific for STAT1 (Figure 6, lower panel). STAT3:3 homodimers and STAT1:3 heterodimers could be supershifted with antibodies specific for STAT3, whereas STAT2-specific antisera had no effect (Figure 6, lower panel). Untreated cells revealed no activation of STAT proteins (Figure 6, on both panels).

In summary, our gel shift data showed that the intracellular STAT pathways are activated in ARPE-19 cells in response to cytokine treatment, with IFN- γ activating STAT1 and IFN- α activating STAT1, STAT2, and STAT3. Similarly, IL-6 activated STAT3, and IL-4 activated STAT6.

Immunofluorescence staining

In the next set of experiments, we confirmed activated STAT protein nuclear localization by immunofluorescence. The ARPE-19 cells were cultured on cover glasses. After stimulation with appropriate cytokines, methanol/acetone fixed cells were probed with antibodies that react

only with phosphorylated STAT1, STAT3, or STAT6 proteins. The immunofluorescence assay confirmed the data obtained from the gel shift experiments. Representative results of the nuclear expression of STAT1 and STAT3 proteins are shown in Figure 7. ARPE-19 cells were left untreated (left panel) or were treated with IFN- γ (500 U/mL) for 20 min (Figure 7A, right panel). Strong nuclear localization of STAT1 was observed in the cells after treatment with IFN- γ . The cytoplasm was stained with anti-cytokeratin 8 antibodies (Figure 7A). Nuclear translocation of phosphorylated STAT3 protein was observed after incubation of ARPE-19 cells with 10 ng/mL of IL-6. The cytoplasm was stained with anti-cytokeratin 18 antibodies (Figure 7B). Strong phospho-STAT6 translocation was observed after incubation with IL-4. (Data are not shown.) IFN- α induced the nuclear translocation of both STAT1 and STAT3 proteins. (Data are not shown.)

Expression of STAT molecules on ARPE cells

To further confirm the above observations, the expression of phosphorylated STAT proteins was measured by flow

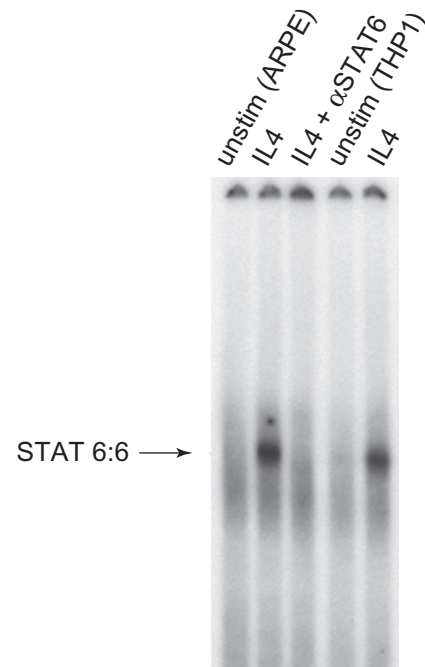


Figure 4 EMSA analysis of nuclear extracts from ARPE-19 cells using the β Cas probe. Nuclear extracts from unstimulated ARPE-19 cells (negative control) were compared with nuclear extracts of ARPE-19 cells treated with IL-4. Supershift experiment: IL-4-treated nuclear extracts were incubated with antisera specific for STAT6 (IL-4 + α STAT6). THP-1 cells, untreated or IL-4 treated, were used as STAT6 positive control. Both ARPE-19 and THP-1 cells were treated with 10 ng/mL of IL-4 for 20 min.

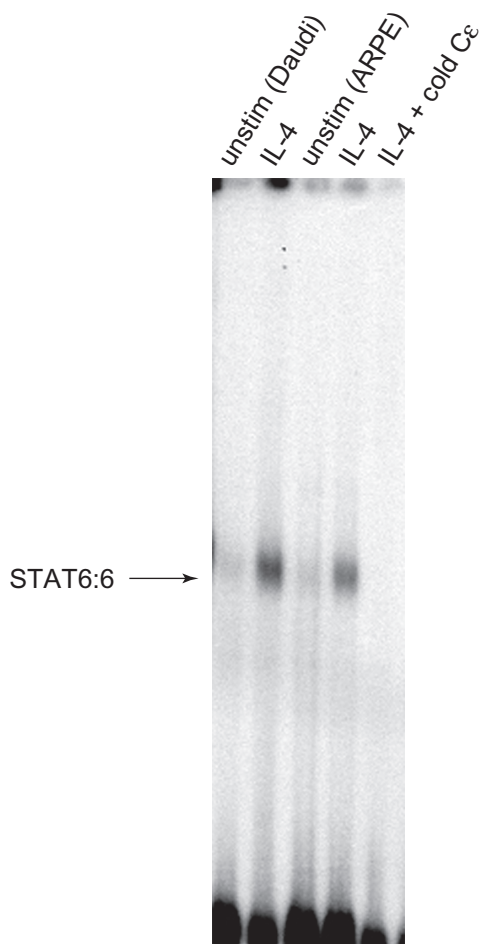


Figure 5 EMSA analysis of nuclear extracts from ARPE-19 cells using the C ϵ probe. Daudi cells stimulated with 10 ng/mL of IL-4 for 20 min were used as a positive control for the C ϵ probe. ARPE-19 cells were unstimulated (negative control) or treated with IL-4. To confirm the specificity of the induced gel shift, the ARPE-19 nuclear extracts treated with IL-4 were incubated with 50-fold excess of the cold C ϵ probe.

cytometry after incubation with IFN- α , IFN γ , IL-4, or IL-6. After 20-min incubation with the appropriate cytokines, the ARPE-19 cells were fixed with 2% paraformaldehyde and treated with 90% methanol. The samples were incubated with anti-phospho-STAT1, STAT3, or STAT6 antibodies (Figure 8). A very strong anti-phospho-STAT1 shift was observed in response to IFN- γ (Figure 8A). Representative results of IFN- α -induced activation of STAT1 and STAT3 are shown in Figure 8B, C. IFN- α induced a strong anti-phospho-STAT1 (Figure 8B) and anti-phospho-STAT3 (Figure 8C) shift. When the cells were probed with anti-phospho-STAT3 antibody after incubation with IL-6 (Figure 8D), the shift was less strong, but still significant (mean fluorescence values of untreated and treated cells were 3.4 and 13.5, respectively). The treatment with IL-4 induced a very strong STAT6 shift as shown in Figure 8E.

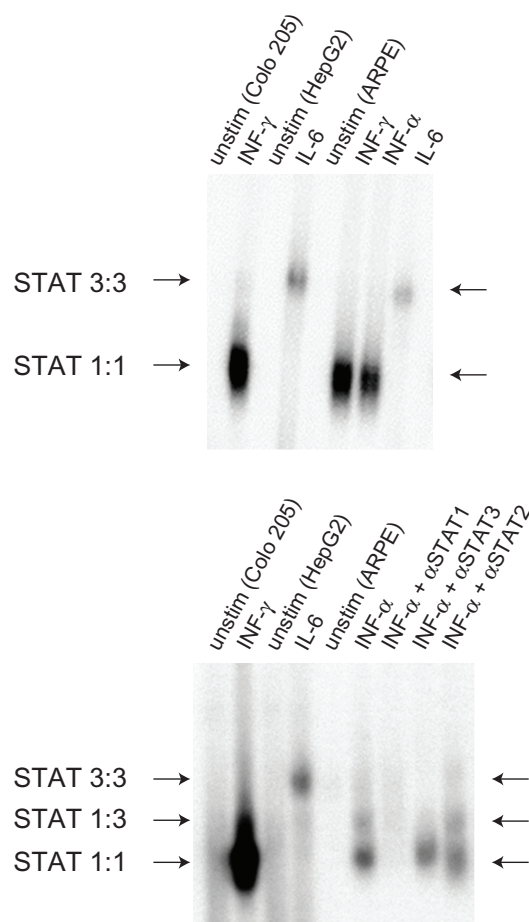


Figure 6 EMSA analysis of nuclear extracts from ARPE-19 cells using the M67 probe. Upper panel: Colo 205 cells were left untreated or stimulated with 500 U/mL of IFN- γ for 20 min (STAT1 positive control). HepG2 cells stimulated with 10 ng/mL of IL-6 were used as STAT3 positive control. ARPE-19 cell extracts were either untreated or treated with 500 U/mL of IFN- γ or with 500 U/mL of IFN- α or with 10 ng/mL of IL-6 for 20 min. Lower panel: supershift experiment. The same controls as shown in the upper panel were used as positive control for STAT1 and STAT3 activation. ARPE-19 cells were left untreated or stimulated with 500 U/mL of IFN- α . IFN- α -treated ARPE-19 cells were incubated with antisera specific for STAT1 (IFN- α + α STAT1), with antisera specific for STAT3 (IFN- α + α STAT3), and with antisera specific for STAT2 (IFN- α + α STAT2).

Discussion

AMD is a leading cause of severe visual impairment and blindness. The immune system can play an important role in AMD pathogenesis.^{8,24–31} Although the eye is an immunologically privileged organ, induction of cytokines in RPE cells could be an early trigger of the inflammatory response, such as is known to occur after viral or bacterial infection.³² RPE cells have been shown to secrete inflammatory and chemoattractant cytokines, including IL-6, IL-8, monocyte chemotactic protein, IFN- γ , and tumor necrosis factor alpha.^{33–38} The biological effects of type I and type 2 IFNs are known to be mediated through activation of JAK–STAT pathways, specifically JAK1, STAT1, and STAT2.³⁹

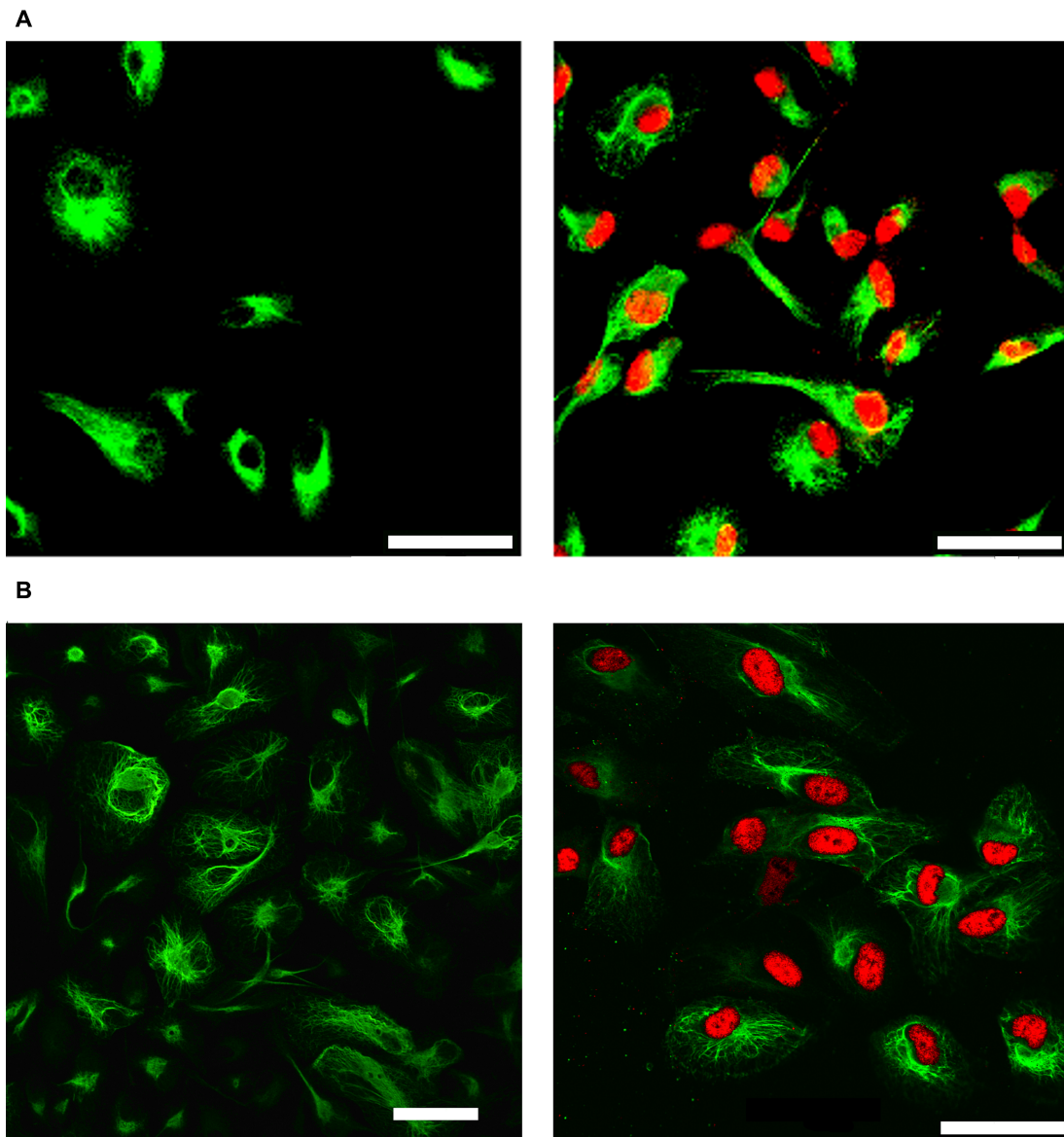


Figure 7 Confocal immunofluorescence analysis of phospho-STAT proteins in ARPE-19 cells. **A)** Phospho-STAT1 staining. ARPE-19 cells were untreated (left panel) or treated with 500 U/mL of IFN- γ for 20 min (right panel). Both untreated and IFN- γ -treated cells were incubated with anti-cytokeratin 8 (green) and anti-phospho-STAT1 (red) antibodies. When ARPE-19 cells were stimulated with IFN- γ , there was intense nuclear immunostaining of phospho-STAT1 (right panel). Magnification 40 \times . Scale bar 50 μ m. **B)** Phospho-STAT3 staining ARPE-19 cells were untreated (left panel) or treated with 10 ng/mL of IL-6 for 20 min (right panel). The cells were incubated with anti-cytokeratin 18 (green) and anti-phospho-STAT3 (red) antibodies. In cells stimulated with IL-6, intense nuclear immunostaining of phospho-STAT3 was observed (right panel). Magnification 40 \times . Scale bar 50 μ m.

In our previous study,²⁰ we demonstrated the presence of activated STAT3 proteins in CNV membranes of AMD patients. Our interest in STAT3 is explained by the fact that STAT3 is a key signaling molecule for multiple cytokines and growth factor receptors (eg, members of IFN, IL-2, and gp130 family). Consistently, the pharmacological blockade of the STAT3 pathway has suppressed CNV.⁴⁰

In this study, we investigated the activation of JAK-STAT signaling pathway in the ARPE-19 cell model and demonstrated that not only the STAT3 protein, but also other STAT

molecules including STAT1, STAT2, and STAT6 are activated in ARPE-19 cells in response to IFN- α , IFN- γ , IL-6, and IL-4. The individual activation patterns were cytokine-specific (Table 1). Taken together, our data indicate that IFN- γ activated STAT1, IFN- α activated STAT1, STAT2, and STAT3. IL-6 and IL-4 activated STAT3 and STAT6, respectively. IFN- α induced a strong activation of STAT1, which was measured by EMSA and FACS. Interestingly, the activation of STAT3 after incubation with IFN- α was weak in EMSA, but strong in FACS experiments (Figures 6 and 8). In contrast, IL-6 induced

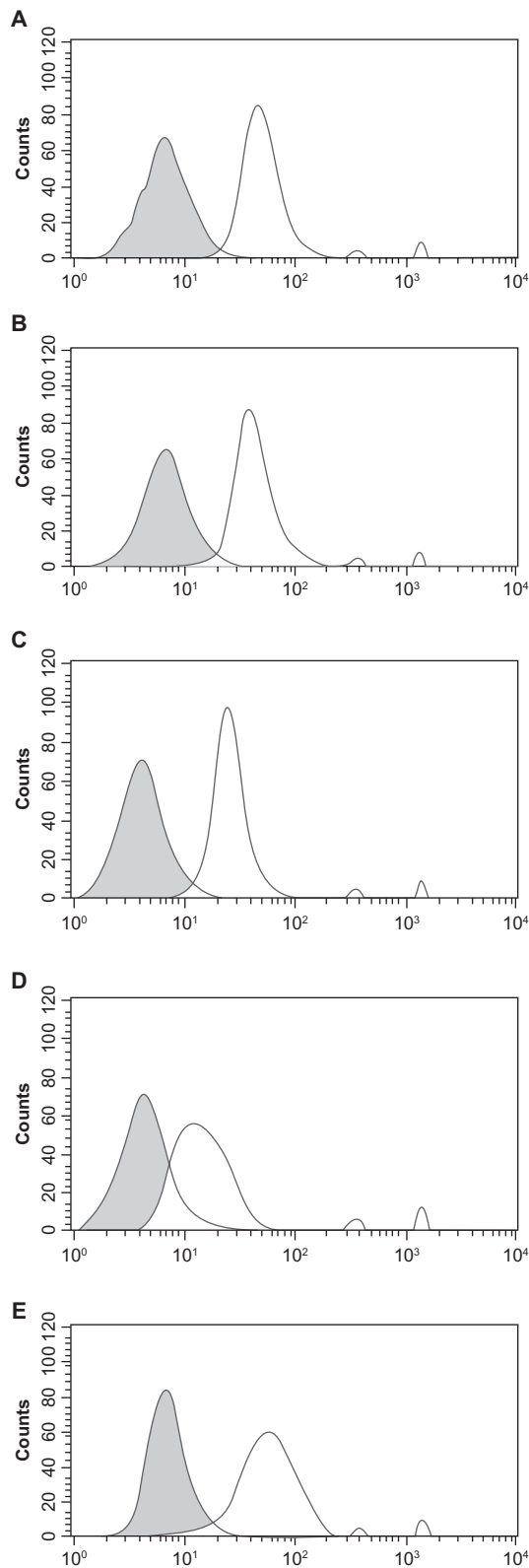


Figure 8 Flow cytometric analysis of phospho-STAT translocation in ARPE-19 cells. Unstimulated cells (gray histograms) are compared to cells stimulated for 20 min with different cytokines (white histograms). **A)** Stimulation with 500 U/mL of IFN- γ and analysis of phospho-STAT1. **B)** Stimulation with 500 U/mL of IFN- α and analysis of phospho-STAT1. **C)** Stimulation with 500 U/mL of IFN- α and analysis of phospho-STAT3. **D)** Stimulation with 10 ng/mL of IL-6 and analysis of phospho-STAT3. **E)** Stimulation with 10 ng/mL of IL-4 and analysis of phospho-STAT6.

Table 1 Summary of cytokine specific activation patterns in ARPE-19 cells

Cytokine	Protein	EMSA	IF	FC
IFN- α	STAT1	++	++	++
IFN- α	STAT2	++	nd	nd
IFN- α	STAT3	+	+++	++
IFN- γ	STAT1	+++	+++	+++
IL-6	STAT3	++	+++	+
IL-4	STAT6	+++	+++	+++

Notes: +++ = strong expression; + = weak expression; nd = not determined.

Abbreviations: EMSA, electromobility shift assay; IF, immunofluorescence staining; FC, flow cytometry.

a strong activation of STAT3 in EMSA experiments, but weak activation of STAT3 in FACS experiments (Figures 6 and 8). Activation of STAT1 upon stimulation with IFN- γ was very strong in all assays, whereas IL-4 induced a very strong activation of STAT6 in all tests (Table 1).

Based on these findings, we hypothesize that upregulation of IFNs and other cytokines in AMD can lead to activation of JAK-STAT pathways and, furthermore, that STATs can contribute to the pathogenesis of AMD (Figure 9). The JAK-STAT signaling pathway (specifically the STAT1 and STAT3 proteins) has been already implicated in neurodegenerative and chronic inflammatory pathogenetic mechanisms.⁴¹⁻⁴⁴

One protein family that appears to be highly susceptible to modulation by inflammatory cytokines is the MHC. RPE

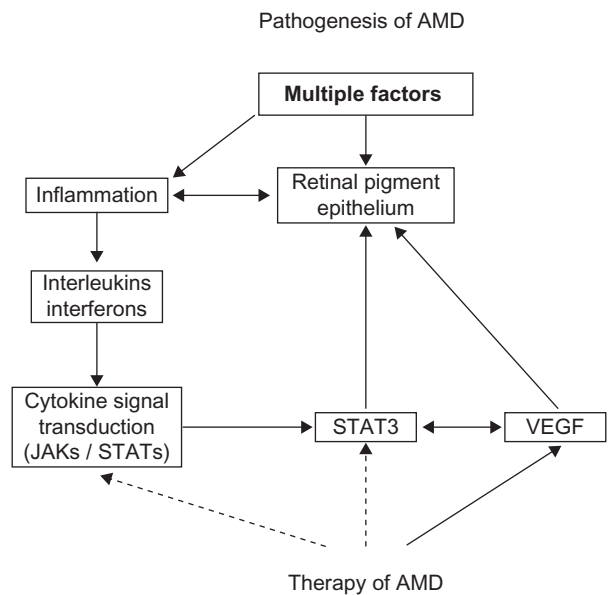


Figure 9 Schematic representation of AMD pathogenesis. Current AMD therapy is based on vascular endothelial growth factor inhibition. Novel approaches for AMD therapy should also consider JAK-STAT signaling as a target. The term 'multiple factors' may include genetic or environmental factors or aging.

cells phagocytose and recycle autoantigen-rich retinal rod outer segments and coexpress HLA-DR and HLA-DQ antigens in response to IFN- γ stimulation. An immunoregulatory role is postulated for RPE cells under conditions implicated in retinal autoimmunity, such as chronic idiopathic posterior uveitis and retinal vasculitis.⁴⁵ In our present study, IFN- γ stimulated HLA-DR antigen expression in ARPE-19 cells (Figure 1C). This observation is consistent with those of Gabrielian et al⁴⁶ and Farrokh-Siar et al⁴⁷ where HLA-DR has been shown to be upregulated in response to IFN- γ on RPE cells. The important role of MHC class II antigen expression in inflammatory eye diseases was emphasized by studies demonstrating that administration of anti-IFN- α antibodies significantly delayed the onset of experimental autoimmune uveitis in rats.⁴⁸ Upregulation of MHC II after stimulation with IFNs further confirms that RPE cells have antigen-presenting capabilities and may be involved in the development of abnormal immune response during AMD. Recent study has shown that proangiogenic effect of IFN- γ is dependent on the PI3 K/mTOR translational pathway.⁴⁹

We speculate that deregulated activation of STAT proteins may lead to elevated angiogenic and apoptotic phenotypes, vascular endothelial growth factor deregulation,^{50,51} and later even lead to retina degeneration (Figure 9). While interferons and cytokines upregulate the expression of MHC molecules and activate the JAK-STAT signaling pathway, activated STAT members, in turn, may lead to the activation of additional growth factors and chemokines. Novel approaches for AMD therapy should therefore consider STAT signaling as a target. Further studies including tissues from patients suffering from AMD are necessary to clarify the precise mechanisms underlying AMD progression and for the further development of new therapies for AMD treatment.

Conclusion

In the present study, JAK-STAT pathway activation in ARPE-19 cells was demonstrated with various cytokines using the techniques of EMSA, flow cytometry, and immunofluorescence. Our current data showed that multiple STAT proteins (STAT1, STAT2, STAT3, and STAT6) are activated in ARPE-19 cells upon stimulation with cytokines.

The individual STAT activation patterns were obviously cytokine-specific. We foresee the ARPE-19 cell line as a model for screening of novel drugs through interference with JAK-STAT signaling pathway and for future research using primary RPE culture.

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Disclosure

The authors report no conflicts of interest in this work.

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