The complement C3a-C3aR and C5a-C5aR pathways promote viability and inflammation of human retinal pigment epithelium cells by targeting NF-κB signaling

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Abstract. Retinal detachment (RD) and its special form of rhegmatogenous RD associated with choroidal detachment (RRDCD) feature similar pathological alterations, including enhanced retinal cell inflammation. Although the importance of the complement components C3a and C5a and their corresponding receptors in retinal maintenance has been demonstrated, the relevance of these molecules to the pathogenesis of RD or RRDCD remains to be investigated. The contents of C3a, C5a and inflammatory factors, such as TNF-α, IL-1β, IL-6 and prostaglandin (PG)E2, in related clinical samples were examined by ELISA. Subsequently, human retinal pigment epithelial (HRPE) cells were subjected to challenge with the C3a and C5a recombinant proteins with or without C3a and C5a antagonists and NF-κB inhibitor, and the cell viability and inflammatory cytokines were then determined by a Cell Counting Kit-8 assay and ELISA, respectively. In addition, reverse transcription-quantitative PCR and western blot analyses were utilized to examine the mRNA or/and protein levels of C3a and its receptor C3aR, as well as C5a and its receptor C5aR, and NF-κB. In addition, the correlation of C3a and C5a with the aforementioned inflammatory factors was analyzed. The inflammatory factor levels of C3a and C5a were considerably elevated in patients with RRDCD compared to those in the controls. Consistently, C3a and C5a treatment led to increased cell viability and aggravated inflammation in HRPE cells. Accordingly, C3a and C5a induced upregulation of their corresponding receptors C3aR and C5aR, which was in turn observed to be linked to the activation of the NF-κB signaling pathway. Furthermore, there was a positive correlation of the complements C3a and C5a with individual TNF-α, IL-1β, IL-6 and PGE2. Taken together, the C3a-C3aR and C5a-C5aR pathways were indicated to promote cell viability and inflammation of HRPE cells by targeting the NF-κB signaling pathway.

Introduction

Debilitating eye diseases, such as age-related macular degeneration, retinal detachment (RD) and rhegmatogenous RD associated with choroidal detachment (RRDCD), represent a series of visual health issues that impact the quality of life of affected patients (1-3). While the etiology and pathogenesis of these disorders remain to be fully elucidated, retinal pigment epithelium (RPE) loss and dysfunction appear to be significant contributors (4-6). Understanding the underlying mechanisms of phenotypic alterations, including cell viability and inflammation in RPE, should greatly assist the therapy of eye diseases (6-8).

Growing evidence has revealed the vital role of complement signaling in retinal physiopathology (9,10). Complement components, such as C2, C4b, C5/C5a and C9, have been indicated to be elevated in the vitreous humor of patients with RRDCD (11). Of note, RPE cells become more susceptible to complement-mediated damage (10). Complement and its receptor signaling may activate oxidative stress, contribute to transcriptional and metabolic homeostasis and promote inflammation in RPE (12,13). C3a and C5a, the bioactive fragments of C3 and C5, may upregulate the secretion of VEGF in RPE (14). In addition, RPE expresses the complement receptors C3aR and C5aR, and exhibits an increase in free cytosolic Ca2+, PI3K/Akt activation and forkhead box (FOX)P3, and FOXO1 phosphorylation and cytokine/chemokine secretion correspond to C3a and C5a (15). In another study, mice with complement factor H (CFH) mutation exhibited retinal degeneration, edema and detachment, which was rescued by pharmacologic blockade of C5 (16). Furthermore, the deficit of C3a-C3aR and C5a-C5aR signaling was confirmed to be linked to abnormal retinal structure and function (9).

However, it has been confirmed that the participation of C3a-C3aR and C5a-C5aR pathways in various cellular processes is mediated by the regulation of distinct signaling
pathways, such as MAPK, NF-κB, PI3K-Akt and nuclear factor of activated T cells (NFAT) (17-20). Of note, both C3a-C3aR and C5a-C5aR pathways may trigger the activation of the NF-κB signaling pathway (9). Accumulating evidence has indicated that phenotypic alterations of RPE, including the junctions between RPE, the expression of inflammatory cytokines within RPE and its antioxidant ability, are frequently related to the NF-κB signaling pathway (21-23). Furthermore, a recent study has established a close association among NF-κB signaling, CFH loss-induced abnormality of the complement system and the occurrence of inflammation in RPE cells (24). However, it remains undetermined whether the C3a-C3aR and C5a-C5aR pathways are able to regulate RPE and what is the underlying mechanism, in particular in the context of RD or RRDCD.

To this end, the present study intended to explore the role of the C3a-C3aR and C5a-C5aR pathways in regulating RPE viability and inflammation during RD or RRDCD progression and investigate the underlying molecular mechanisms, with an emphasis on the NF-κB signaling pathway.

Materials and methods

Data collection. The design of the present study was in compliance with the tenets of the 1975 Declaration of Helsinki and the protocol was approved by the Ethics Committee of the Nanjing Medical University Affiliated Wuxi Second Hospital (Wuxi, China; no. 2019Y-30). Written informed consent was provided by all 40 patients recruited for the purposes of this study. A total of 20 patients with RRDCD and 20 patients with idiopathic epimacular membrane (IEM), used as the control, enrolled at Nanjing Medical University Affiliated Wuxi Second Hospital between January 2020 and July 2021, were included in the cohort. Individuals with recurrent or secondary IEM or RRDCD, or a previous endophthalmitis complication, or a history of eye surgery during the last six months and vitreous hemorrhage, were excluded from this study. The age and sex were matched between the two groups. For all recruited participants, two senior experienced ophthalmologists performed a systematic and comprehensive eye examination. The numbers of detached quadrants and the PVR grades were scored according to the 1983 International Classification of PVR (25) in patients with RRDCD and were then evaluated.

Collection of vitreous samples. Prior to primary pars plana vitrectomy, the vitreous samples were obtained using a three-port 25-gauge transconjunctival suture-less vitrectomy system (TSV25G; Alcon Constellation; Alcon Laboratories) with the aid of a non-contact wide-angle viewing system (Resight; Carl Zeiss Meditec AG) for visualization. The samples were then suctioned directly into a 5-ml syringe, were immediately transferred into microcentrifuge tubes and kept on ice. Following centrifugation at 1,360 x g for 10 min at 4°C, the supernatants were collected and stored at -80°C prior to analysis.

Cell culture. Human RPE (HRPE) cells (ARPE-19; CRL-2302) purchased from the American Type Culture Collection were maintained in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator with 5% CO₂ at 37°C.

Cell treatment. HRPE cells were divided into six groups and treated as follows. In the first group, HRPE cells (3x10⁵ or 3x10⁶ per well) were subjected to 0.5, 1, 2, 4 and 8 µg/ml of recombinant endotoxin-free human complement component C3a protein (R&D Systems, Inc) for 24 or 48 h. In the second group, HRPE cells (3x10⁵ or 3x10⁶ per well) were exposed to 0.5, 1, 2, 4 and 8 µg/ml of recombinant endotoxin-free human complement component C5a protein (R&D Systems, Inc.) for 24 or 48 h (26). In the third group, HRPE cells (3x10⁵ per well) were treated with recombinant human complement component C3a protein (2 µg/ml) and NF-κB inhibitor pyrroolidine dithiocarbamate (PDTC; 10 µM; MilliporeSigma) for 24 h. In the fourth group, HRPE cells (3x10⁵ per well) received treatment of recombinant human complement component C5a protein (1 µg/ml) and PDTC (10 µM) for 24 h. In the fifth group, HRPE cells (3x10⁶ per well) were treated with recombinant human complement component C3a protein (2 µg/ml) and C3aR antagonist SB290157 (20 µM; MilliporeSigma) for 24 h. In the sixth group, HRPE cells (3x10⁶ per well) were treated with recombinant human complement component C5a protein (1 µg/ml) and C5aR antagonist CCX168 (2 µM; Abmole Bioscience, Inc.) for 24 h.

Measurement of cell viability. Approximately 3x10⁵ HRPE cells were seeded in each well of a 96-well/plate and incubated overnight. Following 0, 12, 24 and 48 h of treatment, 10 µl Cell Counting Kit-8 solution (Signalway Antibody LLC) was applied to the cells for 1 h. The viability of the HRPE cells was evaluated by measuring the absorbance at 450 nm on a microplate reader (Hua Dong).

ELISA. Approximately 3x10⁵ HRPE cells were seeded in each well of a 6-well/plate and incubated overnight. Following 24 h of treatment, the contents of various cytokines and other compounds were analyzed in HRPE cell supernatants and/or human vitreous humor were determined using the following ELISA kits in accordance with the manufacturers’ protocols: Tumor Necrosis Factor-α Assay Kit (cat. no. H052-1), Interleukin-1β Assay Kit (cat. no. H002), Interleukin-6 Assay Kit (cat. no. H007-1-1) and Interleukin-10 Assay Kit (cat. no. H009-1; all from Nanjing Jiancheng Bioengineering Institute); prostaglandin E2 (PGE2) ELISA Kit (cat. no. E-EL-0034c; Elabscience Biotechnology Co., Ltd.); Human Complement Fragment 3a ELISA Kit (cat. no. CSB-E08509h) and Human Complement Fragment 5a ELISA Kit (cat. no. CSB-E08512h; both from Cusabio).

Reverse transcription-quantitative (RT-q)PCR. Approximately 3x10⁵ HRPE cells were seeded in each well of a 6-well/plate and incubated overnight. Following 24 h of treatment, TRIzol® reagent (Thermo Fisher Scientific, Inc.) was used for the extraction of total RNA from the HRPE cells, while the PrimeScript RT reagent kit (Takara Bio, Inc.) was employed according to the manufacturer’s protocol for cDNA synthesis. qPCR was performed with the SYBR Green
PCR master mix on an ABI 9700 PCR machine (both from Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. The following thermocycling conditions were used for qPCR: 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 45 sec; final extension at 95˚C for 15 sec, 60˚C for 1 min, 95˚C for 15 sec and 60˚C for 15 sec. The PCR primer pairs were as follows: C3aR forward, 5'-TCTTTATGCCTCTTGG-3' and reverse, 5'-GAACCGCTGGATTGATTCC-3'; C5aR forward, 5'-TAGGGAGACCAAATGTAGGT-3' and reverse, 5'-TGGAGGAGATTGTAGTC-3'; and GAPDH forward, 5'-AATCCCATCACCACCTCTC-3' and reverse, 5'-AGGGCTTGTTGATCATCTTC-3'. The 2^ΔΔCq method was used to calculate the expression levels (13).

**Western blot analysis.** Approximately 3x10^5 HRPE cells were seeded in each well of a 6-well/plate and incubated overnight. Following 24 h of treatment, RIPA buffer (Beyotime Institute of Biotechnology) was employed to prepare the cell lysates. The protein concentration was measured using a bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.), and absorbance was measured using a microplate reader (SM600 Labsystem; Shanghai Utrao Medical Instrument Co., Ltd.). Proteins (20 µg) were fractioned by 10 or 15% SDS-PAGE and were subsequently transferred onto polyvinylidene fluoride membranes (EMD Millipore), which were then exposed to primary antibodies against C3aR (cat. no. ab126250), C5aR (cat. no. ab234757; both from Abcam), phospho (p)-NF-κB (cat. no. 3033), NF-κB (cat. no. 8242) and GAPDH (cat. no. 5174; all from Cell Signaling Technology, Inc.) at 4˚C overnight, washed three times with Tris-buffered saline containing Tween-20 and then incubated with the secondary antibody (cat. no. A0208; Beyotime Institute of Biotechnology) for 1 h at 37˚C. The chemiluminescence signals of the targets were generated with an Enhanced Chemiluminescence Detection kit (Pierce; Thermo Fisher Scientific, Inc.). GAPDH was used as an internal control.

**Statistical analysis.** Prism v.8.0.2 (GraphPad Software, Inc.) was used to perform all statistical analyses. The mean ± standard deviation was used to denote data from triplicates. The Shapiro-Wilk normality test was used to evaluate the normal distribution of the collected data. When data followed a normal distribution, a two-sided Student's t-test or one-way ANOVA with Tukey's post-hoc test were adopted. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Complement C3a and C5a promote cell viability and inflammation of HRPE cells.** Table I presents the demographic and clinical data of the patients enrolled in the study (20 patients with RRDCD and 20 patients with IEM). Patients with RRDCD or IEM included 10 males and 10 females, aged 63.1±5.22 and 64.1±6.69, respectively. To determine the possible role of complement C3a and C5a in the progression of RD or RRDCD, their contents were examined in patients with RRDCD (n=20) and in individuals with IEM as controls (n=20) by ELISA. The results revealed that the levels of both C3a and C5a in patients with RRDCD were markedly higher than those in patients with IEM (Fig. 1A and B). To investigate the relevance of this C3a and C5a alteration to visual disorders, HRPE cells were treated with various doses of purified recombinant human complement C3a and C5a protein. The application of C3a or C5a triggered a considerable increase in HRPE cell viability (Fig. 1C and D), suggesting that these factors influence cell survival. Considering the relevance of these factors to the occurrence of inflammation, the secretion of inflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-10 and PGE2, in C3a- or C5a-treated cells, was evaluated by ELISA. As indicated in Fig. 1E-H, the secretion of TNF-α, IL-1β, IL-6 and PGE2 by HRPE cells was markedly enhanced by C3a or C5a stimulation and a dose-dependent effect was observed. However, C3a or C5a stimulation significantly decreased IL-10 secretion (Fig. 1I). These results indicated that elevated C3a or C5a levels may be associated with increased cell viability and inflammatory response in HRPE cells.

**Complement C3a and C5a enhance the expression of C3aR and C5aR and activate NF-κB signaling.** It is established that C3a and C5a act through binding to their corresponding receptors. Indeed, markedly increased mRNA levels of C3aR or C5aR were observed in the presence of the C3a or C5a ligands, respectively (Fig. 2A and B). Considering the vital role of the NF-κB signaling pathway in mediating complement-triggered responses, the activation of this pathway was investigated by western blot analysis. Of note, the protein levels of C3aR and the p-NF-κBp65/NF-κBp65 ratio gradually increased with increasing C3a concentrations (Fig. 2C), and a similar trend was observed in the presence of various C5a concentrations (Fig. 2D). These results demonstrated the upregulation of C3aR and C5aR and activation of the NF-κB pathway in response to C3a and C5a challenge.

**Complement C3a and C5a promote inflammation of HRPE cells via the NF-κB signaling pathway.** To confirm that C3a and C5a triggered inflammation in HRPE cells is indeed mediated by NF-κB signaling, the cytokine levels were...
analyzed following incubation with or without the NF-κB inhibitor PDTC. Administration of C3a or C5a induced the release of TNF-α, IL-1β, IL-6 and PGE2, while this promoting effect was inhibited by the application of PDTC in HRPE cells (Fig. 3A-D). However, C3a or C5a administration inhibited the release of IL-10, while this effect was inhibited by the application of PDTC in HRPE cells (Fig. 3E). These findings demonstrate that C3a and C5a triggered inflammatory responses in a process that is largely mediated by the NF-κB pathway.

Complement C3aR and C5aR antagonists repress inflammation and NF-κB signaling in HRPE cells challenged with complement C3a and C5a. To further confirm the role of the C3a-C3aR and C5a-C5aR pathways in the occurrence of inflammation and the activation of NF-κB signaling in HRPE cells, antagonists of these receptors were also applied. C3a-mediated secretion of TNF-α, IL-1β, IL-6, IL-10 and PGE2 was inhibited by the presence of the C3aR antagonist SB290157 (Fig. 4A-E). Likewise, the C5a-mediated release of these cytokines was repressed by the application of the
C5aR antagonist CCX168 (Fig. 4F-J). In addition, activation of NF-kB induced by C3a or C5a was robustly blocked by their corresponding receptor antagonists (Fig. 4K and L). These findings validate that the C3a-C3aR and C5a-C5aR pathways participate in the subsequent inflammatory responses but also in NF-kB activation in HRPE cells.

Complement C3a and C5a are positively correlated with the contents of TNF-α, IL-1β, IL-6 and PGE2 in patients with RRCD. Finally, the cytokine levels in patients with RRCD and the controls were analyzed and the relationship of C3a and C5a with these inflammatory factors was assessed. As presented in Fig. 5A-E, the patients with RRCD had significantly higher contents of TNF-α, IL-1β, IL-6 and PGE2 and a lower content of IL-10 compared to the controls, indicating a stronger inflammatory response in these individuals. Furthermore, the levels of TNF-α, IL-1β, IL-6 and PGE2 exhibited a positive correlation, as opposed to IL-10, which had a negative correlation with C3a (Fig. 5E-J) and C5a (Fig. 5K-O). These data suggested that progression of RD or
RRDCD disorders may be accompanied by higher levels of inflammation. In addition, it may be inferred that this condition is associated with elevated C3a and C5a contents.

Discussion

Loss or dysfunction of photoreceptors or the adjacent RPE is strongly associated with the progression of retinal diseases, which affect millions of individuals globally (24,27), highlighting the necessity to investigate the pathogenesis and develop novel therapeutic strategies. In the present study, C3a and C5a contents were higher in patients with RRDCD than in control subjects. Consistently, elevated C3a and C5a levels led to increased cell viability and aggravated inflammation in HRPE cells. The results of the present mechanistic study indicated that higher C3a and C5a contents were able to enhance the expression levels of their corresponding receptors C3aR and C5aR, which in turn resulted in NF-κB signaling activation in vitro. This was further supported by the fact that the patients with RRDCD had higher levels of inflammatory factors, such as TNF-α, IL-1β, IL-6 and PGE2. Furthermore, the C3a and C5a levels were positively correlated with these cytokines.

Figure 3. Complement C3a and C5a aggravate inflammation in HRPE cells via the NF-κB signaling pathway. HRPE cells were treated with recombinant human complement component C3a (2 µg/ml) or C5a (1 µg/ml) with or without NF-κB inhibitor PDTC (10 µM), and the release of (A) TNF-α, (B) IL-1β, (C) IL-6, (D) PGE2 and (E) IL-10 was measured by ELISA. ***P<0.001 relative to control; ###P<0.001 relative to C3a or C5a treatment. PGE2, prostaglandin E2; HRPE, human retinal pigment epithelium.
It has been demonstrated that inflammatory responses are present in common pathological alterations of photoreceptor and accessory cells in RD and other related disorders (28). Previous studies have indicated that the C3a-C3aR and C5a-C5aR signaling axes are crucial pathways for triggering and modulating inflammatory response; accordingly, they are linked to numerous inflammation-related disorders (29-31). Of note, these pathways have also been validated to be essential for preserving normal retinal structure and function (9). The present study revealed significantly enhanced levels of both C3a and C5a under the pathological conditions of a retinal disorder, i.e. RRDCD. Furthermore, the elevation of these complement components was accompanied by an increase in a series of inflammatory factors. A previous study reported increased inflammatory mediators were inconsistent with the observations of the present study, this finding may be due to the different scope of the examined targets. The present results highlight the role of C3a-C3aR and C5a-C5aR signaling in cell viability and inflammation in HRPE cells.

It is well-known that the NF-κB signaling pathway is a vital modulator of cell survival and inflammation (24,32-34). When the NF-κB signaling pathway is activated, the NF-κB protein undergoes phosphorylation modification (35). C3a-C3aR signaling significantly enhances T-cell proliferation and IL-17A expression, a process that is mediated by the activation of NF-κB in T cells (36). C5a-C5aR signaling enhances the IL-8 promoter and activates NF-κB in the human leukemia cell line HL-60, peripheral blood mononuclear cells and RAW264.7 macrophage cells (37). Consistently, it was observed in the present study that increases of C3a or C5a were accompanied by an increase in the level of p-NF-κBp65, suggesting that the NF-κB signaling pathway mediates the phenotypic alterations of
HRPE cells induced by these complement components. These findings were further confirmed by the application of C3aR and C5aR antagonists or NF-κB inhibitor. C3a was previously reported to promote the production of chemokine through NFAT activation in mast cells (19), and it was indicated that its regulation on its receptor gene was mediated by activator protein-1 in glial cells (19,20). Furthermore, the activation of the PI3K/Akt pathway by both C3a-C3aR and C5a-C5aR in T cells has been previously described in the literature (29). The present results robustly demonstrated that both the C3a-C3aR and C5a-C5aR pathways were able to activate NF-κB signaling, thereby regulating the phenotypes of HRPE cells, which is, to a certain degree, consistent with their important role in retinal structure and function (9). Furthermore, NF-κB regulation by the C3a-C3aR pathway in tubular epithelial cells has been previously described in the literature (18); therefore, whether C3a-C3aR has the same role in other epithelial cells and whether the C5a-C5aR pathway has a similar function require to be investigated. In addition, the RPE is a physical barrier and provides immune-suppressive molecules, which contribute to the privileged immune status of the eye (38). Damage to the RPE layer and the underlying choroid may promote infiltration of immune cells and induce retinal microglia and choroidal inflammatory cells to release pro-inflammatory cytokines. A previous study suggested that the anti-inflammatory cytokine IL-10 inhibits RPE cell proliferation and inflammation through the regulation of VEGF in RRD rats (39), a finding that is consistent with the role of IL-10 in C3a-C3aR- or C5a-C5aR-induced RPE cells. However, the role of C3a-C3aR or C5a-C5aR in the regulation of RPE and immune cells at the retina-choroidal interface remains to be investigated. In the present study, C3a, C5a, TNF-α, IL-1β, IL-6 and PGE2 contents were higher, while the IL-10 content was lower in patients with RRDCD than in control subjects. Although no public datasets are currently available for patients with RRDCD, the complement anaphylatoxins and inflammatory factors detected in the present study may be further supplemented with data from our institution including additional patients. Meanwhile, selection bias may be another potential limitation. Future studies will aim to determine the correlation between complement anaphylatoxins and inflammatory factors in patients with recurrent or secondary RRDCD.

In conclusion, the present results revealed the significance of the complement components C3a and C5a, as well as their pathways, in the pathogenesis of retinal disorders such as RRDCD. These molecules and their pathways influenced the viability of HRPE cells and enhanced the cellular inflammatory level through regulation of the NF-κB signaling pathway. This finding suggests that C3a and C5a are both involved in the progression of retinal diseases and that the C3a-C3aR and C5a-C5aR pathways, as well as their downstream NF-κB signaling, may be considered novel targets for the development of new drugs to treat RD and related disorders.
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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

SL and HX designed this study. HX, XG and JS performed the experiments. SL, XC and ZW analyzed and interpreted the data. XG and JS checked and approved the authenticity of the raw data. SL and ZW wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study is in compliance with the tenets of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of Nanjing Medical University Affiliated Wuxi Second Hospital (Wuxi, China; no. 2019Y-30). Written informed consent was obtained from each patient.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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