The complement system and toll-like receptors (TLRs) are two major components of the innate immune system that recognize and respond rapidly to pathogens and serve as important mediators between innate and adaptive immunity [1]. The two systems have the ability to recognize pathogen-associated molecular patterns (PAMPs) and to destroy microbial invaders. It is becoming increasingly clear that there is crosstalk between TLRs and complement pathways, including the widespread regulation of TLR signaling by complements [2,3].

In ocular immunity, the complement system is implicated in the development and progression of several immune-mediated ocular conditions, including age-related macular degeneration (AMD) and uveitis [4]. Nozaki et al. showed that C5a is present in the drusen of patients with AMD, and it promotes choroidal neovascularisation (CNV), which is the hallmark of wet AMD, by increasing the expression of vascular endothelial growth factor (VEGF) [5,6]. Systemic and local anti-C5 therapies reduce the disease severity in experimental autoimmune uveoretinitis [7]. C5a induces the increased expressions of IL-1beta, IL-6, MCP-1, GM-CSF, and IL-8 in RPE cells [8]. Lipopolysaccharide (LPS), as a well-established ligand for TLR4 [9], can elicit acute ocular inflammation in animals and can lead to uveitis [10,11].

Many PAMPs activate both complements and TLRs, and recent studies have revealed a marked synergistic interaction between the two systems. Heiko and colleagues showed that C5a negatively regulates the TLR4-induced synthesis of IL-12 family cytokines (IL-12, IL-23, and IL-27) from inflammatory macrophages by extracellular signal-regulated kinase- and phosphoinositide 3 kinase-dependent pathways [2]. C5a controls TLR4-induced IL-10 and IL-12 production in mouse macrophages, and it was shown to depend on the extracellular signal-regulated kinase (ERK) 1/2 pathway [12]. A recent report demonstrated that C5a reduced the LPS-induced production of IL-12, IL-23, and IL-6 in immature dendritic cells (DCs), but the suppressive effect was time dependent [13].

Although it is reported that there is crosstalk between TLRs and complements in other cell types, whether and how these two systems when co-activated in the eye interact with each other has not been well studied. RPE cells have been shown to express both TLR4 [14] and C5a receptors [15], and they were therefore chosen in this study to investigate the interaction between these systems. It was observed that C5a leads to an enhanced TLR4 expression in RPE cells.

C5a and toll-like receptor 4 crosstalk in retinal pigment epithelial cells

Yi Zhu,1 Bingling Dai,2 Yongguo Li,3 Hui Peng2
(The first two authors contributed equally to this study.)

1The People’s Hospital of Kai County, Chongqing, China; 2The First Affiliated Hospital of Chongqing Medical University, Chongqing Key Laboratory of Ophthalmology, Chongqing Eye Institute, Chongqing, China; 3Chongqing Medical University, Chongqing, China

Purpose: To investigate the effect of the complement activation product C5a on toll-like receptor (TLR) 4-induced responses in RPE cells.

Methods: Confluent cultures of human RPE cells (ARPE-19) were stimulated with C5a, lipopolysaccharide (LPS), or a combination of the two. The expression of TLR4 was determined by real-time PCR and flow cytometry. Cytokine profiles were determined by real-time PCR and enzyme-linked immunosorbent assay (ELISA). The phosphorylation of p38, ERK 1/2, and JNK was measured by flow cytometry.

Results: C5a stimulation enhanced the expression of TLR4 in a dose- and time-dependent manner. C5a was able to stimulate the production of TLR4-induced IL-6 and IL-8 by ARPE-19 cells. Blocking experiments showed that the effect of C5a on cytokine production was mediated via C5aR. ERK1/2, but not JNK or p38, were involved in the production of IL-6 and IL-8.

Conclusions: The results indicate that C5a can induce the TLR4 expression and enhance the production of TLR4-induced IL-6 and IL-8 by ARPE-19. The effect of C5a on cytokine production was mediated by C5aR and the phosphorylation of ERK1/2.

Correspondence to: Hui Peng, 1 Youyi Road, Yuzhong District, Department of Ophthalmology, Chongqing 400016 China; Phone: 86 23 68485440; FAX:86 23 89012851; email: pengh9@aliyun.com
C5a furthermore significantly increased the TLR4-induced synthesis of IL-6 and IL-8 by RPE cells, and evidence of the involvement of the C5aR signaling pathways and the activation of ERK was provided.

METHODS

Cell culture: The human RPE (ARPE-19) cell line was obtained from the American Type Culture Collection (ATCC). Cells were cultured in a medium (Dulbecco’s modified Eagle’s medium: nutrient mixture F12 [DMEM/ F12], 1:1; Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS; Invitrogen), 100 U/mL penicillin, and 100 ng/ml streptomycin. Cells were incubated in a humidified 5% CO2 atmosphere at 37 °C and they were passaged every 5 to 7 days. After reaching confluence, the cells were detached with a trypsin-EDTA solution, diluted at 1:3 to 1:4, and plated for subculture. The ARPE-19 cells used in the experiments were confluent. Before the experimental procedures, the ARPE-19 cells were kept under serum-free conditions for 24 h. Cells used in these experiments were validated as authentic ARPE-19 cells by STR analysis (Appendix 1).

Stimulation of RPE: Cells were primed with LPS (100 ng/ml, Sigma, E. coli O127:B8) for 16 h in a total volume of 1 ml. Recombinant human C5a (50 ng/ml, Sigma) was added to the culture 10 min before LPS challenge. Inhibitors of JNK (SP600125, 10 uM), ERK1/2 (PD98059, 20 uM), and P38 (SB239063, 20 uM; all from Calbiochem, Darmstadt, Germany) were added 1 h before LPS stimulation. An antagonist for C5aR (W-54011, 10 ng/ml, Calbiochem) was added 4 h before LPS stimulation.

Real-time quantitative PCR analysis: Total RNA was extracted with TRIzol (Invitrogen) following the manufacturer’s instructions. RNA concentrations were determined with a Nano instrument (NanoDrop Technologies, Wilmington, DE). The first-strand cDNA was synthesized for each RNA sample using the Superscript III Reverse Transcriptase system (Invitrogen). The concentrations of IL-6 and IL-8 were determined using human enzyme-linked immunosorbent assay (ELISA) development kits (R&D Systems) according to the manufacturer’s instructions, with detection limits of 9.4 pg/ml and 15.6 pg/ml, respectively.

Statistical analysis: Data were expressed as mean±standard deviation (SD) and a one-way analysis of variance (ANOVA) was applied using SPSS 17.0 software (SPSS Inc.). Significant differences are indicated for p<0.05 and p<0.01, respectively.

RESULTS

C5a upregulates the TLR4-Induced expressions of IL-6 and IL-8: To evaluate the effects of C5a on the TLR4-induced expressions of IL-6 and IL-8, ARPE-19 cells were incubated with 100 ng/ml of LPS for 16 h in the presence or absence of recombinant C5a. Real-time PCR was performed to investigate the mRNA expressions of IL-6 and IL-8. As shown in Figure 1, 50 ng/ml of a C5a treatment of ARPE-19 cells has
a minimal impact on IL-6 (Figure 1A) and IL-8 (Figure 1B) production following 16 h of in vitro stimulation, while it significantly increased IL-6 and IL-8 production induced by LPS (100 ng/ml). IL-6 and IL-8 levels increased by more than 4-fold in the co-presence of C5a and LPS in comparison to LPS and C5a alone, suggesting a strong enhancing effect of C5a on LPS-induced IL-6 and IL-8 production. Surprisingly, C5a did not enhance TLR3-induced IL-6 and IL-8 production after PolyI:C stimulation (Appendix 2a).

It was then tested whether the IL-6 and IL-8 mRNA expressions resulted in a difference in protein production. Consistent with the mRNA data of this study, the ELISA showed that C5a significantly increased TLR4-induced IL-6 and IL-8 secretions from ARPE-19 cells.

C5a induces changes in the expression levels of TLR4: The experiments shown above could be explained by the fact that C5a upregulated the TLR expression on RPE cells, and this hypothesis was therefore tested in the following series of experiments. It was found that C5a did not seem to change the TLR4 expression after stimulation for 16 h (Appendix 3). RPE cells were then stimulated with C5a for shorter time periods ranging from 0 to 120 min (Figure 2A). TLR4 gene transcripts were significantly upregulated at 40 min and peaked at 80 min. At 120 min, the expression of TLR4 reached preincubation levels (Figure 2A).

Incubation of ARPE-19 cells with varying concentrations showed that the addition of 50 ng/ml and 100 ng/ml of C5a enhanced the mRNA expression of TLR4 (Figure 2B). No significant upregulation of the mRNA levels of TLR4 was detected when cells were treated with a concentration of 25 ng/ml C5a.

The study data thus demonstrated that the TLR4 mRNA expression in RPE cells was significantly elevated by C5a in a time- (Figure 2A) and dose-dependent (Figure 2B) manner. In the following experiments, 30 ng/ml of C5a and 80 min of incubation were chosen to investigate whether C5a can affect the TLR4 protein expression.
Flow cytometry of RPE cells showed that the cell-surface TLR4 expression was significantly increased at 80 min after C5a or LPS treatment (Figure 2F). The TLR4 expression after LPS treatment was higher than that seen after C5a treatment (Figure 2F).

C5a-enhanced TLR4-induced IL-6 and IL-8 production is mediated via C5aR: C5a binds to two trans-membrane receptors: C5aR/CD88 and C5L2 (GPR77). Previous studies suggest that C5aR is the predominant receptor facilitating the ability of C5a to induce cytokine release in LPS-stimulated macrophages [16]. Pre-treatment of ARPE-19 cells with the C5aR antagonist W-54011 (10 ng/ml) for 4 h before LPS stimulation showed that the enhanced effect of C5a was abrogated (Figure 3), which is in agreement with a previous study using macrophages [11]. These results indicated that...
the effect of C5a on IL-6 and IL-8 production was mainly mediated by C5aR.

**C5a actions are dependent on the ERK1/2 pathway:** As it was demonstrated previously that C5a is engaged in the ERK1/2, p38, and JNK pathways for cytokine production [2], it was investigated whether the same pathways are also responsible for the cytokine production observed upon the C5a stimulation of ARPE-19 cells. RPE cells were stimulated with LPS, C5a, or a combination of both for 30 min, and flow cytometry was used to measure the phosphorylation of ERK1/2, p38, and JNK. As shown in Figure 4B, the phosphorylation of ERK1/2, but not JNK or p38, was higher when LPS and C5a were used in combination as compared to LPS or C5a alone.

To investigate further the contribution of these pathways to IL-6 and IL-8 production, experiments were conducted in which the RPE cells were pre-incubated with ERK1/2 (PD98059), P38 (SB239063), and JNK (SP600125) inhibitors for 1 h before C5a/LPS stimulation. IL-6 and IL-8 production was significantly inhibited by the ERK1/2 inhibitor PD98059, while the JNK inhibitor SP600125 and p38 inhibitor SB239063 had no inhibitory effect on IL-8 and IL-6 production (Figure 4C, D). These results suggested that the enhancing effect of C5a on LPS-induced IL-6 and IL-8 production by RPE cells might be mediated by the phosphorylation of ERK1/2, but not of the JNK or p38 signaling pathways.

**DISCUSSION**  
This study provided evidence that C5a can induce the TLR4 expression, and it enhances the production of TLR4-induced IL-6 and IL-8 by ARPE-19 cells. The effect of C5a on cytokine production was mediated by C5aR and the phosphorylation of ERK1/2.

This study is in agreement with earlier findings that have suggested a regulatory role of C5a in the expression of TLR4-induced inflammatory mediators in a variety of cells [2,3]. To the best of the authors’ knowledge, crosstalk between C5a and TLR4 in RPE cells has not yet been reported.

It was found that C5a could significantly enhance the expression of TLR4-induced IL-6 and IL-8 by RPE cells, whereas a recent report demonstrated that C5a reduced the LPS-induced production of IL-12, IL-23, and IL-6 in immature DCs [13]. In this latter study, the suppressive effect was time dependent, which implies that signaling via C5aR can only affect TLR-induced signaling when initiated around the same time [13]. Another study showed that C5a could strongly amplify the IL-8 expression from human whole blood cells induced by LPS [17]. Complements can enhance LPS-induced IL-6 and TNF-alpha production in wild type (WT) mice [18]. In some studies, however, C5a treatment enhanced IL-6 production by LPS-stimulated mouse neutrophils but suppressed LPS-induced IL-6 production by macrophages [19]. These experiments point out an interesting perspective concerning the cell-type-specific regulatory effects of complements on TLR responses.

It was recently reported that the incubation of bovine neutrophils with a high concentration of C5a resulted in changes in the transcription of selected genes of the TLR4 pathway and resulted in a higher expression of the LPS receptor CD14 [20]. Similarly, a clear increase was found in mRNA and the protein level of TLR4 upon C5a incubation in a time- and dose-dependent manner.
Figure 4. C5a regulates TLR4-induced IL-6 and IL-8 expressions through the activation of ERK1/2. ARPE-19 cells were stimulated with LPS (100 ng/ml) in the absence or presence of C5a (50 ng/ml) for 30 min. A: Representative histograms for the phosphorylation of MAPK are shown at 30 min. B: The MFI of phosphor-ERK1/2, JNK, and P38. C5a was added to the culture 10 min before LPS challenge. Inhibitors of JNK (SP = SP600125, 10 uM), ERK1/2 (PD = PD98059, 20 uM), and P38 (SB = SB239063, 20 uM) were added 1 h before LPS stimulation. C and D: Contribution of ERK1/2, JNK, and P38 to the effect of C5a on TLR4-induced IL-6 (C) and IL-8 (D) expressions. The data are expressed as the mean±SD of three independent experiments. Statistical analysis was performed using a one-way ANOVA (* indicates p<0.05 and ** indicates p<0.01).
C5a stimulates cells via an interaction with the C5a receptors (C5aR), which belong to a family of G-protein-coupled receptors with seven transmembrane segments [21]. Another receptor for C5a, C5L2, was originally termed a ‘default receptor,’ but its exact function is not yet clear. Recently, C5L2 has been reported as a negative modulator of C5aR activity, thereby ensuring the regulatory control of the biologic activities of C5a [22]. C5aR is expressed in RPE cells [15], but the expression of C5L2 on RPE cells has not yet been reported. The two receptors are expressed on various immune and non-immune cells, such as granulocytes and dendritic cells [22,23]. Consistent with previous studies that showed that C5a induces cytokine release mainly via C5aR in LPS-stimulated macrophages, it was found that the C5a-mediated increase of LPS-induced IL-6 and IL-8 production by ARPE cells was susceptible to the C5aR antagonist W-54011, which indicates that the observed effect of C5a on TLR-induced cytokine production is mediated via the C5aR. In a recent study, pre-exposure of PBMCs to LPS enhanced the expression of C5a-induced IL-6 and IL-8. The effect of TLR4 on C5a was not mediated via the C5aR, but this was caused by a reduced expression of C5L2 [24]. The authors suggested that the reduction of the competing C5L2 receptor for C5a enhanced the interaction of the available C5a for C5aR. Whether a similar mechanism might be operative in RPE cells deserves further study. It should also be noted that the regulation of C5a on TLR4-induced immune responses might be amplified by TLR4-induced cytokines, such as IFN-γ [25], which may promote the expression of C5aR in RPE cells [15].

It has been previously shown that the enhancing effect of C5a on LPS-induced cytokine production is dependent on the activation of the ERK1/2, JNK, and p38 signaling pathways [2]. These findings prompted the investigation of whether C5a could regulate TLR4-reduced responses by RPE cells through these three signaling pathways. ERK1/2 phosphorylation, but not JNK or p38 phosphorylation, was higher when LPS and C5a were used in combination, as compared to LPS or C5a alone, which is consistent with the finding that LPS plus C5a resulted in higher levels of IL-6 and IL-8 released from RPE cells. More importantly, the blockade of the ERK1/2 signaling pathway with the specific inhibitor PD98059 resulted in a significant inhibition of IL-6 and IL-8 production by RPE cells.

In conclusion, this study revealed that the effect of C5a on the TLR4 expression, which may lead to an increased production of TLR4-induced IL-6 and IL-8, was mediated by C5aR and it was involved in the phosphorylation of ERK1/2. Manipulation of the crosstalk pathways between the TLR and complement system may offer future opportunities to control intraocular inflammation.

APPENDIX 1. STR ANALYSIS.
To access these data, click or select the words "Appendix 1".

APPENDIX 2. C5A COULD NOT ENHANCE TLR-3 INDUCED IL-6 AND IL-8 PRODUCTION.
ARPE-19 cells were stimulated with poly(I:C) (1 μg/ml) in the absence or presence of C5a (50 ng/ml) for 16h. C5a was added to the culture 10 min prior to poly(I:C) challenge. Effect of C5a on TLR3-induced IL-6 (A) and IL-8 (B) mRNA expression in ARPE-19 cells. The data are expressed as mean±SEM of three independent experiments. Statistical analysis was performed using one-way ANOVA. (* indicates p < 0.05 and ** indicates p < 0.01). To access these data, click or select the words "Appendix 2".

APPENDIX 3. C5A DID NOT CHANGE TLR4 EXPRESSION AFTER STIMULATION FOR 16 H.
ARPE-19 cells were stimulated with C5a (50ng/ml) for 16 h. TLR-4 mRNA expression in ARPE-19 cells. The data are expressed as mean±SEM of three independent experiments. Statistical analysis was performed using one-way ANOVA. (* indicates p < 0.05 and ** indicates p< 0.01). To access these data, click or select the words "Appendix 3".

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