

# The Ingenious Interactions Between Macrophages and Functionally Plastic Retinal Pigment Epithelium Cells

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**PURPOSE.** The purpose of this study was to clarify the interactions between macrophages (MPs) and RPE cells in coculture systems to investigate the functional plasticity of RPE cells.

**METHODS.** Adherent peritoneal cells or murine MP cell line Raw 264.7 was cocultured with primary RPE cells taken from C57BL/6 mice, with or without lipopolysaccharide (LPS) or TNF- $\alpha$  stimulation. The cytokine levels of the culture supernatants (CSs) were then analyzed with the Bio-Plex murine 23-Plex Panel Assay Kit (Bio-Rad Laboratories). Monocyte chemoattractant protein-1 (MCP-1), IL-6, VEGF, and TNF- $\alpha$  in CS were further quantified by ELISA. The expression profiles, in cocultures, of complement-associated genes, TNF- $\alpha$ , and angiogenesis-associated genes were analyzed by quantitative real-time PCR.

**RESULTS.** The production of MCP-1, IL-6, and VEGF was synergistically elevated when primary MPs or RAW264.7 cells and RPE cells were cocultured compared with those derived from sole cultures of MPs and RPE cells. The synergistic effect was confirmed without direct cell contact and was more prominent in the presence of LPS or TNF- $\alpha$ . TNF- $\alpha$  production by MPs was suppressed by RPE cells. Coculture of RPE cells with RAW264.7 cells increased the gene expression of *C3*, *CFB*, and *VEGF* genes, whereas it reduced those of complement regulatory factors *CFH*, *CD59*, *clusterin*, and TNF- $\alpha$  and antiangiogenic pigment epithelium-derived factor (*PEDF*).

**CONCLUSIONS.** Our findings indicate the presence of ingenious interactions between MPs and RPE cells that forces the inflammation and complement activation in the vicinity of RPE cells under pathologic conditions.

**Keywords:** age-related macular degeneration, coculture of macrophages with RPE cells, complement regulatory factors

Age-related macular degeneration is a leading cause of blindness in elderly persons in developed countries.<sup>1-3</sup> The pathology of AMD is characterized by abnormal extracellular deposits known as drusen, which accumulate beneath the RPE and along the Bruch's membrane.<sup>4,5</sup> Retinal pigment epithelium performs numerous important roles, such as phagocytosis, the formation of the blood-retinal barrier, and the secretion of VEGF or pigment epithelium-derived factor (PEDF).<sup>6-9</sup> It has long been theorized that RPE cells are a source of the material that accumulates in drusen.<sup>4,10,11</sup> Previous studies have reported that drusen recruits macrophages (MPs) to the sub-RPE space, where they are in close contact with RPE cells.<sup>12-15</sup> Numerous reports have shown that ocular-infiltrating MPs play a role in the pathogenesis of AMD-associated inflammation.<sup>13,16-21</sup> The proinflammatory cytokine environment triggers inflammatory responses around the RPE layer,<sup>22-25</sup> and when secreted into the posterior chamber, these inflammatory mediators seriously impair critical functions of the RPE.<sup>22,26,27</sup> Of note, a series of previous studies described the immunosuppressive effects of MPs in *in vivo* experiments, including the induction of immune privilege.<sup>28-30</sup>

Activated MPs are known to produce TNF- $\alpha$ , which is capable of stimulating the production of monocyte chemoattractant protein (MCP-1, Ccl2) via RPE cells, thus recruiting more MPs to the vicinity of the sub-RPE space.<sup>31,32</sup> In ocular inflamma-

tion, monocytes/MPs and T-lymphocytes are sources of TNF- $\alpha$ , which can subsequently activate RPE cells.<sup>22-24,33</sup> Tumor necrosis factor- $\alpha$  is reportedly capable of disrupting the RPE-cell barrier function, and there are indications that it prompts RPE cells to adopt a proinflammatory phenotype.<sup>1,22,24,34,35</sup> To the best of our knowledge, the findings of this study demonstrate for the first time that the coculture of RPE cells with peritoneal MPs or RAW264.7 cells results in a marked up-regulation of proinflammatory cytokines, such as MCP-1, IL-6, and VEGF, and complement-activating factors and down-regulation of TNF- $\alpha$ , PEDF, and complement regulatory molecules, thus suggesting the presence of the ingenious interaction between RPE cells and MPs.

## MATERIALS AND METHODS

### Materials and Mice

For the experiments conducted in this study, Hanks' balanced salt solution (HBSS), Dulbecco's modified Eagle's medium (DMEM), penicillin, and streptomycin were purchased from Life Technologies, Inc. (Grand Island, NY, USA). Male C57BL/6J mice (8-12 weeks old) were purchased from Shimizu Animal Technology Co., Ltd. (Kyoto, Japan), and they were maintained under specific pathogen-free conditions. In all experiments, the mice were



housed and treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Phase Contrast Microscopy

Phase contrast microscopy images were taken via the use of an inverted microscope system (CKX41; Olympus Corporation, Tokyo, Japan).

### Primary RPE Preparation and Passage

Eyes from euthanized healthy mice were enucleated and placed in HBSS on ice for 3 hours. The eyes were then cut with scissors along a circumferential line posterior to the ciliary process to create posterior eyecups. Using forceps, the retina was then gently peeled off from the posterior eyecups. After incubating in 0.2% trypsin for 1 hour at 37°C, the eyecups were placed in a 6-cm dish with some blots of DMEM, and the RPE was scraped off using the side of the forceps. For the purpose of making a single cell suspension, the tissues were triturated through a 22-gauge needle with a 10-mL syringe by drawing and releasing the fluid a few times. The tissues were then placed in a 15-mL tube and were washed with 3 mL DMEM, thus ensuring that all of the RPE cells were removed. The 15-mL tube was then centrifuged at 110g for 8 minutes, the culture supernatants (CSs) were removed, and the tube was washed a second time. The RPE cells were then plated into 6-well plates with 3 mL DMEM and incubated at 37°C for 7–10 days until reaching confluence.

Primary RPE cells taken from C57BL/6 mice, as described above, were cultured and propagated in DMEM containing 10% fetal bovine serum, 50 U/mL penicillin, and 50 µg/mL streptomycin in a humidified atmosphere at 37°C in 5% CO<sub>2</sub>. RPE cells at passages 5–10 were grown to 70%–90% confluence and then used.

### Resident Peritoneal MPs

Peritoneal cells (PCs) were harvested by injecting 5 mL ice-cooled HBSS without antibiotics into a peritoneal cavity of C57BL/6 mice. The collected PCs from three or more mice were plated into 96-well cell culture plates (Corning Japan, Tokyo, Japan) at  $5 \times 10^5$  cells/mL. After a 4-hour incubation, the culture plates were carefully washed three times, and the nonadherent cells were removed. The adhered cells were used as resident peritoneal MPs. Murine macrophage cell line RAW 264.7 was purchased from Riken Bio Resource Center (Ibaraki, Japan).

### Coculture of MPs and RPE

Immediately after preparation of the peritoneal MPs in the 96-well cell culture plates, RPE cells at a density of 176,000 cells/mL were added to the cultured MPs. They were then cocultured for 24 hours in the absence or presence of 100 ng/mL of *Escherichia coli* lipopolysaccharides (LPSs; Sigma-Aldrich Corp., St. Louis, MO, USA) or recombinant TNF-α (eBioscience, San Diego, CA, USA). After 24-hour incubation, the CSs were collected and stored at –80°C. For the culture in 24-well plates (Corning Japan),  $5 \times 10^5$  of peritoneal resident cells or RAW264.7 cells were seeded to the plates and cultured for 4 hours. After washing,  $2 \times 10^5$  of RPE cells were added and cultured for 24 hours. In a separate experiment, 700 µL RAW264.7 cells ( $5 \times 10^5$  cells/mL) in the lower chamber and 300 µL RPE cells ( $2 \times 10^5$  cells/mL) in the upper chamber were cocultured in Transwell (3460; Corning Japan) cell culture inserts for 24 hours to investigate the requirement of direct cell-to-cell contact. In all experiments, RAW264.7 cells were used at the passage between 5 and 10.

### Integrated Analysis of Cytokines by Bio-Plex

The CS were harvested after 24-hour culture and then immediately frozen and stored at –80°C until analysis. The cytokine levels in the CS were analyzed by Luminex Corporation (Austin, TX, USA) xMap Technology (Bio-Plex 200; Bio-Rad Laboratories, Inc., Hercules, CA, USA) with the Bio-Plex murine 23-Plex Panel Kit (Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions. The measured cytokines were as follows: eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17A, KC, MCP-1, MIP-1α, MIP-1β, and RANTES. Standard curves for each cytokine (in duplicate) were generated using the reference cytokine concentrations supplied in the kit and then used to calculate the cytokine concentrations in the CS.

### Enzyme-Linked Immunosorbent Assay

The CSs were harvested and centrifuged at 1580g at room temperature for 10 minutes to remove detached cells. CSs were collected and filtered through 0.22-µm filters (Millex-GV; EMD Millipore Corporation, Temecula, CA, USA). The ELISA kit for mouse TNF-α was obtained from eBioscience, the kit for VEGF was obtained from R&D Systems (Minneapolis, MN, USA), and the kits for MCP-1 and IL-6 were obtained from BD Biosciences (San Diego, CA, USA). The kit for PEDF was obtained from Cloud-Clone Corporation (Houston, TX, USA). The measurements were performed in quadruplicate and repeated as three independent experiments. The average of the quadruplicate is depicted.

### Quantitative Real-Time PCR

Cultured cells were lysed by use of QIAzol Lysis Reagent and then stored at –80°C until total RNA extraction. Total RNA was extracted by use of an miRNeasy Mini kit (QIAGEN N.V., Venlo, The Netherlands). The quality of the purified total RNA was analyzed by use of a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The cDNA was synthesized by use of a High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA, USA). Polymerase chain reaction was performed via TaqMan Fast Advanced Master Mix (Applied Biosystems) and TaqMan Gene Expression Assays (inventoried) (Applied Biosystems) under the following conditions: activation of enzyme at 95°C for 20 seconds, 40 cycles of denature at 95°C for 1 second, and annealing/elongation at 60°C for 20 seconds. The StepOnePlus Real-Time PCR system (Applied Biosystems) was used for PCR amplification and analysis. The levels of gene expression were normalized to that of 18S rRNA. Results are presented as  $2^{-\Delta\Delta Ct}$  (relative units of expression).

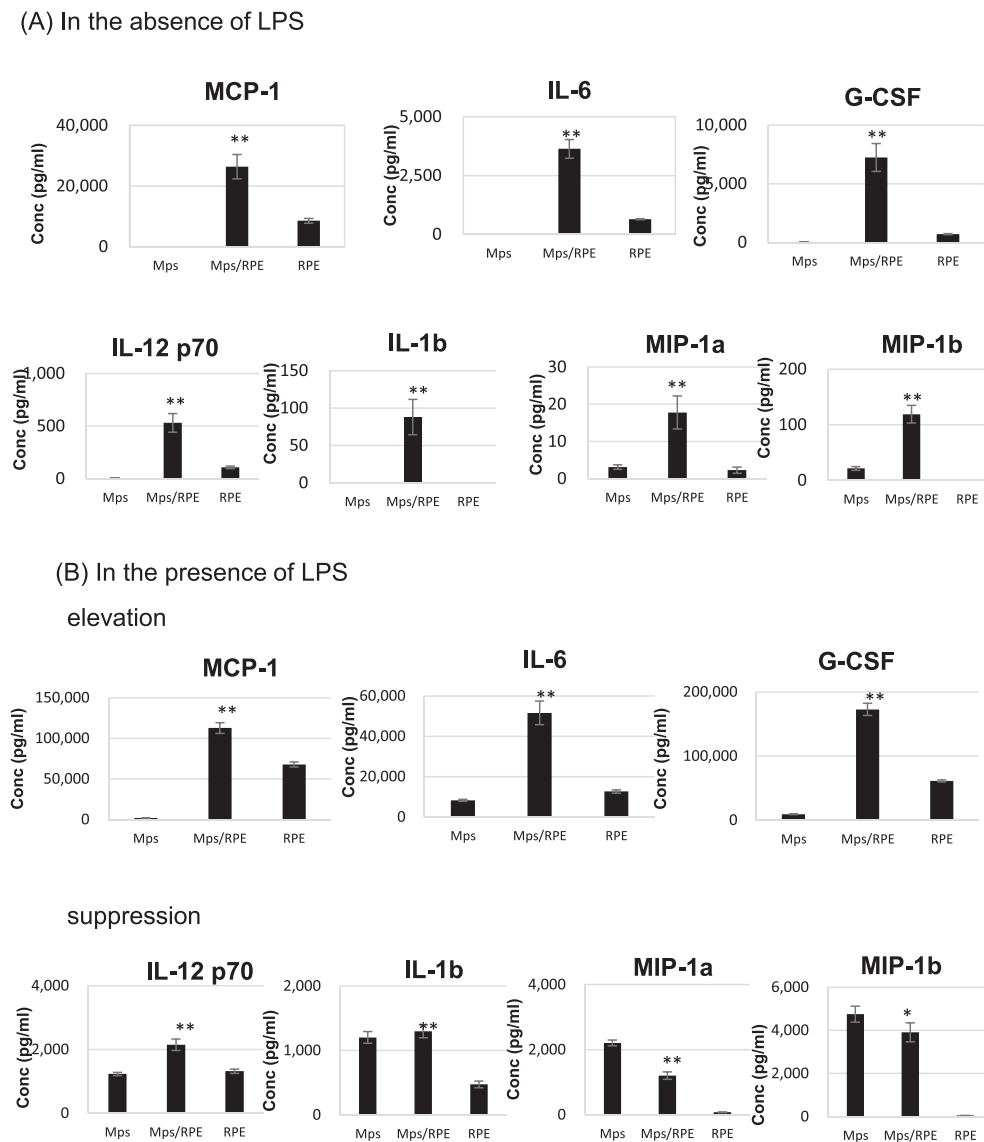
### Statistical Analysis

Statistical analyses were performed with the Student's *t*-test. Values shown in the figures are expressed as the mean ± SE. All reported *P* values were two sided, and *P* < 0.05 was considered statistically significant.

## RESULTS

### Integrated Analysis of Cytokines in Cocultures of MPs and RPE Cells

Using suspension array technology, protein levels of secreted cytokines and growth factors taken from CS of the MPs and RPE cell cocultures were examined. Of the 23 cytokines and



**FIGURE 1.** Synergistic elevation of cytokine production in cocultures of peritoneal resident MPs and murine RPE cells for 24 hours, analyzed by the Bio-Plex murine 23-Plex Panel Kit (Bio-Rad Laboratories). Production of cytokines in the absence (A) or presence (B) of LPS (0.1  $\mu\text{g}/\text{mL}$ ). A portion of the data is presented. \*\* $P < 0.01$ , \* $P < 0.05$ .

growth factors tested, MCP-1 and IL-6 were synergistically elevated in the coculture in the absence of LPS stimulation (Fig. 1A). Moreover, IL-1 $\beta$ , IL-12, MIP-1 $\alpha$ , and MIP-1 $\beta$  were also synergistically elevated (Fig. 1A). In the presence of LPS stimulation (0.1  $\mu\text{g}/\text{mL}$ ), the profiles of the interactions between these two cells were greatly varied (Fig. 1B); MCP-1, IL-6, and G-CSF were synergistically elevated, whereas IL-1 $\beta$ , IL-12, MIP-1 $\alpha$ , and MIP-1 $\beta$  were significantly reduced in the cocultures. All of the latter four cytokines reduced in the cocultures were mostly produced by MPs. Conversely, the former cytokines with synergistic elevation in the cocultures were produced in larger amounts by RPE cells than by MPs. Interleukin-1 $\alpha$ , IL-13, IFN- $\gamma$ , and KC exhibited only the additive increment in the coculture (data not shown).

#### Augmented Production of MCP-1, IL-6, and VEGF in Cocultures of Peritoneal MPs and RPE Cells

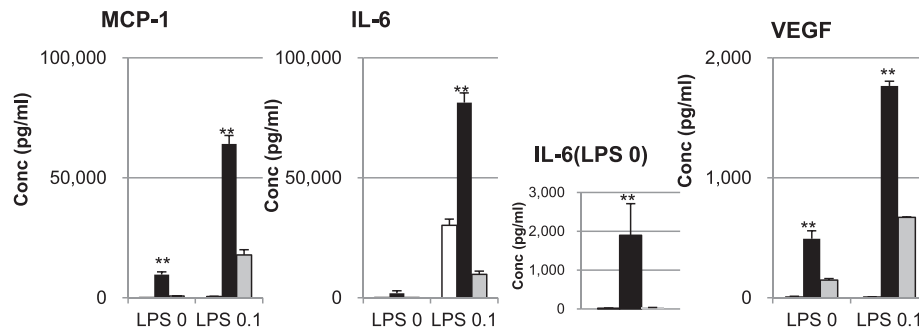
In consideration of the unique features of polarized production of cytokines in the above-described cocultures, we further

investigated in detail the influence of cocultures on the selected cytokines using the ELISA method. The production of MCP-1 and IL-6 was synergistically up-regulated in the coculture of primary peritoneal resident MPs and RPE cells than in the sole cultures of the MPs or RPE cells (Fig. 2A). This synergistic effect was confirmed in all cases when the RPE cells were between passages 4 and 24, although the production of MCP-1 and IL-6 was greatest when RPE cells were at passage 4 (Fig. 2B). Retinal pigment epithelial cells between passages 5 and 10 were used in all of the following coculture experiments with MPs. The synergies in the production of MCP-1 and IL-6 were also evident in the presence of LPSs in the cocultures (Fig. 2A).

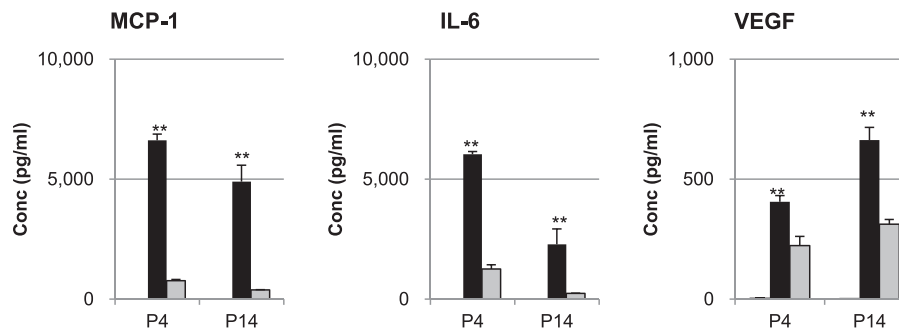
#### Augmented Production of MCP-1, IL-6, and VEGF in Cocultures of RAW 264.7 and RPE Cells

For all further analyses, it was a prerequisite to confirm the similar synergistic effect in coculture systems between MP cell lines with cultured RPE cells, due to the ethical problems of

## (A). Augmentation of the cytokine production assayed by ELISA.



## (B) Influence of the passages of RPE upon the cytokine production.



**FIGURE 2.** Synergistic elevation of cytokine production in cocultures of peritoneal resident MPs and murine primary RPE cells for 24 hours, analyzed by ELISA. (A) Production of cytokines in the absence or presence of LPS (0.1 µg/mL). (B) The efficacy of synergism was confirmed for murine primary RPE cells at the different culture passages. *White bar* represents MPs, *black bar* represents MPs/RPE, and *gray bar* represents RPE cells. \*\* $P < 0.01$ , \* $P < 0.05$ .

animal affairs and the contamination of other cells in the peritoneal MP cells. Mouse peritoneal MPs may contain dendritic cells, neutrophils, and other bone marrow-derived cells. With this in mind, we next investigated whether the synergistic effects observed would also be reproducible between the MP cell line RAW264.7 and RPE cells. The synergistically elevated production of MCP-1, IL-6, and VEGF was confirmed in the cocultures, both in the 96-well and 24-well culture plates (data not shown). Similar synergies for these three cytokines were clearly confirmed when coculturing RPE cells and RAW 264.7 were between passages 5 and 15 (data not shown). Therefore, RPE cells between passages 5 and 10 and Raw 264.7 cells between passages 5 and 10 were used in all of the following coculture experiments. The synergistically elevated production of MCP-1, IL-6, and VEGF were nearly at the same levels through the coculture periods from 24 to 72 hours (Fig. 3A). To investigate the necessity of direct contact between Raw264.7 cells and RPE cells in the observed elevated production of MCP-1, IL-6, and VEGF, the coculture of both cells in the Transwell system was explored. The cocultures also exhibited marked upregulation of MCP-1, IL-6, and VEGF (Fig. 3B). These observations indicate that secreted factors may play critical roles in the induction of the synergy observed.

To clarify whether these cytokines are secreted from RPE cells or MPs, quantitative PCR (qPCR) in transwell coculture experiments was performed. Gene expression of MCP-1 and IL-6 was up-regulated only in RPE cells cocultured (13.1 and 9.5 times elevation, respectively), whereas the expressions in RAW264.7 cells were nearly null (the standard 1.0 was assigned for the expression of each cytokine in RPE cells without coculture). The elevated production of VEGF only by RPE cells, but not RAW264.7 cells, was confirmed, but the expression

was elevated only by 50%. The results indicate that the cytokines produced mainly by one cell, either RPE cells or MPs, did not change these profiles even in cocultures.

### Tumor Necrosis Factor- $\alpha$ -Stimulated Production of MCP-1, IL-6, and VEGF in the Cocultures

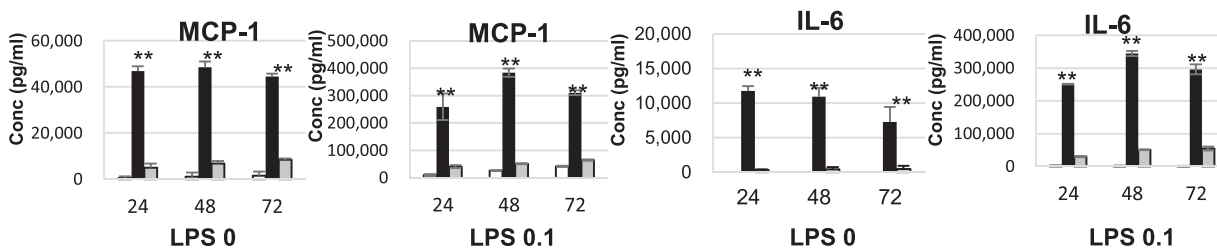
Lipopolysaccharide is a major component of the cell membrane of gram-negative bacteria and triggers Toll-like receptor 4 (TLR4) activation; however, it may not directly play a role in the pathogenesis of AMD. Thus, we next investigated the effect of TNF- $\alpha$  in the modulation of MCP-1, IL-6, and VEGF production. Tumor necrosis factor- $\alpha$  is a representative cytokine produced by MPs triggered by TLR4. We found that the production of IL-6 and VEGF was synergistically increased in the presence of TNF- $\alpha$  (Fig. 4). However, the production of MCP-1 in cocultures was not elevated more than the sole culture of RPE cells, because TNF- $\alpha$  stimulation itself strongly elevated the production of MCP-1 by RPE cells. The addition of IL-6, instead of TNF- $\alpha$ , induced neither elevation nor reduction of MCP-1 and VEGF (data not shown).

### Nondegenerated RPE Cells Reduced the Production of TNF- $\alpha$ by MPs

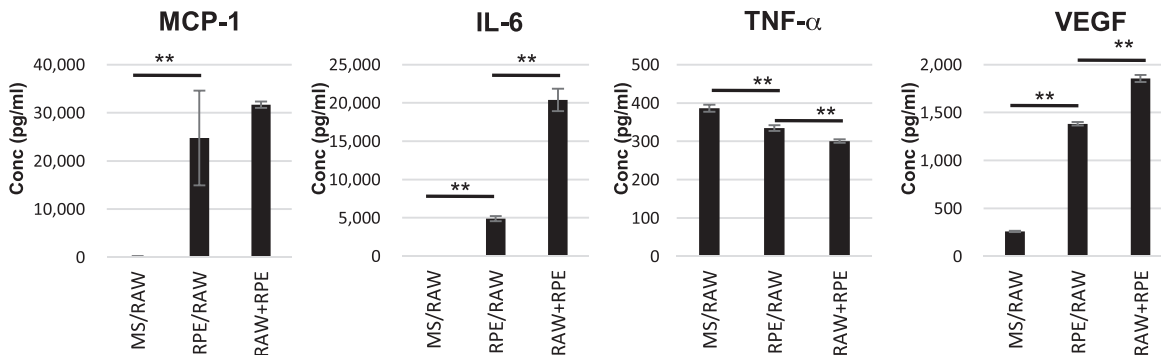
The production of TNF- $\alpha$  from MPs has long been well known, and it is stimulated by LPS via TLR4 triggering. In this study, we observed the remarkable reduction of LPS-stimulated TNF- $\alpha$  production by RPE cells. Retinal pigment epithelial cells reduced the LPS-stimulated production of TNF- $\alpha$  by MPs (Fig. 5A). This result was also confirmed when LPS-stimulated



(A) Co-culture periods



(B) Transwell®



**FIGURE 3.** Synergistic elevation of cytokine production in cocultures of murine MP cell line RAW264.7 cells and murine primary RPE cells. (A) Confirmation of the synergy between RAW264.7 cells and murine primary RPE cells at prolonged culture periods (24, 48, and 72 hours, RPE cells and RAW 264.7 cells, passages P12, P5). (B) The synergies between RAW264.7 cells and RPE cells were also confirmed in the Transwell system (Corning Japan). A total of 700  $\mu$ L RAW264.7 cells ( $5 \times 10^5$  cells/mL) in the lower chamber and 300  $\mu$ L of RPE cells ( $2 \times 10^5$  cells/mL) in the upper chamber were cocultured for 24 hours. White bar represents RAW264.7 cells, black bar represents RAW264.7/RPE cells, and gray bar represents RPE cells. \*\* $P < 0.01$ , \* $P < 0.05$ .

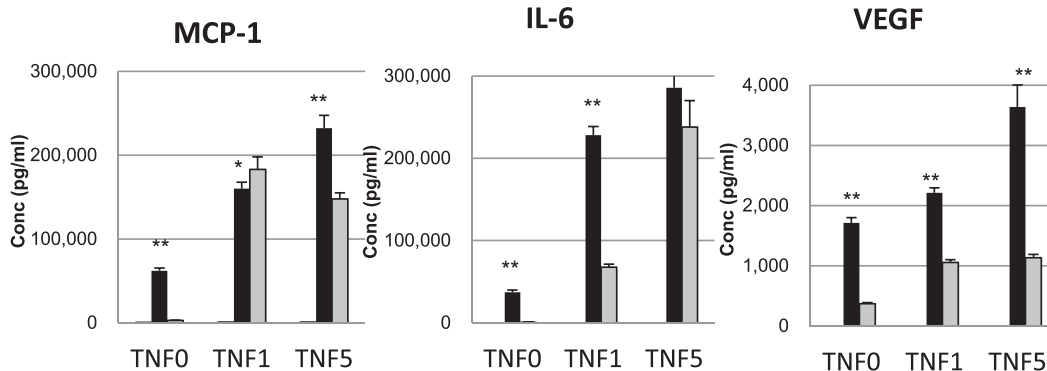
RAW264.7 cells were used throughout the culture between 24 and 72 hours (Fig. 5B). The reduction was found to depend on the number of RPE cells added to the coculture (Fig. 5C), indicating the absolute dependence of this suppressive effect on RPE cells. Consistent with this observation, we also observed the remarkable reduction of LPS-stimulated TNF- $\alpha$  gene expression by RPE cells. Retinal pigment epithelial cells reduced the LPS-stimulated gene expression of TNF- $\alpha$  by MPs both in the presence and absence of 0.1  $\mu$ g/mL of LPS (Fig. 5D). These observations suggest that the production of TNF- $\alpha$

from MPs may be strictly regulated by immune-regulatory RPE cells under the homeostatic physiologic condition.

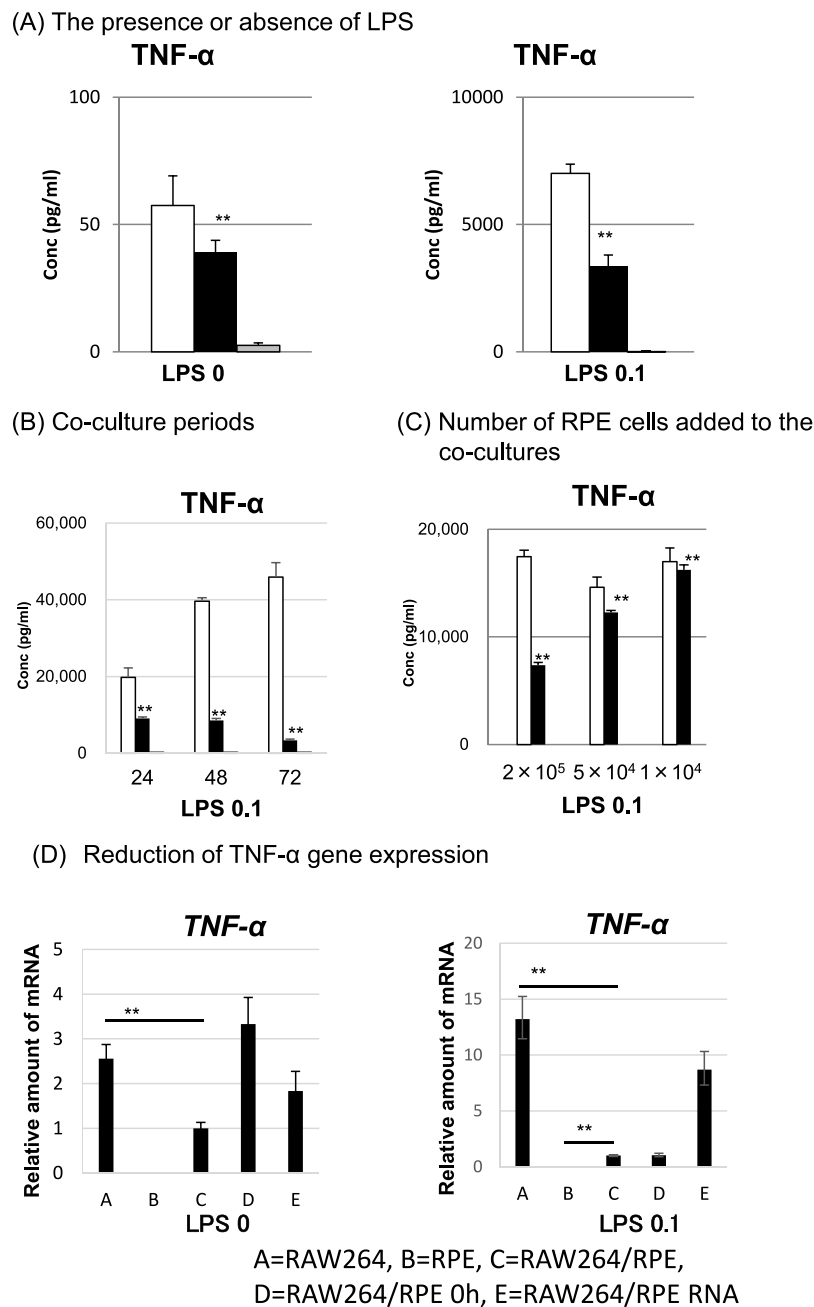
**Up- Versus Down-Regulated Gene Expression of Complement- or Angiogenesis-Associated Factors in the Cocultures of RPE Cells and MPs**

The alternative complement pathway (APC) is known to be an important component of the pathogenesis of AMD. It has been reported that the decay of complement regulators can potentially impair complement regulation on RPE cells, subsequently

**Production of cytokines in the presence of TNF- $\alpha$**



**FIGURE 4.** Tumor necrosis factor- $\alpha$ -stimulated synergistically elevated production of MCP-1, IL-6, and VEGF in cocultures of RAW264 cells and murine primary RPE cells. Cultured for 24 hours; 24-well culture plates; RPE cells and RAW 264.7 cells, passages: P10, P13. TNF 1, 5, TNF- $\alpha$  1 or 5 ng/mL in the cultures. \*\* $P < 0.01$ .



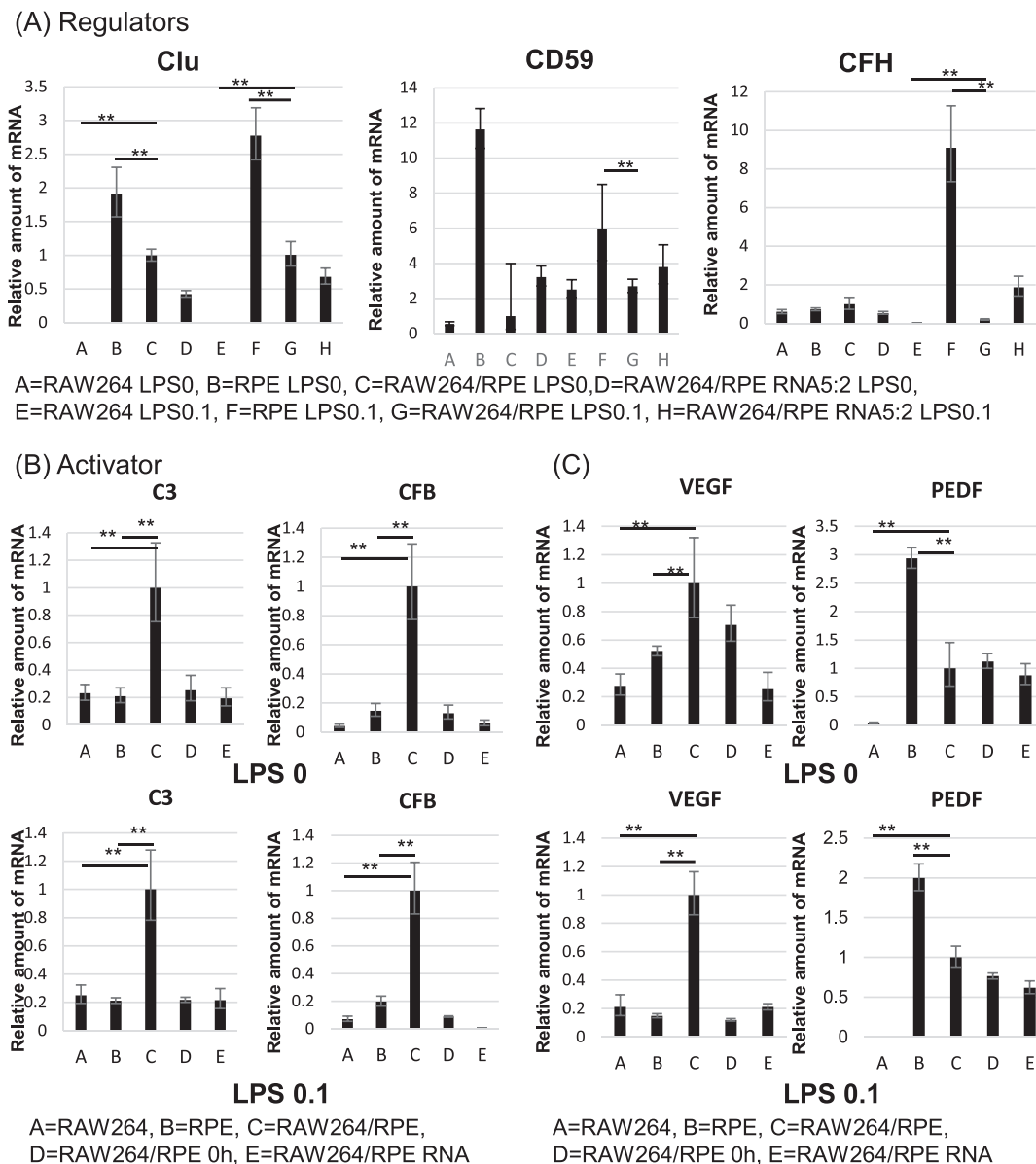
**FIGURE 5.** Nondegenerated RPE cells reduced the production of TNF- $\alpha$  by MPs in the coculture with RPE cells. (A) The reduction by RPE cells (24 hours, 96-well culture plates, RPE cells and peritoneal resident MPs, either nonstimulated or stimulated with LPS). (B) The reduction by RPE cells during the cultures in the presence of LPS (0.1  $\mu$ g/mL) for 24 to 72 hours (RPE cells and RAW 264.7, passages: P12, P5). (C) The reduction induced by RPE cells was dependent on RPE cell numbers added onto the cocultures in the presence of LPS (0.1  $\mu$ g/mL) (24 hours, RPE cells and RAW 264.7, passages: P7, P10). (D) Regulated gene expression of TNF- $\alpha$  in co-cultures of RPE cells and RAW 264. Results are presented as  $2^{\Delta\Delta Ct}$  (relative units of expression; cocultures: 24 hours, RPE cells and RAW 264.7, passages: P9, P12). \*\* $P < 0.01$ . All were performed in 24-well culture plates. LPS 0.1, LPS 0.1  $\mu$ g/mL. \*\* $P < 0.01$ .

contributing to lesion formation in the outer retina and Bruch's membrane.<sup>36</sup> Therefore, we investigated the alteration of the gene expression of C3 and CFB as factors participating in the activation of APC and CFH, CD59, and Clu as factors regulating APC activation. Surprisingly, the coculture of RPE cells with RAW264.7 cells greatly elevated the gene expression of *C3* and *CFB* (Fig. 6B). On the contrary, the expression of the complement regulators investigated (i.e., *CFH*, *CD59*, and *Clu*) was strikingly down-regulated in the coculture, irrespective of the absence or presence of LPS (Fig. 6A). In addition, a contrasting effect of the

cocultures was confirmed for the augmented and reduced gene expression of *VEGF* and *PEDF* (Fig. 6C).

### DISCUSSION

Immuno-histochemical analysis has implicated that MPs play a critical role in the pathogenesis of AMD,<sup>37</sup> and numerous studies have reported that ocular-infiltrating MPs play an active role in the pathogenesis of AMD-related inflammation.<sup>13,16,17</sup> However, there are conflicting opinions as to whether or not



**FIGURE 6.** Regulated gene expression of complement-activating or regulating factors and angiogenesis-associated factors in cocultures of RPE cells and RAW 264.7. **(A)** Complement regulatory factors CFH, CD59, and clusterin. **(B)** Complement activating factors C3 and CFB (complement factor B in alternative pathway of complement activation). **(C)** Angiogenesis-associated factors, VEGF and PEDF. The levels of gene expression were normalized to that of 18S rRNA. Results are presented as  $2^{\Delta\Delta Ct}$  (relative units of expression; cocultures: 24 hours, RPE cells and RAW 264.7, passages: P9, P12).  $^{**}P < 0.01$ . RAW264/RPE: RAW264.7 ( $5 \times 10^5$  cells) and RPE ( $2 \times 10^5$  cells) were cocultured for 24 hours, and RNA was extracted. RAW264/RPE 0h: RAW264.7 ( $5 \times 10^5$  cells) and RPE ( $2 \times 10^5$  cells) were mixed, and RNA was extracted immediately without culture. RAW264/RPE RNA: RAW264.7 ( $5 \times 10^5$  cells) and RPE ( $2 \times 10^5$  cells) were cultured separately for 24 hours, and then extracted RNA was mixed at the ratio of 5:2 and qRT-PCR was performed. RAW264: Cells were cultured at the cell density of  $5 \times 10^5$  cells/mL, and thereafter RNA was extracted. RPE: Cells were cultured at the cell density of  $2 \times 10^5$  cells/mL, and thereafter RNA was extracted. LPS 0.1, LPS 0.1  $\mu\text{g/mL}$ .

the accumulation of dysfunctional subretinal MPs results in retinal degeneration.

Our results appear to contradict those of previous in vivo experiments by directly injecting LPS into the ocular microenvironment,<sup>28-30</sup> as they showed that MPs can suppress the intraocular inflammation and that the inflammation mediated by the injection of LPS lasts only 48 hours, with the eye re-establishing immune privilege. Our preliminary data showed that the observed augmented production of MCP-1, IL-6, and VEGF is mostly born by M1 MPs and not by M2 induced by IL-4 (Supplementary Fig. S1). In this context, our results are not contradictory. The other possibility is that the cultured RPE

might not functionally be the same as the intact RPE monolayer in vivo.

It has been reported that the proinflammatory cytokine milieu triggers and aggravates inflammatory responses around RPE layers.<sup>22-25</sup> However, and to the best of our knowledge, coculture system investigation between MPs and RPE cells has been scarcely reported, except for the very recent report by Devarajan et al.,<sup>21</sup> which appeared during the course of the preparation of this manuscript. There have been many published studies describing the effect of MP CSs, but not the study of cocultures. In this study, the elevated production of MCP-1, IL-6, and VEGF was repeatedly confirmed in

cocultures, indicating that the interaction between recruited MPs and RPE cells may function to aggravate the ocular inflammation. The synergistically enhanced cytokine production may be mediated by the production of third cytokines, mediators, and/or exosomes. The result that the elevated production of IL-6 and VEGF was partly reconstituted by TNF- $\alpha$  (Fig. 4) supports this hypothesis, but further precise extensive studies will be needed to validate the hypothesis.

It has been reported TNF- $\alpha$  is a key mediator of ocular inflammation and that its interaction with RPE cells may be a driving force in vitreoretinal disorders.<sup>21</sup> Hence, the present finding that RPE cells down-regulate the production of TNF- $\alpha$  and the gene expression TNF- $\alpha$  by TLR4-triggered MPs is of interest (Fig. 5). In addition, preliminary experiments by our group indicate that the degeneration of RPE cells induced by oxidized lipid products result in the failure to down-regulate TNF- $\alpha$  production by MPs (Ito EI, unpublished data, 2016). These observations suggest that the production of TNF- $\alpha$  from MPs may be regulated by nondegenerated RPE cells under the homeostatic physiologic condition. However, under pathologic conditions,<sup>38</sup> such as exposure to oxidative stress involving oxidized lipid products, this down-regulation of TNF- $\alpha$  will be broken up, causing the augmented infiltration of MPs and the production of a far higher amount of MCP-1, IL-6, and VEGF by RPE cells to aggravate AMD pathogenesis.

Macrophages can reportedly be polarized to exhibit either proinflammatory M1 or proangiogenic M2 phenotypes.<sup>39,40</sup> The pathologic shift away from protective M2 toward proinflammatory M1 may be implicated in the pathogenesis of diverse tissue inflammatory diseases<sup>41-43</sup> and AMD,<sup>44</sup> specifically in the early stages of the disease.<sup>18-20</sup> However, in later stages, M2 may actually play a harmful role by promoting fibrosis and angiogenesis.<sup>20,44</sup> Moreover, it has been reported that VEGF production is closely associated with M2<sup>45,46</sup> and that the profibrotic roles of M2 may accelerate neovascularization and fibrosis.<sup>20</sup> For the interpretation of the findings presented here, further detailed investigations will be needed to elucidate the role of distinctly polarized MPs.

Dysregulated complement activation is thought to play one of the central roles in the pathogenesis of AMD.<sup>47</sup> The coculture of RPE cells and RAW264.7 cells resulted in the marked up-regulation of the complement activating factor genes *C3* and *CFB*, whereas the complement regulatory factor genes *CFH*, *CD59*, and *Clu* were strikingly down-regulated (Figs. 6A, 6B). The striking contrast of the gene expression by RPE cells between VEGF and PEDF (Fig. 6C) is also remarkable when considering the critical roles of these two factors in the later stage of AMD.<sup>48,49</sup> Accordingly, we postulate the presence of an ingenious interaction between RPE cells and MPs. In this context, these observations provide us with the hypothesis that pathologically degenerated RPE cells, with reduced ability to down-regulate TNF- $\alpha$  production, and the infiltrated MPs, which in turn might be activated in an amplification loop of APC by complement C3 and CFB,<sup>50</sup> may orchestrate the pathologic microenvironments.

In conclusion, the findings in this study demonstrate that TNF- $\alpha$  derived from MPs and MCP-1 derived from RPE cells, together with the components of APC, may orchestrate an ingenious interaction between infiltrated MPs and RPE cells to aggravate inflammatory changes in the subretinal space. The aggravated inflammatory changes may be modulated by the disordered activation of complement in the subretinal RPE layers.

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