

Detection of Complement Activators in Immune Attack Eyes After iPS-Derived Retinal Pigment Epithelial Cell Transplantation

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PURPOSE. To determine whether human induced pluripotent stem (iPS) cell-derived retinal pigment epithelial (RPE) cells (iPS-RPE) can express complement factors.

METHODS. To confirm expression of complement factors in human iPS-RPE cells, we performed flow cytometry, immunohistochemistry, ELISA, and quantitative RT-PCR for the following: C3, C5, CFB (Factor B), C5b-9 (membrane attack complex [MAC]), CFH (Factor H), CFI (Factor I), CD46, CD55, CD59, clusterin, and vitronectin. We also prepared iPS-RPE cells in the presence of recombinant IFN- γ , recombinant TNF- α , lipopolysaccharide, supernatants of naïve T cells, and T helper 1 (Th1) cells. For the transplantation, after preparation of iPS-RPE cells from cynomolgus monkeys, the iPS-RPE cells (allografts) were transplanted into the subretinal space in monkeys. After surgery, monkeys were euthanized for IHC evaluation of the retinal section and determination of complement factors (C3, C5, CFB, MAC, and C1q), cytokines, and immunoglobulin G (IgG).

RESULTS. Human iPS-RPE cells expressed complement activators and inhibitors. iPS-RPE cells highly expressed complement factors during inflammatory conditions, especially IFN- γ exposure including Th1 cell supernatants. In immune attack eyes after allogeneic iPS-RPE cell transplantation, complement activators such as C3, CFB, C5, and MAC were detected around the host RPE layer, grafted RPE cells, inflammatory retinal lesions, and transplanted subretinal space. In addition, we observed a large number of C1q and IgG double positive and IFN- γ positive inflammatory cells in the retinal sections.

CONCLUSIONS. iPS-derived RPE cells greatly expressed complement factors. Thus, RPE cells might be activated and produce complement factors after exposure to infiltrating inflammatory cells in the eye.

Keywords: complement factors, iPS cells, inflammation, retinal transplantation

Complement activation is involved in many retinal diseases, such as age-related macular degeneration (AMD). Complement activation has also been implicated in the pathology of the retinal pigment epithelium (RPE) in retinal diseases. Several research groups have previously shown that a number of complement activators and regulators (inhibitors) can be produced locally in the eye, with RPE cells the major source of complement expression at the retina-choroid interface and subretinal space.¹⁻⁴ Representative complement activators include complement component 3 (C3), complement component 5 (C5), complement factor B (CFB: Factor B), and C5b-9 (MAC). In addition, representative complement inhibitors include complement factor H (CFH: Factor H), complement factor I (CFI: Factor I), CD46 (membrane cofactor proteins [MCP]), CD55 (decay-accelerating factor [DAF]), CD59, clusterin, and vitronectin. Inflammatory mediators regulate the expression of complement components by RPE cells.¹⁻⁴ After an infection (e.g., bacterial, viral), in vivo RPE cells and retinal microglia can produce inflammatory cytokines and complement activators, which indicates that these are involved in the immunological defense in the retina. To protect the eye from infection, ocular tissues (including the RPE cells) express

inflammatory factors such as complement proteins. Moreover, the RPE cells also produce complement inhibitors that are designed to stop complement activation.

Many studies examined the relationship between patients with AMD and complement factors have been published in the literature.⁵⁻⁸ Furthermore, AMD has recently been considered a chronic inflammatory disorder. Genome-wide association studies (GWAS) have suggested that dysregulated complement may play a central role in AMD pathogenesis, that is, genetic polymorphism in Factor H and ARMS2/HTRA1 may indicate a risk of AMD in patients.⁶ GWAS in Japan have also reported finding a relationship between AMD and the novel gene *TNFRSF10A*.⁹ Additionally, it has been shown that Factor H risk alleles, smoking history, and hypertension can affect standard treatments, for example, changes in the mid-term response to ranibizumab in exudative AMD patients.¹⁰ Moreover, older age, smoking, and ultraviolet lights can promote complement activation. Thus, it appears that the pathogenesis of AMD is associated with chronic local retinal inflammation, including continual complement activation. However, it remains unknown whether human RPE cells—including induced



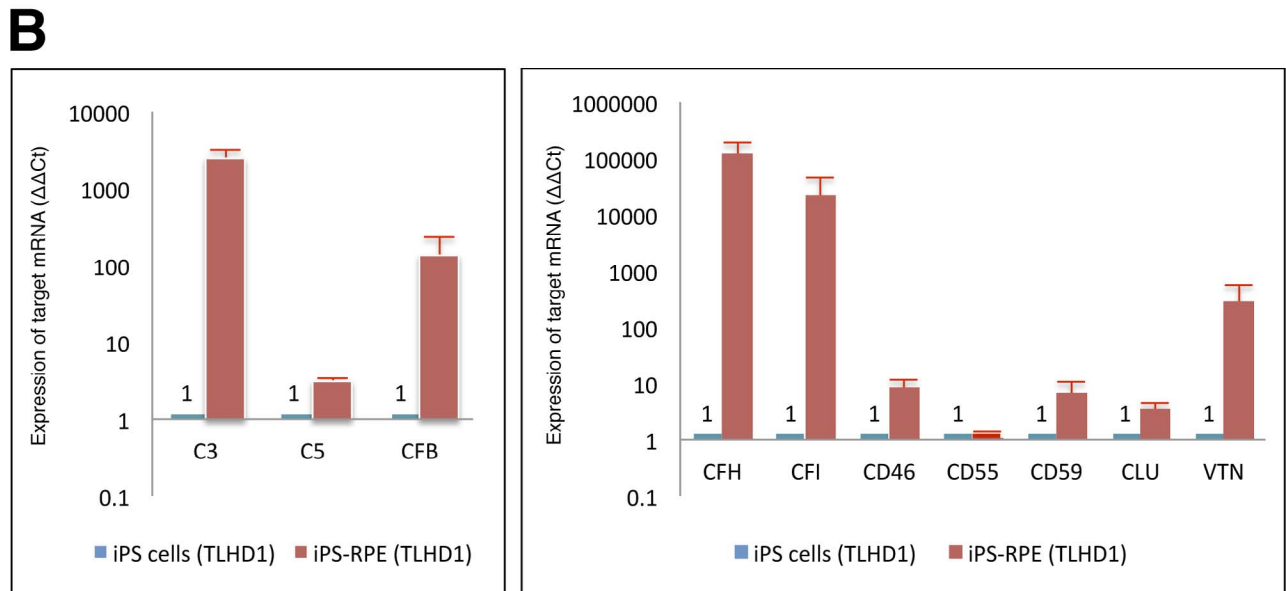
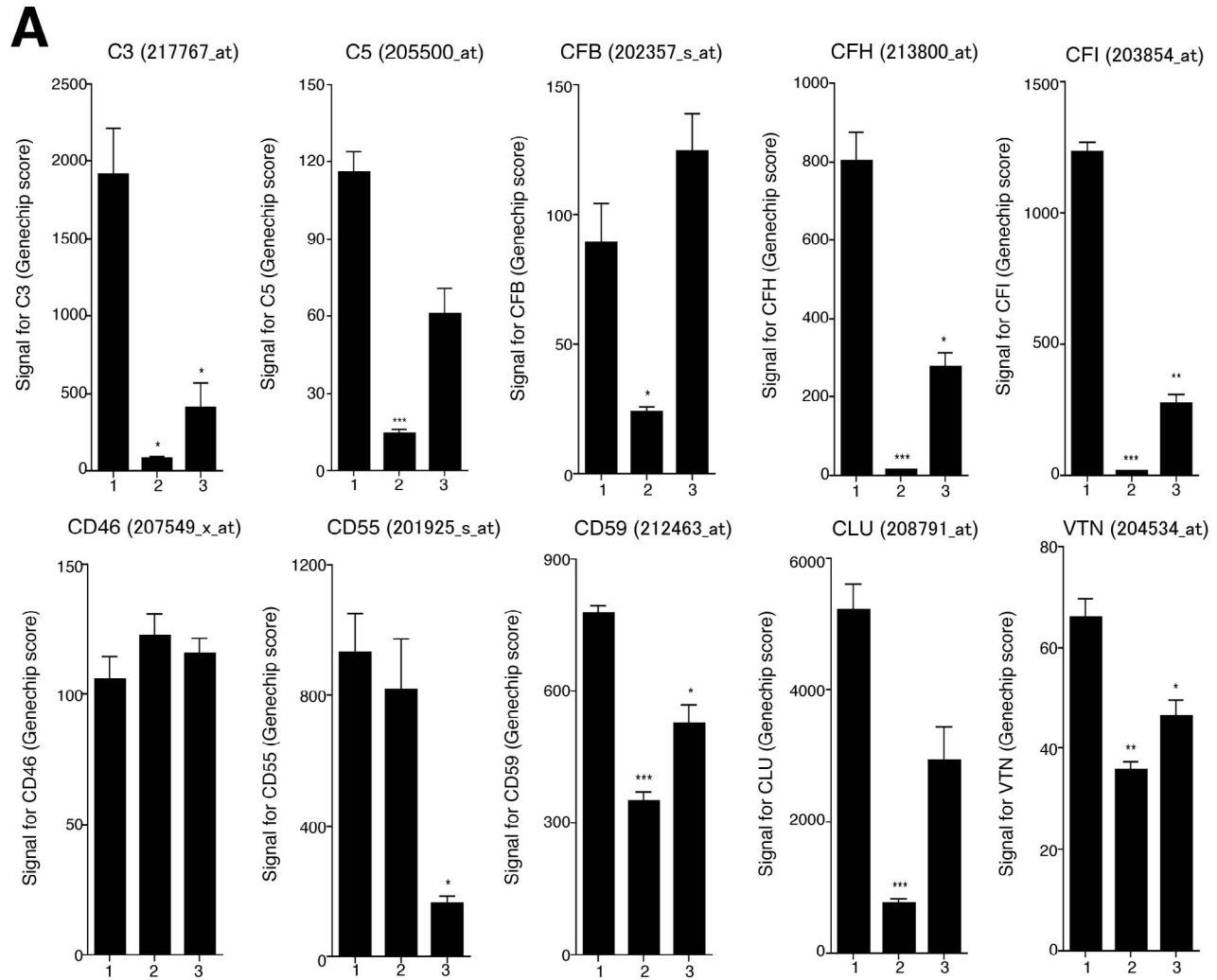


FIGURE 1. Expression of mRNA for complement factors in human iPS cell-derived RPE cells. **(A)** RNA for 59 iPS-RPE cells ($n = 6$) were harvested and analyzed by the GeneChip microarray and the data for several complement factors were collected. For the controls, RNA from 59 iPS cells ($n = 5$) and human primary RPE cells ($n = 3$) were also analyzed. (1) iPS-RPE cells, (2) iPS cells, (3) primary RPE cells. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, when compared to the data of the iPS-RPE cells. **(B)** Expression of complement factor genes in iPS-RPE cells during qRT-PCR analysis. *Right panel:* complement activators (C3, C5, CFB). *Left panel:* complement inhibitors (CFH, CFI, CD46, CD55, CD59, clusterin [CLU], vitronectin [VTN]). iPS cells (TLHD1) were also prepared for use as the controls. Results indicate the relative expression ($\Delta\Delta C_t$: iPS cells = 1). Data are shown as mean \pm SEM of three PCR determinations. Data are representative of three experiments performed with the TLHD1 iPS-RPE cells (all similar patterns).

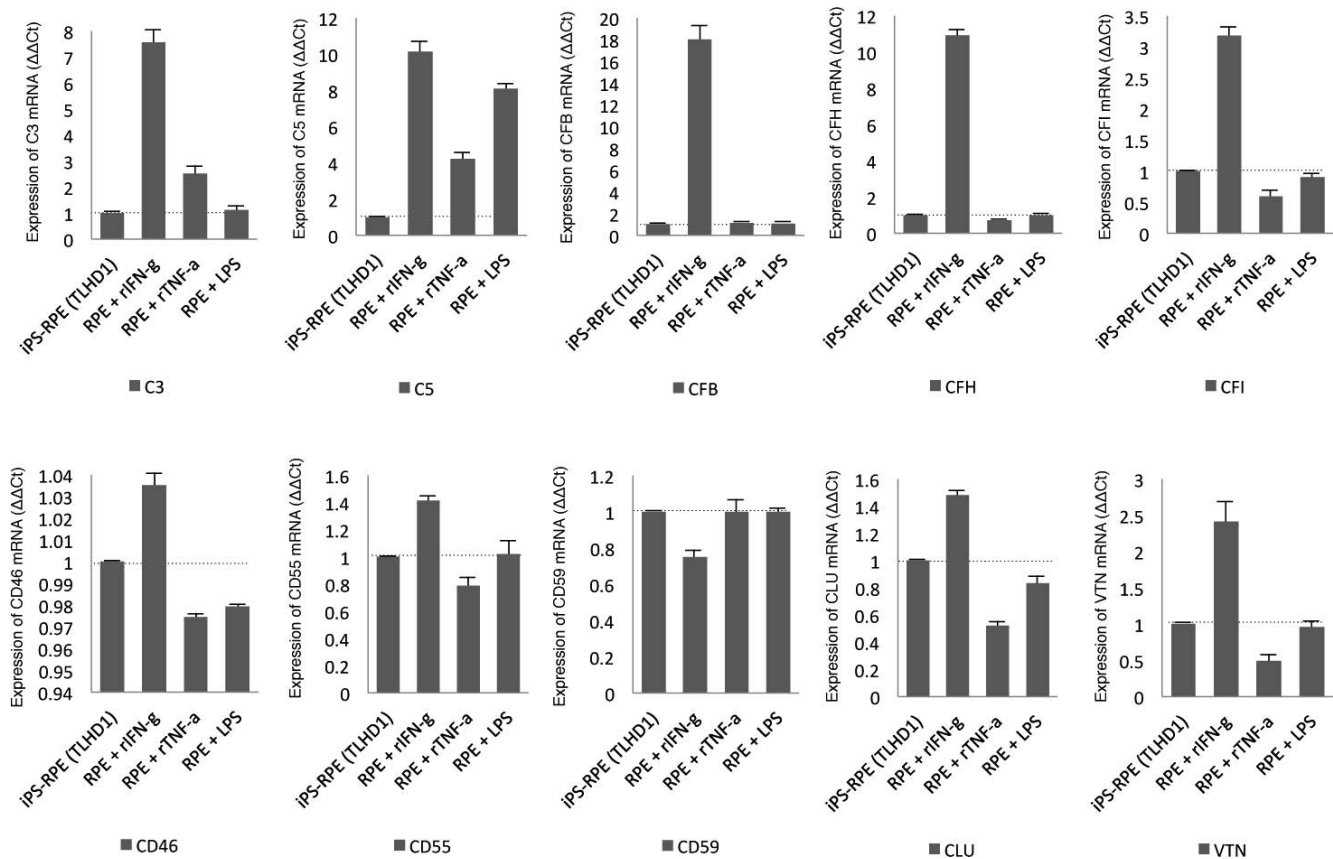


FIGURE 2. Expression of mRNA for complement factors in human iPS-RPE cells in the presence of inflammatory proteins. Expression of complement factor genes in iPS-RPE cells (TLHD1) in the presence of recombinant IFN- γ , recombinant TNF- α , and lipopolysaccharide (LPS) during qRT-PCR analysis. Results indicate the relative expression ($\Delta\Delta C_t$: iPS-RPE cells without proteins = 1). Data are shown as mean \pm SEM of three PCR determinations. Data are representative of three experiments that used other human iPS-RPE lines (all similar patterns).

pluripotent stem (iPS) cell-derived RPE cells—are able to express complement factors.

Previously, several investigators have reported that RPE cells express complement factors^{1–4} and that there is a relationship between the complement alternative pathway and RPE cells in AMD patients, which suggests there is activation of a complement alternative pathway. In addition, allogeneic iPSCs-derived RPE cell transplantation might be due to the activation of the complement classical pathway, as the transplanted animal models exhibit B-cell associated inflammation,¹¹ as well as T cell and antigen presenting cell immunity.¹² Therefore, it is possible that the pathogenesis of AMD is associated with complement activation and that this activation in the retina might be upregulated by the allogeneic immune responses that occur after RPE transplantation.

In this study, we examined iPS cell-derived RPE cells, as well as primary RPE cells, and investigated the expression and secretion of complement factors. Furthermore, we also investigated whether complement could be detected in retinal sections from immune attack eyes after iPS cell-RPE cell transplantation in an experimental in vivo animal model.

MATERIALS AND METHODS

Establishment of Human iPS Cell-Derived RPE Cells

The human iPS cells and the iPS cell-derived RPE cells were established from normal healthy donors (HD), as previously

described.^{13,14} For the assay, iPS cell-derived RPE cells were used (TLHD1 or 454E2 lines), while iPS cells (TLHD1 or 454E2 lines) and TLHD1 fibroblasts were prepared for use as controls. Other human RPE lines, such as 59 iPS-RPE (control: 59 iPS cells), were used for the GeneChip analysis.¹⁵ Primary cultured human RPE cells were also prepared for use as a control. Detailed information on the RPE-specific medium and signal inhibitors has been described in our previous studies.^{13,14} This current research study followed the tenets of the Declaration of Helsinki, and the study was approved by the Institutional Ethics Committee of the Center for Developmental Biology (now Center for Biosystems Dynamics Research), RIKEN.

Preparation of Monkey iPS-RPE Cells and Transplantation of RPE Cells Into Monkey's Eyes

For the transplantation, we prepared iPS cells from a normal cynomolgus monkey (*Macaca fascicularis*), 1121A1 iPS cells from a HT-1 MHC homozygote monkey, and 46a iPS cells from a Cyn46 MHC heterozygote monkey, as previously described.¹² Monkey RPE cells were then established from these iPS cells.

The normal control monkeys (S2-4, K-247, S3-2, TLHM1, TLHM6, and Iwana) used for the transplantation were purchased from Shin Nippon Biomedical Laboratories, Ltd. (Tokyo, Japan) and Eve Bioscience Ltd. (Wakayama, Japan). The iPS-RPE cells/sheets (all allografts) were transplanted into the subretinal space, as previously described.¹² For transplantation of iPS-RPE cells, a complete vitrectomy was performed after the animals were anesthetized. The transplanted monkeys were euthanized at 4, 12, and 16 weeks or at 6 months after

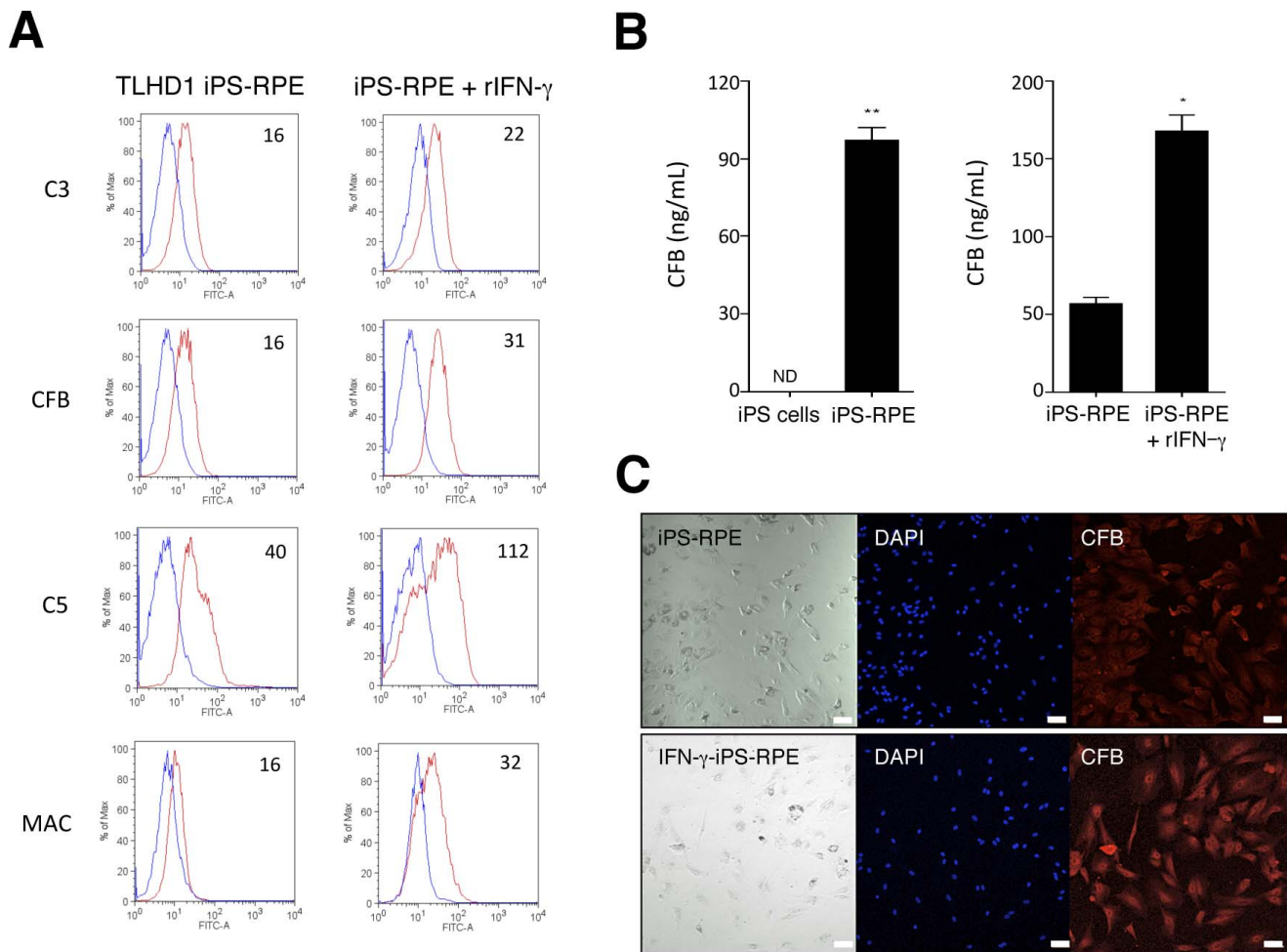


FIGURE 3. Detection of complement activators in human iPS-RPE cells. Expression of complement factors genes and proteins on iPS-RPE cells in the presence of recombinant IFN- γ by FACS analysis, ELISA, and immune staining. **(A)** We prepared iPS-RPE cells and recombinant IFN- γ treated iPS-RPE cells for the expression of C3, CFB (Factor B), C5, and membrane attack complex (MAC) in FACS analysis. Number in the graph indicates mean fluorescence intensity. Blue histograms represent isotype control staining. Data are representative of three experiments. **(B)** Supernatants of iPS-RPE cells ($n = 3$) and iPS cells ($n = 3$) were collected and measured CFB (Factor B) by ELISA. Supernatants of iPS-RPE cells ($n = 3$) and recombinant IFN- γ treated iPS-RPE cells ($n = 3$) were also collected. Data are means \pm SEM of three ELISA determinations. * $P < 0.05$, *** $P < 0.005$, as compared to two groups. ND, not detected. **(C)** Photomicrographs showing labeling of iPS-RPE cells (upper) and recombinant IFN- γ treated iPS-RPE cells (lower) with anti-human CFB antibody (red). Cell nuclei were counterstained with DAPI (blue). Scale bars: 50 μ m.

the surgery to evaluate the retinal section by immunohistochemistry (IHC). The care and maintenance of the monkeys conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the Use of Laboratory Animals, as well as to the Guidelines of the RIKEN CDB Animal Experiment Committee.

IHC

After collecting the transplanted monkeys' eyes at 4, 12, and 16 weeks or at 6 months, specimens were fixed and embedded in paraffin (Sigma-Aldrich Corp., St. Louis, MO, USA). As a positive control for the section, experimental autoimmune uveitis (EAU) models in primate were immunized using bovine interphotoreceptor retinoid binding protein (IRBP) peptides, as has been described in a previous report.¹⁶ After we found inflammation in the retina and the choroid (10 weeks after immunization), we prepared the retinal sections as a positive control.

Paraffin sections were sliced into 10 μ m-thick sections and prepared as a series of five sequential slides through the use of an auto slide preparation system (Kurabo Techno System Ltd.,

Osaka, Japan). Sections were blocked with 5% goat serum in PBS for 1 hour at room temperature. Primary antibodies against the following antigens were added: C1q (host: mouse, Abcam, Cambridge, UK), C3 (host: goat, R&D Systems, Minneapolis, MN, USA), C5 (host: rabbit, Abcam), CFB (Factor B; host: goat, R&D Systems), MAC (host: mouse, Dako, Glostrup, Denmark), immunoglobulin G (IgG; host: rabbit, Abcam), and IFN- γ (host: mouse, R&D Systems). All antibodies were incubated in cultures at 4°C overnight. After rinsing with Tween 20 in PBS three times, sections were incubated with secondary antibodies for 1 hour at room temperature and counterstained with DAPI (Life Technologies, Carlsbad, CA, USA). Images were acquired with a confocal microscope (LSM700; Zeiss, Jena, Germany).

Expression of CFB (Factor B) in cultured iPS-RPE cells (TLHD1) was evaluated by IHC. Cultured iPS cell-derived RPE cells or IFN- γ pretreated RPE cells were fixed with 4% PFA-PBS for 15 minutes at room temperature, washed three times with PBS, and permeabilized with 0.3% Triton X-100-PBS. Anti-human CFB antibody (R&D Systems) was used as the primary antibody, and anti-goat IgG was used as the secondary antibody (Supplementary Table S2). Cell nuclei were counterstained with DAPI.

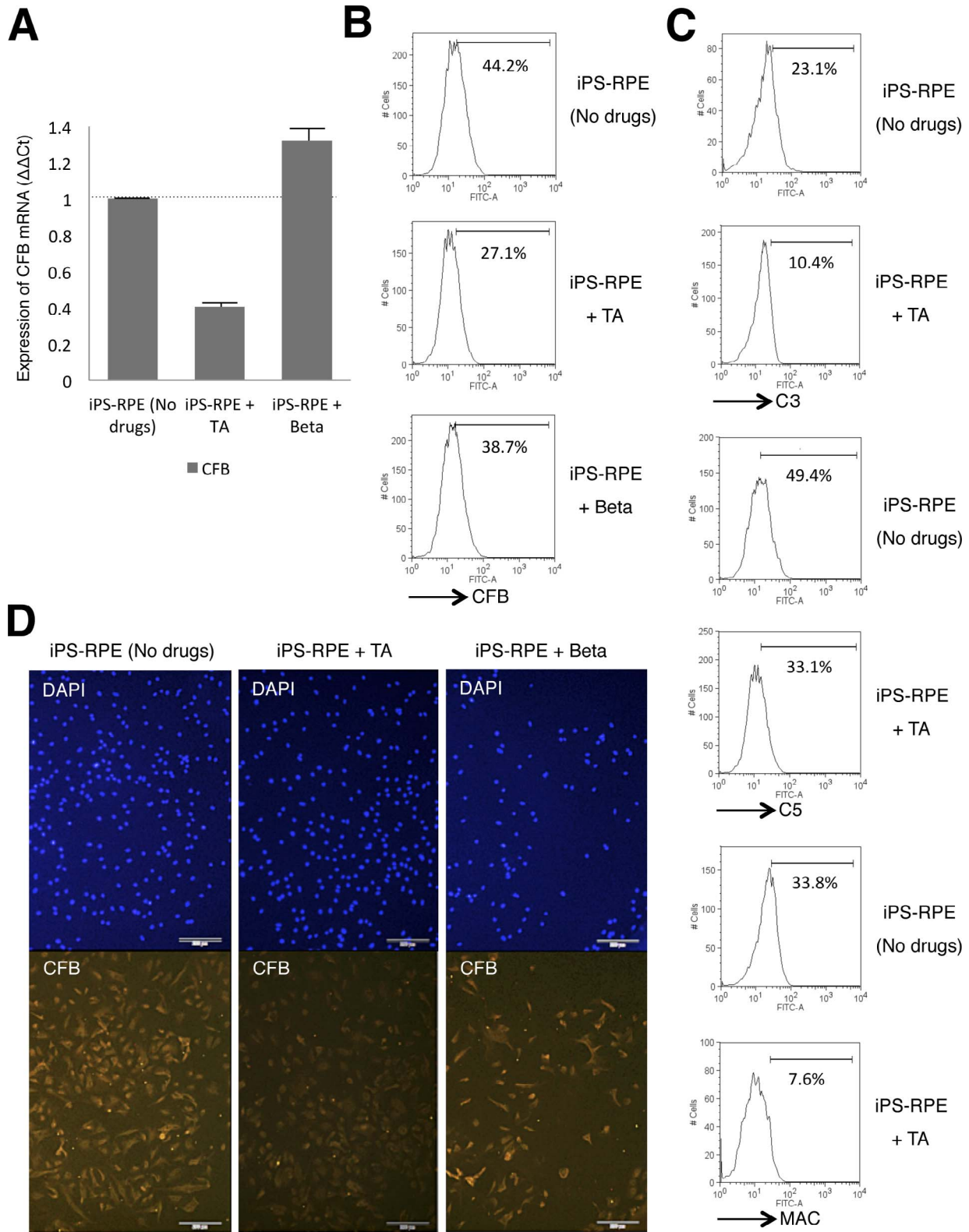


FIGURE 4. Detection of CFB in iPS-RPE cells in the presence of steroid. Expression of complement factors on iPS-RPE cells in the presence of recombinant IFN- γ plus steroid during (A) the qRT-PCR analysis, (B, C) FACS analysis, and (D) immune staining. Human iPS-RPE cells in the presence of IFN- γ were cultured with triamcinolone (TA) or betamethasone (Beta) for 48 hours. (C) Expression of C3, C5, and MAC (C5b-9) on iPS-RPE cells in the presence of IFN- γ plus TA by FACS analysis. (D) For the IHC analysis, cell nuclei were counterstained with DAPI (blue) and stained with anti-human CFB antibody (red). Scale bars: 200 μ m.

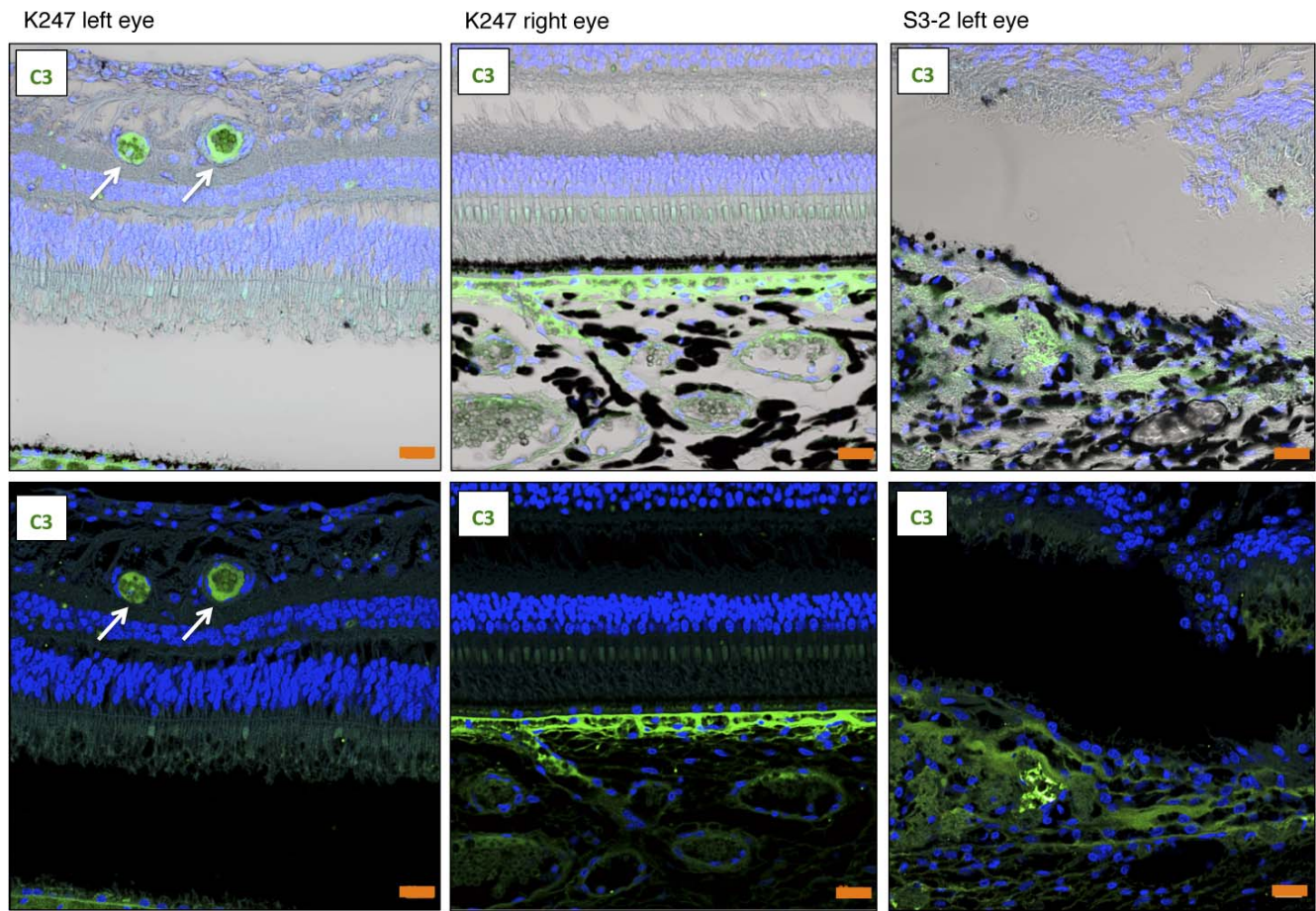


FIGURE 5. Detection of C3 in retinal sections from the immune attack animal models with allogeneic iPS-RPE cells. Photomicrographs showing labeling of the K247 and S3-2 retina in monkeys grafted with iPS-RPE cells into the eyes with anti-human C3 antibody. Cell nuclei were counterstained with DAPI (blue). Enhanced staining of C3 was seen around the iPS-RPE cell sheet graft (arrow; left panels), and host RPE layer (middle panels) in the eyes. In addition, enhanced staining of C3 was seen in the granuloma-like mass with the iPS-RPE cell sheet graft (right panels). Enhanced staining of C3 was also seen in other retinal sections, such as S3-2 right, TLHM1 right and left, and TLHM6 right. Scale bars: 20 μ m.

Quantitative RT-PCR

Expression of mRNA for C3, C5, CFB (Factor B), CFH (Factor H), CFI (Factor I), CD46 (MCP), CD55 (DAF), CD59, clusterin, and vitronectin in the iPS-RPE cells was evaluated using quantitative RT-PCR (qRT-PCR). Total RNA was isolated from the iPS-RPE cells, iPS cells, and fibroblasts (all TLHD1 donors). iPS-RPE cells in the presence of recombinant human IFN- γ (100 ng/mL; R&D Systems), recombinant human TNF- α (100 ng/mL; R&D Systems), lipopolysaccharide (LPS, 1 μ g/mL; Sigma-Aldrich Corp.), supernatants of naive T cells (CD4), and supernatants of Th1 cells (CD4)¹⁷ were also prepared for the PCR assay. After cDNA synthesis, the expressions of the above complement factors and β -actin in triplicate samples were analyzed by qRT-PCR with a LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany) through the use of the qPCR Master Mix (Roche Diagnostics) and Universal ProbeLibrary assays (Roche Diagnostics). The primers and the Universal Probe are described in Supplementary Table S3. The qRT-PCR was performed as per a previous report.¹⁸ Results indicated the relative expression of the molecules ($\Delta\Delta$ Ct: control cells = 1).

Flow Cytometry and ELISA

Expression of C3, C5, CFB (Factor B), and membrane attack complex (MAC) by iPS-RPE cells (TLHD1 or 454E2) was

evaluated by FACS analysis. Expression of complement inhibitors such as CFH (Factor H), CFI (Factor I), CD46 (MCP), CD55 (DAF), CD59, clusterin, and vitronectin were also evaluated in the iPS-RPE cells (TLHD1 or 454E2 lines).

For the complement factor expression analyses, the RPE cells were treated with an intracellular staining material (BD Cytofix/Cytoperm Kits; BD PharMingen, San Diego, CA, USA). Before staining, these cells were incubated with a human Fc block (Miltenyi Biotec, Auburn, CA, USA) at 4°C for 15 minutes. After the human Fc block staining, the RPE cells were then stained with anti-human C3, C5, CFB, or MAC antibodies (listed in Supplementary Table S2) at 4°C for 30 minutes. RPE cells were also stained with FITC-labeled anti-goat IgG or anti-mouse IgG at 4°C for 30 minutes. All samples were analyzed using a FACSCanto II or a FACSaria II flow cytometer (BD Biosciences, San Jose, CA, USA). Data were analyzed using FlowJo software (version 9.3.1; Tree Star, Inc., Ashland, OR, USA).

The concentration of CFB (Factor B) in the supernatants of iPS-RPE cells (TLHD1), control cells (iPS cells; TLHD1) or IFN- γ treated iPS-RPE cells (TLHD1) was measured by using human CFB ELISA (Abcam).

Drug Assay

Expression of CFB (Factor B) by iPS-RPE cells (TLHD1 or 454E2) in the presence of other medication was evaluated by

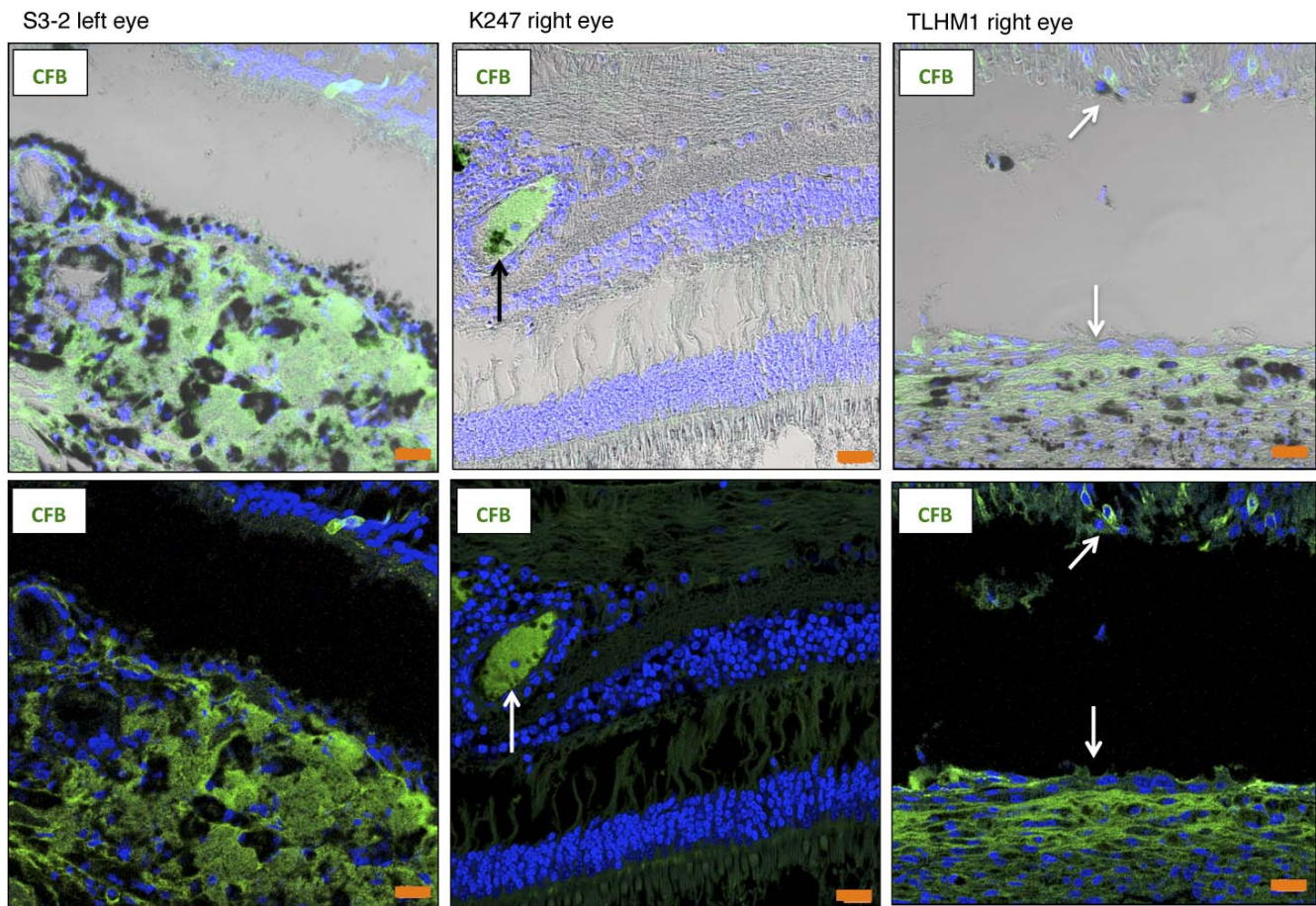


FIGURE 6. Detection of CFB (Factor B) in the immune attack animal models. Photomicrographs showing labeling of the K247, S3-2, TLHM1 retina in monkeys grafted with iPS-RPE cells into the eyes with anti-human CFB antibody. Enhanced staining of CFB was seen in the inflammatory granuloma (*left panels*) or grafts with iPS-RPE sheet (*middle panels*). In addition, enhanced staining of CFB was seen in the granuloma with the iPS-RPE cell sheet graft (*right panels*). Enhanced staining of CFB was also seen in other retinal sections, such as K247 left, S3-2 *right*, TLHM1 *left*, and TLHM6 *right*. Cell nuclei were counterstained with DAPI (*blue*). Scale bars: 20 μ m.

FACS, quantitative RT-PCR or IHC analysis. IFN- γ treated iPS-RPE cells exposed to triamcinolone or betamethasone (0.01, 0.1, 1, 10 μ g/mL; Sigma-Aldrich Corp.) were used for the *in vitro* assay.

Statistical Evaluation

All experiments were repeated at least twice with similar results. All statistical analyses were conducted using a Student's *t*-test (paired *t*-test). Values were considered statistically significant if *P* was less than 0.05.

RESULTS

Detection of mRNA for Complement Factors and Inhibitors by Human iPS Cell-Derived RPE Cells

Several groups have previously reported that cultured RPE cells expressed complement factors.¹⁻⁴ Therefore, we first confirmed whether human iPS cell-derived RPE cells express complement factors. In our previously published GeneChip analysis study,¹⁵ we reported that human iPS-RPE cells (as well as human primary RPE cells) significantly expressed mRNA for complement activators such as C3, C5, and CFB (Factor B; Fig. 1A). Complement inhibitors such as CFH (Factor H), CFI

(Factor I), CD46 (MCP), CD55 (DAF), CD59, clusterin, and vitronectin were also expressed on the iPS-RPE cells. There was no significant difference between the iPS-RPE cells and the iPS cells, as the iPS cells also expressed mRNA for CD46 and CD55 (Fig. 1A). Similarly, in qRT-PCR analysis (Fig. 1B), iPS-RPE cells significantly expressed mRNA for complement factors (except for CD55) as compared with the iPS cells. In addition, when compared with human fibroblasts from the same donor, iPS-RPE cells significantly expressed mRNA for complement factors (Supplementary Fig. S1). These results indicated that our established iPS cell-derived RPE cells constitutively expressed complement factors.

Influence of Expression of Complements in iPS Cell-Derived RPE Cells in the Presence of Inflammatory Proteins or T-Cell Supernatants

Subsequently, we examined whether human iPS-RPE cells could produce complement factors during inflammatory conditions. For this assay, we used inflammatory proteins, recombinant IFN- γ , recombinant TNF- α , lipopolysaccharide (LPS), or T-cell supernatants collected from naïve T cells and activated Th1 cells. For the qRT-PCR, iPS-RPE cells in the presence of recombinant IFN- γ proteins (but not TNF- α or LPS) greatly expressed mRNA for complement factors (except for

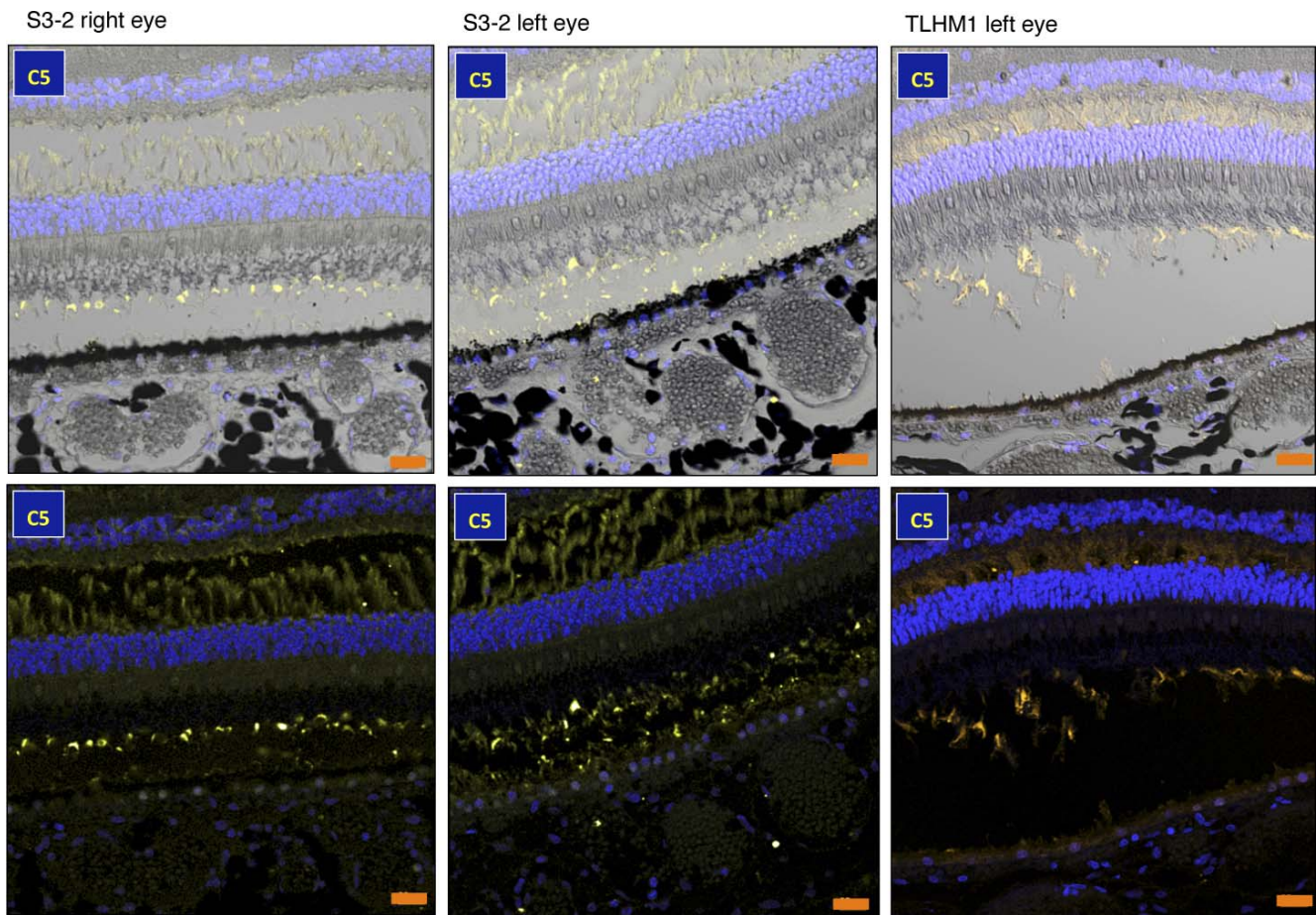


FIGURE 7. Detection of C5 in the immune attack animal models. Photomicrographs showing labeling of the S3-2 and TLHM1 retina in monkeys grafted with iPS-RPE cells into the eyes with anti-human C5 antibody. There was C5 deposit staining in the subretinal space of the retinal sections, but not in the retina and choroid. C5 deposit staining was also seen in other retinal sections such as K247 *right* and *left*, TLHM1 *right*, and TLHM6 *right*. Cell nuclei were counterstained with DAPI (blue). Scale bars: 20 μ m.

CD59; Fig. 2) as compared with the iPS-RPE cells without proteins. Recombinant TNF- α promoted the expression of some of the complement activators, such as C3 and C5. However, LPS only promoted the expression of C5 (Fig. 2). In addition, iPS-RPE cells in the presence of Th1 cell supernatants (but not naïve T cells) greatly expressed mRNA for complement factors (except for vitronectin; Supplementary Fig. S2) as compared to the iPS-RPE cells without T-cell supernatants. Taken together, these results indicated that human iPS-RPE cells inducibly expressed complement factors during inflammatory conditions, especially after IFN- γ exposure.

Detection of Complement Activators and Inhibitors in Human iPS-RPE Cells

We next examined whether human iPS-RPE cells expressed complement activators at the protein level. As seen in Figure 3A, iPS-RPE cells expressed complement activators such as C3, CFB (Factor B), C5, and MAC in the FACS analysis. iPS-RPE cells in the presence of recombinant IFN- γ greatly expressed these complement activators as compared with the iPS-RPE cells without recombinants. Furthermore, human iPS-RPE cells also expressed complement inhibitors such as CFH, CFI, CD46, CD55, CD59, clusterin, and vitronectin at the protein level (Supplementary Fig. S3). ELISA results showed that human iPS-RPE cells significantly secreted CFB proteins as compared to

the iPS cells, and that human iPS-RPE cells in the presence of recombinant IFN- γ significantly secreted these proteins as compared to the non-treated RPE cells (Fig. 3B). We also found that human iPS-RPE cells exposed to recombinant IFN- γ greatly expressed CFB during immune staining (Fig. 3C).

Detection of Factor B (CFB) in iPS-RPE Cells in the Presence of Steroid

Next, we examined whether the administration of medication for complement factors would be able to suppress the expression of Factor B on iPS-RPE cells in vitro. The qRT-PCR (Fig. 4A) and FACS (Fig. 4B) analyses showed that human iPS-RPE cells in the presence of recombinant IFN- γ greatly expressed the complement activators, CFB. However, the expression of CFB in iPS-RPE cells was suppressed when the cells were treated with triamcinolone but not with betamethasone. Moreover, FACS analysis showed that triamcinolone also suppressed other complement activators such as C3, C5, and MAC (C5b-9) (Fig. 4C). Furthermore, IHC showed that the expression of CFB in iPS-RPE cells was clearly suppressed when exposed to triamcinolone. In contrast, there was no suppression of the expression in the iPS-RPE cells after being exposed to betamethasone (Fig. 4D). Several concentrations (0.01, 0.1, 1, 10 μ g/mL) of betamethasone did not suppress the CFB expression on RPE cells (data not shown). Thus, some

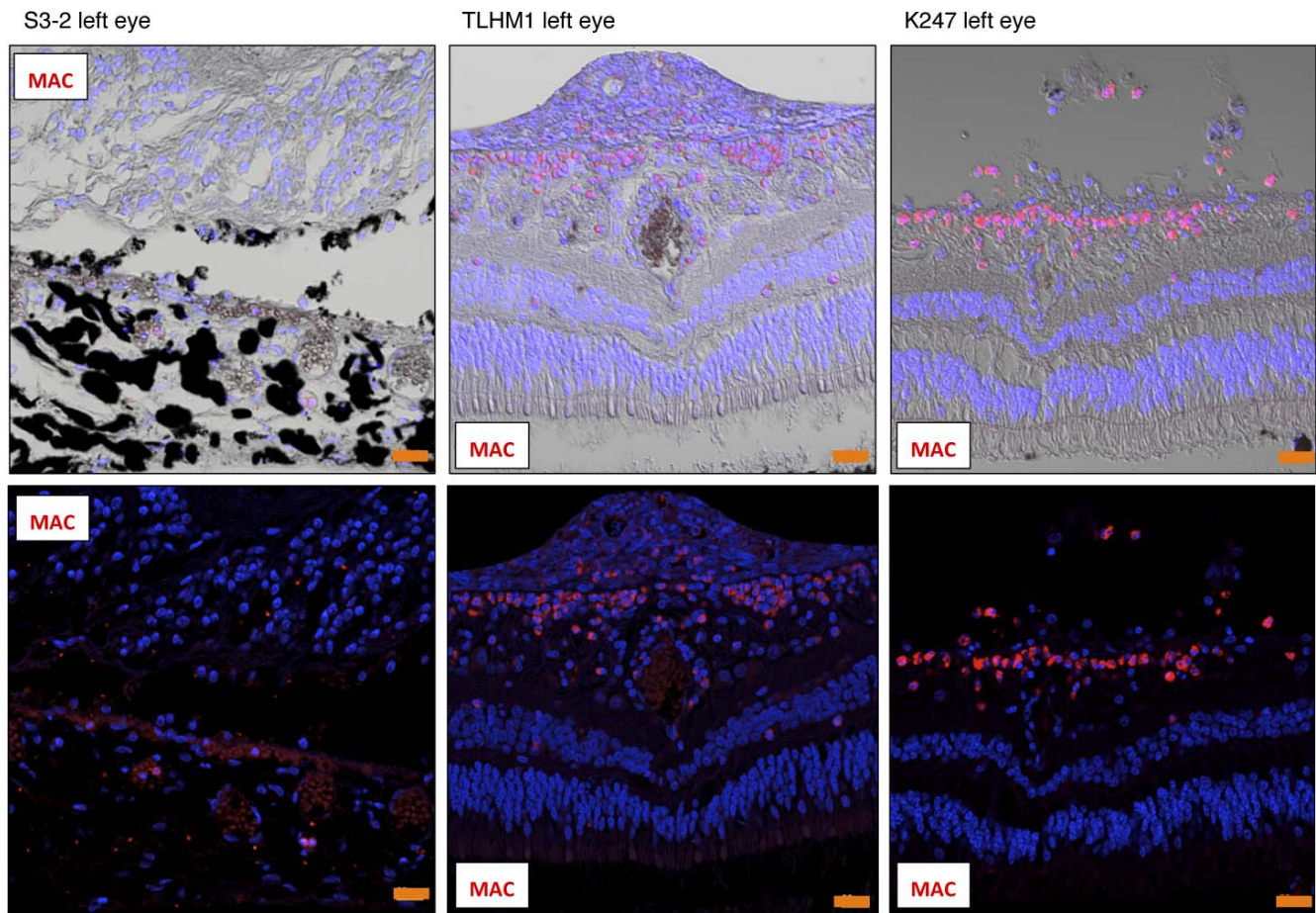


FIGURE 8. Detection of membrane attack complex (MAC) in the immune attack animal models. Photomicrographs showing labeling of the K247, S3-2, TLHM1 retina in monkeys grafted with iPS-RPE cells into the eyes with anti-human MAC antibody. There were a large number of MAC⁺ inflammatory cells observed in the inflammatory lesions of the retina and choroid. We also found MAC⁺ inflammatory cells in other retinal sections, such as K247 *right*, S3-2 *right*, TLHM1 *right*, and TLHM6 *right*. Cell nuclei were counterstained with DAPI (*blue*). Scale bars: 20 μ m.

steroids might be able to effectively suppress complement activators in the eye.

Detection of Complement Activators in Retinal Sections Obtained From Immune Attack Animal Models With iPS Cell-Derived RPE Cells

During the first step, we examined whether a normal retina can express complement factors. In the eye of a normal monkey (S2-4), we found staining for both C3 and Factor B (CFB) under the RPE layer, slight staining for C5 in the subretinal space, and slight staining for MAC on the RPE cells, which indicates that there was no retinal staining. As a positive control, we used the monkey experimental autoimmune uveitis (EAU) models.¹⁶ As compared to the normal sections (Supplementary Fig. S4), we found that there was greater C3 staining under the RPE layer and the choroid in the inflammatory eye. IHC of the CFB also showed that there was greater staining under the RPE layer and the choroid. IHC for C5 also showed that there was staining in the subretinal space, and there was MAC staining for the RPE cells and the infiltrating inflammatory cells in the choroid (Supplementary Fig. S4).

After the allogeneic iPS-RPE cell transplantation in the immune attack eyes,¹² complement activators such as CFB, C3, C5, and MAC were detected in the retinal sections. Supple-

mentary Table S1 summarizes current information on transplanted primates and the immune attacks after iPS-derived RPE cell sheet transplantation. For example, we found that there was greater C3 staining around the graft RPE cells, under the host RPE layer, and in the inflammatory granuloma in the subretinal space (Fig. 5). Similar results were found for the CFB staining, for example, there was greater CFB staining around the graft cells, under the RPE layer, and in the inflammatory granuloma (Fig. 6). For the S3-2 and TLHM1 eyes of the immune attack eye model, there was greater C5 staining in the subretinal space but not in the retina and choroid (Fig. 7). For the MAC staining, there were a large number of MAC⁺ inflammatory cells found in the retina and choroid (Fig. 8) similar to a positive control retinal section. An important finding was that the immune attack eyes from the K251 monkey included IFN- γ positive cells (probably T cells) in the retina (Supplementary Fig. S5), which suggests that cytokine existence in the immune attack eyes might be responsible for the complement activation in these local microenvironments.

In the last step of this part of the experiment, we examined whether other complement pathways, such as the classical pathway, were involved after the allogeneic iPS cell-derived RPE cell transplantation. To confirm this, we checked for the expression of the C1q complement factor and IgG in the retinal sections from the transplanted primate *in vivo* animal models. Our results showed that there were a large number of C1q and

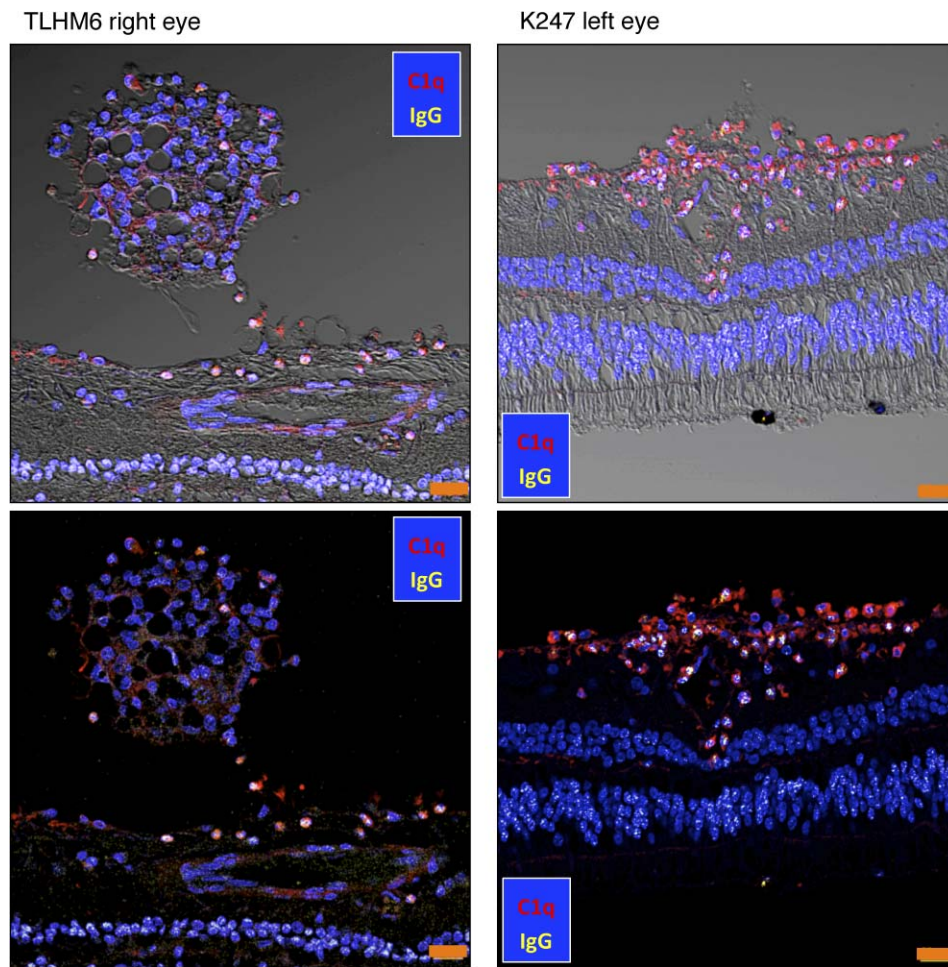


FIGURE 9. Detection of C1q and IgG in the immune attack animal models. Photomicrographs showing labeling of the TLHM6 and K247 retina in monkeys grafted with iPS-RPE cells into the eyes with anti-human C1q and IgG antibody. There were a larger number of C1q and IgG double positive inflammatory cells observed in the inflammatory retina. (Left panels show the granuloma-like mass on the retina.) We also found C1q and IgG⁺ inflammatory cells in other retinal sections such as K247 right, S3-2 left, and TLHM1 right. Cell nuclei were counterstained with DAPI (blue). Scale bars: 20 μ m.

IgG double-positive inflammatory cells in the retinal sections (Fig. 9), which suggests that these might activate the classical complement pathway as well as the alternative pathway. Taken together, our results appear to indicate that allogeneic iPS-RPE cell transplantation that was subjected to immune attacks could promote the activation of the complement pathway in the eye.

DISCUSSION

In the present study, we demonstrated that human iPS cell-derived RPE cells expressed complement factors, both activators and inhibitors. During inflammatory conditions, especially IFN- γ exposure, there was a significant increase in the production of the complement factors by the iPS-RPE cells. Based on these findings, the question that then needed to be answered is whether *in vivo* RPE cells express complement factors. If so, could complement factors be detected in retinal sections, including in the immune attack eyes after iPS-RPE cell transplantation? Our results showed that complement activators, such as CFB, C3, C5, and MAC, were detected in the retinal sections, including in the immune attack eyes after iPS-RPE cell transplantation. Although the normal RPE cell layer

also expressed the complement activators, we found an upregulation of the expression of complements around the host RPE layer without inflammation and in grafted iPS-RPE cells in inflammatory eyes. Moreover, we also found there was enhanced staining of the complement factors in the retina. Therefore, after being exposed to retinal invading inflammatory cells, RPE cells—including both explanted RPE cells and host RPE cells—might be activated and produce complement factors.

Previously, we reported that allogeneic iPS-RPE cell transplantation might be activated by the complement classical pathway, as the transplanted animal models were shown to exhibit B-cell associated inflammation¹¹ and IgG deposits along with C1q (Fig. 9) in the retina. In addition, we also found T cell and antigen presenting cell (APC) immunity.¹² APCs included retinal microglia and choroidal macrophages. It has been shown that the first step in the complement cascade (classical pathway) is the binding of C1q to the antibody.¹⁹ Although complement factors are considered to be a branch of the innate immune systems, they are also involved in the regulation of adaptive immune systems. For example, this regulation can be achieved through activation of T cells, B cells, and APC by C3b opsonization, which can then result in the release of some complement factors. In fact, our current

study demonstrates the existence of these immune cells and the inflammatory cytokine IFN- γ (see Supplementary Fig. S5) in the immune rejection of the retina after RPE transplantation. Thus, the pathogenesis of AMD is associated with the activation of complement factors in the retina that might be upregulated by immune responses occurring after allogeneic RPE cell transplantation.

RPE cells can produce inflammatory cytokines/chemokines during inflammatory conditions. Human RPE cells with activated T cells significantly expressed IFN- γ associated chemokines such as CXCL9, 10, and 11.²⁰ Immune attack eyes with iPS-RPE cells in animal models have also been found to have activated T lymphocytes as well as APC.¹² Moreover, the human in vitro immune response against iPS-RPE cells is also important for T-cell immunity, especially for the Th1 immune response.¹⁴ While activated T cells such as Th1 cells can produce significant amounts of IFN- γ , naïve T cells cannot. In addition, APC and NK cells can also secrete IFN- γ . Moreover, it has also been reported that human ocular inflammatory disorders²⁰⁻²² and experimental uveitis^{23,24} involve Th1 cells and IFN- γ . Therefore, IFN- γ is a key cytokine involved in the mechanisms of ocular inflammatory disorders, including RPE-related immune rejections. Another previous study has demonstrated that activated macrophages promoted the alternative pathway of complement activation in the retina via induction of Factor B and C3 expression on RPE cells during inflammatory conditions.²⁵ Juel et al.³ investigated the effect of the co-culture of human RPE cells with activated T cells on the complement expression. Their results demonstrated that the co-culture increased mRNA for C3, CFB, CFH, CD46, CD55, CD59, and clusterin. The findings from these previous reports, as well as our present study, are important in furthering our understanding of inflammatory ocular disorders and retinal degeneration such as AMD.

Clinicians have recently tried to use complement inhibitors for the treatment of AMD patients. In animal AMD models, Collier et al.²⁶ reported that administration of a 5-HT_{1A} agonist (AL-8309A) prevented retinal lesions and decreased the activation of retinal microglia and complement deposition in the retina after light exposure. Therapies that are currently in clinical trials for AMD patients include complement inhibitors such as ARC1905 (target = C5), FCFD4514S (target = Factor D), and POT-4 (target = C3).²⁷ These potential therapies that target the underlying disease mechanisms of AMD and stem cell therapies, including iPS cells, all show promise in being able to stabilize or improve visual acuity in AMD patients. In fact, we have recently reported on a successful transplantation that used RPE cells established from iPS cells to treat and stabilize the visual acuity of an AMD patient.²⁸ In addition, we are currently investigating whether complement inhibitors or local steroid administration (e.g., intravitreal triamcinolone injection) can control the retinal inflammation that occurs in RPE-related immune attacks in the in vivo animal models. Moreover, the results of our present study demonstrated that the expression of Factor B in iPS-RPE cells was suppressed when these cells were pretreated with triamcinolone (Fig. 4). In fact, we have clinically administered triamcinolone injections in the eyes of patients with macular edema secondary to branch retinal vein occlusions, diabetic macular edema, and uveitis in Japan. Since a previous study has also reported that triamcinolone vitreous injection treatments resulted in an anti-angiogenic effect for three months in AMD patients,²⁹ this suggests that local steroid therapy might be effective for treating recurrence in AMD as well. Based on these findings, we might want to consider using these medications for patients with AMD after performing iPS cell-derived RPE cell transplantation, that is, both for treating AMD itself and for

controlling inflammation including complement activation due to the RPE transplantation.

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