A local complement response by RPE causes early-stage macular degeneration

Rosario Fernandez-Godino1, Donita L. Garland1 and Eric A. Pierce1,2,*

1Ocular Genomics Institute, Department of Ophthalmology and 2Berman-Gund Laboratory for the Study of Retinal Degenerations, Department of Ophthalmology, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, MA, USA

*To whom correspondence should be addressed at: Ocular Genomics Institute, Massachusetts Eye and Ear Infirmary, 243 Charles Street, Boston, MA 02114, USA. Tel: +1 617 573 6917; Fax: +1 617 573 6920; Email: eric_pierce@meei.harvard.edu

Abstract

Inherited and age-related maculopathies (AMRs) are important causes of vision loss. An early hallmark of these disorders is the formation of sub-retinal pigment epithelium (RPE) basal deposits. A role for the complement system in MDs was suggested by genetic association studies, but direct functional connections between alterations in the complement system and the pathogenesis of MD remain to be defined. We used primary RPE cells from a mouse model of inherited MD due to a p.R345W mutation in EFEMP1 to investigate the role of the RPE in early MD pathogenesis. Efemp1R345W RPE cells recapitulate the basal deposit formation observed in vivo by producing sub-RPE deposits in vitro. The deposits share features with basal deposits, and their formation was mediated by EFEMP1R345W or complement component 3a (C3a), but not by complement component 5a (C5a). Increased activation of complement appears to occur in response to an abnormal extracellular matrix (ECM), generated by the mutant EFEMP1R345W protein and reduced ECM turnover due to inhibition of matrix metalloproteinase 2 by EFEMP1R345W and C3a. Increased production of C3a also stimulated the release of cytokines such as interleukin (IL)-6 and IL-1B, which appear to have a role in deposit formation, albeit downstream of C3a. These studies provide the first direct indication that complement components produced locally by the RPE are involved in the formation of basal deposits. Furthermore, these results suggest that C3a generated by RPE is a potential therapeutic target for the treatment of EFEMP1-associated MD as well as AMD.

Introduction

Age-related macular degeneration (AMD) causes irreversible vision loss in millions of people (1). AMD has a complex etiology, but MD can also be inherited in a Mendelian fashion and affect individuals in early adulthood (2). Inherited MD and AMD share drusen as an early indicator of disease (3–6). Drusen are extracellular deposits of protein and lipid that accumulate between the retinal pigment epithelium (RPE) and Bruch’s membrane (BrM) (7–9).

Although the sources of drusen components and the mechanisms of drusen formation are not fully understood, many components of drusen have been identified, among them, complement components such as C3, complement component H (CFH) and C5 (3,10,11). Based in part on these findings, it has been suggested that the formation of drusen is an inflammatory process (3,7–15). Consistent with this idea, genetic variants in complement genes are considered to be one of the main risk factors for AMD, but how alterations in complement components contribute to the MD pathology remain to be defined (16–18). In the last decade, many potential therapies for MDs have been designed to target complement components (19). However, it remains unclear if regulation of the complement system locally or systemically is needed for the most effective treatment of MD, or at what stage of disease these treatments can be most usefully applied (13).

Some authors consider confluent basal laminar deposits as the histological definition of AMD (20). Basal laminar deposits...
are microscopic lesions associated with RPE/choroid dysfunction that also contain activated complement proteins (6,8,20–26). RPE cells are critical for the visual cycle and retinal homeostasis (27). Briefly, RPE cells reduce oxidative stress by the absorption of light, recycle outer segments by phagocytosis, supply nutrients to the photoreceptors and form the outer component of the blood retinal barrier (27–29). RPE cells also secrete most of the extracellular matrix (ECM) components in BrM including matrix metalloproteinases (MMPs) responsible for the turnover of this ECM (30–34). Alteration of the ECM turnover in BrM has been associated with complement activation in late stages of AMD (6,27,25).

The inherited form of MD clinically most similar to AMD is the EGF-containing fibulin-like ECM protein 1 (EFEMP1)-associated MD, caused by the dominant p.R345W mutation in the EFEMP1 (4,36). EFEMP1 belongs to the fibulin family of ECM proteins and is thought to be involved in elastogenesis (37). As for the potential role of EFEMP1 in AMD, it (i) accumulates beneath the RPE over- laying drusen and basal deposits (38–40), (ii) binds the tissue inhibitor of metalloproteinases 3 (TIMP-3) (40), which is associated with another inherited MD and with AMD (41,42), and (iii) binds CFH, with higher affinity for the AMD-associated variant (39). Therefore, understanding how the p.R345W mutation in EFEMP1 leads to the RPE pathology in EFEMP1-associated MD will help elucidate the early mechanisms responsible for RPE pathology in AMD.

As previously described, Efemp1<sup>R345W/R345W</sup> mice (henceforth denoted Efemp1<sup>R345W</sup>) develop extensive basal deposits only in the presence of an active complement system (43–45). To investigate the role of RPE, the complement system and ECM in the early aspects of MD pathogenesis, we developed a culture model using primary RPE cells from Efemp1<sup>R345W</sup> mice that recapitulate the formation of sub-RPE deposits in vitro in 2 weeks. Studies performed using this model system show that the secreted mutant EFEMP1 forms an abnormal ECM that stimulates a local inflammatory process, mediated primarily by C3a and altered ECM turnover, causing the formation of basal deposits. This is not only a valuable model to study the early mechanisms responsible for deposit formation in AMD, but also a unique system to discover new targets and test drugs that might inhibit the formation of deposits.

**Results**

**RPE cells from Efemp1<sup>R345W</sup> mice recapitulate deposit formation in vitro**

Efemp1<sup>R345W</sup> mice develop basal deposits between the RPE and BrM and have been characterized as a model to study deposit formation in MD (43–45). To investigate the molecular events associated with the RPE pathology in early MD, we developed a primary RPE culture model from 2-month-old Efemp1<sup>R345W</sup> mice. Culture procedures were optimized in order to reproduce in vitro the properties of RPE cells in vivo. By 72 h, RPE cells were present as a confluent monolayer of polygonal bi-nucleated cells (Fig. 1A). Polarization of the cells was demonstrated by (i) transspheleial electrical resistance (TER) that reaches over 200 Ω cm² by 72 h and increases to nearly 300 Ω cm² at 1 week (Fig. 1B), (ii) expression of the tight junction-specific protein zona oculdens 1 (ZO-1) at 1 week (Fig. 1C) and (iii) transmission electron microscopy (TEM) that showed the presence of apical microvilli, basal infoldings, pigment granules (Figs 1D and 2A–C) and tight junctions between adjacent cells (Fig. 2C). Other cell types such as fibroblasts in the RPE cell cultures were not identified by morphological examination or immunostaining of ZO-1 (Supplementary Material, Fig. S1). Additionally, RT-PCR for the markers Von Willebrand factor (Vuf), cluster of differentiation 68 (Cd68), cluster of differentiation 163 (Cd163) and rhodop- sin (Rho) ruled out the presence of endothelial cells, macrophages and photoreceptors.

Of note, as observed for other RPE culture systems, RPE cells cultured on transwells developed cell projections that passed through the pores to the bottom surface of the transwells (46–49) (Fig. 2D–F). Using scanning electron microscopy (SEM), we observed that the cell projections generated by wild-type (WT) cells were a mixture of thin, filamentous projections and flat processes which covered only a portion of the bottom surface of the transwell (Fig. 2G and J). Efemp1<sup>R345W</sup> cells showed the same cell projections as WT cells at 1 week (Supplementary Material, Fig. S1). However, at 2 weeks, the bottom surface of transwells with Efemp1<sup>R345W</sup> RPE cells was completely covered by thicker more amorphous deposits (Fig. 2H and K). The deposits did not exhibit the shape or structure of cell projections seen in WT cultures, but was spread across the whole transwell surface forming overlapping layers of disorganized, clumped material. Since this was thicker than the material produced by WT cells and it covered more of the insert, it was also noticeable by TEM on transverse sections (Fig. 2E). The RPE cells from Efemp1<sup>R345W</sup> mice thus recapitulate in vitro the formation of basal deposits that was observed in vivo via the production of the deposits (43–45).

The role of complement in the formation of deposits was tested in cultures of primary RPE cells from homozygous double-mutant Efemp1<sup>R345W,C3<sup>−/−</sup></sup> mice. Interestingly, Efemp1<sup>R345W,C3<sup>−/−</sup></sup> RPE cells did not develop deposits on the bottom side of the insert, but showed normal cellular projections similar to WT (Fig. 2I and L). This suggests that the formation of deposits by Efemp1<sup>R345W</sup> RPE cells is complement-dependent, as was observed for the formation of basal laminar deposits in vivo in Efemp1<sup>R345W</sup> mice (45).

**Sub-RPE deposits share features with basal laminar deposits**

Based on the TEM images, the apical side of the cultured RPE cells appeared normal, and thus, the projections and the deposits formed by Efemp1<sup>R345W</sup> RPE cells were likely extensions derived from the basal sides of the cells. Although structurally different, sub-RPE deposits share features with basal laminar deposits in humans and mice (25,45). The bottom side of the transwells was mounted as flatmounts for immunostaining to analyze the composition of the deposits. Staining with Hoechst dye demonstrated that the projections were not migrated cells, as no nuclei were detected on the bottom surfaces of the transwells. The deposits contained increased amounts of EFEMP1, collagen I, elastin, TIMP-3, fibrillin-1 and fibronectin relative to the projections of the WT RPE cells (Fig. 3A). Interestingly, these are major constituents of BrM and basal laminar deposits (25). Increased levels of ECM proteins found in the deposits were not associated with an increased production of their mRNAs (Supplementary Material, Fig. S2). These results suggest that sub-RPE deposits mimic deposits in Efemp1<sup>R345W</sup> mice, and is primarily comprised of normal ECM proteins that are present in abnormal amounts (45). The increased levels of EFEMP1 protein observed in the deposits were not associated with increased production or secretion of the mutant EFEMP1<sup>R345W</sup> by the Efemp1<sup>R345W</sup> cells compared with WT cells (Fig. 3B–D).
Complement is locally produced by RPE cells

The finding that an intact complement system is required for the formation of deposits by Efemp1R345W RPE cells in culture suggests that the RPE cells produce complement themselves. To evaluate this possibility, we measured the expression of complement by the RPE cells in vitro. We detected mRNAs for complement components belonging to the three complement pathways in the RPE cells grown in the absence of fetal bovine serum (Supplementary Material, Fig. S2). Levels of the mRNAs for these complement components were similar in WT and Efemp1R345W RPE cells with the exception of C3, which was 2-fold higher in the Efemp1R345W cells (t-test, P = 0.0299, n = 18 WT and 13 knock-in (KI); Fig. 4A). Increased C3 was also demonstrated by ELISA, with C3 detected in media being 3.8-fold higher in Efemp1R345W cultures compared with WT cultures (t-test, P = 0.0387, n = 5 per genotype; Fig. 4B).

Consistent with the increased production of C3 by the Efemp1R345W RPE cells, increased levels of C3 were detected in the deposits produced by these cells (Fig. 4C). Of note, the activation product of C3, C3a, was detected in deposits but not in normal cellular material produced by WT cells (Fig. 4C). In contrast, CFH, C3d, and membrane attack complex (MAC) were detected on the bottom surface in both WT and Efemp1R345W cultures (Fig. 4C). CFH and C3 appeared to be co-localized with EFEMP1 in deposits (Supplementary Material, Fig. S2). Taken together, these data support the idea that complement components produced by the RPE are also activated locally.

Secreted EFEMP1R345W and C3a act in a non-cell autonomous fashion to stimulate the production of deposits

Having established that RPE cells from Efemp1R345W mice produce deposits in culture, we next used this in vitro system to investigate the mechanisms by which the mutant EFEMP1 protein causes deposit formation. We hypothesized that factors secreted by the Efemp1R345W cells rather than intracellular stress were responsible for deposit formation. To test this hypothesis, we performed a media exchange experiment, in which WT RPE cells were grown in conditioned media (CM) from Efemp1R345W cells, and vice versa for 2 weeks. As shown in Figure 5A, WT cells grown in CM from Efemp1R345W cells developed deposits. On the other hand, Efemp1R345W cells grown in CM from WT cells, or for which media were changed every day, showed a substantial inhibition of deposit formation (t-test, P = 0.0337 and P = 0.0130, respectively; Fig. 5A).

These results demonstrate that factors secreted by the Efemp1R345W cells are necessary and sufficient to stimulate deposit formation by WT cells. To help identify these potential candidate factors, we fractionated the CM from Efemp1R345W cells by molecular weight. WT RPE cells grown in either the 3–10 or
30–100 kDa fractions of CM from Efemp1R345W cells made deposits after 2 weeks, whereas no deposits were observed in WT cells cultured in the 10–30 or >100 kDa fractions (Fig. 5B). Based on molecular weight, the best candidate proteins that could be responsible for deposit formation were EFEMP1 (55 kDa) and C3a or C5a (9 kDa each). We therefore tested the ability of these proteins to stimulate formation of deposits by WT RPE cells.

Cultured WT RPE cells treated with recombinant mouse EFEMP1R345W protein (rmEFEMP1R345W; 10, 50 and 100 ng/ml) for 2 weeks formed deposits in a dose-dependent manner (Fig. 5C), with an EC50 of 11.4 ng/ml. Conversely, the formation of deposits was inhibited in a dose-dependent manner in cultures of Efemp1R345W RPE cells treated with EFEMP1-neutralizing antibodies (0.01, 0.1, 1 and 10 µg/ml) for 2 weeks. At high concentrations of the antibody, mutant cells did not show any deposits (Fig. 5D).

In contrast, cultured WT RPE cells treated with recombinant mouse C3a (5, 10, 50 and 100 ng/ml) for 2 weeks did not form deposits. These data demonstrate that secreted EFEMP1R345W acts in a non-cell autonomous fashion, likely mediated in part by C3a but not by C5a, to stimulate deposit formation.

**EFEMP1R345W protein increases extracellular aggregates in Efemp1R345W RPE cultures**

We next asked how the secreted mutant EFEMP1R345W protein stimulates deposit formation. EFEMP1 levels were increased in
both BrM of Efemp1R345W mice and the deposits produced by Efemp1R345W RPE cells, and thus, one possibility is that incorporation of the mutant protein into the ECM results in an abnormal ECM (45). One metric of ECM status is the level of extracellular protein aggregates; we therefore stained the bottom flatmounts from WT and Efemp1R345W RPE cells with the Proteostat protein aggregation dye (Enzo Life Sciences, Farmingdale, NY, USA), designed to detect aggregation of a broad range of proteins (50,51). As shown in Figure 6, increased levels of protein aggregates were detected in the deposits produced by Efemp1R345W cells (t-test, $P = 0.0357$). The EFEMP1 signal co-localized with the aggregated protein in the deposits of Efemp1R345W RPE cells, which was also observed in WT cells (Fig. 6A and B). This is consistent with a recent study, showing that some aggregation is normal for EFEMP1 (37).

Decreased MMP-2 activity is associated with the formation of deposits

BrM is an ECM that is regulated in part by MMP/TIMP-3 activity (30–32). Since EFEMP1 interacts with TIMP-3 (40), it is possible that in addition to promoting protein aggregation, EFEMP1R345W could also contribute to abnormal ECM structure and deposit formation by reducing ECM turnover. To determine if EFEMP1R345W alters ECM turnover, we measured MMP activity in Efemp1R345W cultures. As shown in Figure 7A, the MMP-2 activity was decreased in media from Efemp1R345W or double-mutant Efemp1R345W.C3−/− RPE cells compared with WT (ANOVA, $P = 0.047$ and $P = 0.0062$). This appears to be due to an effect of the EFEMP1R345W protein on MMP-2 activity, as Mmp-2 mRNA levels were not significantly different from WT cells (Fig. 7B).

To determine if the decrease in MMP-2 activity observed in the Efemp1R345W cells was due to the EFEMP1R345W protein itself, we treated WT RPE cultures with increasing concentrations of rmEFEMP1R345W, which resulted in a dose-dependent inhibition of MMP-2 activity (Fig. 7C). Conversely, Efemp1R345W cultures showed a rescue of MMP-2 activity after being treated with EFEMP1-neutralizing antibody (Fig. 7C). These results also suggest that EFEMP1R345W can directly modulate the activity of MMPs.

Since C3a can stimulate the production of deposits by WT RPE cells, we also evaluated the effect of C3a on MMP activity. As observed for EFEMP1R345W protein, rmC3a also decreased MMP-2 activity in the media of WT cells in a dose-dependent manner (Fig. 7D). However, neutralization of C3a in cultures of Efemp1R345W cells did not increase the activity of MMP-2 (Fig. 7D).

These data suggest that decreased MMP-2 activity and the associated alteration in ECM turnover are mechanisms by which deposits are formed. To determine if decreased ECM turnover can result in the formation of deposits, we treated WT RPE cells with ARP100, a specific inhibitor of MMP-2 (52). After 2 weeks the cells did not show any deposits, although MMP-2 activity was effectively inhibited (Supplementary Material, Fig. S3), and

Figure 3. EFEMP1R345W expression and secretion. (A) EFEMP1, TIMP-3, elastin (ELN), Colla1, fibronectin (FN) and FBN-1 were detected in the deposits by immunostaining of the bottom side of the inserts mounted as flat mounts. (B) mRNA levels of Efemp1 measured by qRT-PCR ($n = 12$ WT, $11$ KI and $7$ KIC3KO). (C) Western blot (apical media) and (D) ELISA (apical and basal media) of WT, KI and KIC3KO cultures at 2 weeks ($n = 4$ per genotype) showed that WT and mutant EFEMP1 are secreted to the same degree. Scale bars, 50 µm. Data represented as mean ± SD.
thus decreased MMP-2 activity alone is not sufficient to induce deposit formation by WT RPE cells.

Interleukins contribute to the process of deposit formation

Since C3a can stimulate the production of interleukin (IL)-1B and IL-6, cytokines that are likely involved in the pathogenesis of RPE in MD, we evaluated the potential roles of these interleukins in deposit formation by Efemp1<sup>R345W</sup> RPE cells (53–55). IL-1B did not show a significant change in WT culture media after the addition of rmC3a; however, IL-1B decreased after neutralization of C3a in Efemp1<sup>R345W</sup> media (Fig. 7E). IL-6 levels increased following the addition of rmC3a in WT cultures and significantly decreased in Efemp1<sup>R345W</sup> media after the addition of C3a-neutralizing antibody (Fig. 7F). In contrast, no change in IL-1B or IL-6 concentration was observed after the addition of rmEFEMP1<sup>R345W</sup> in WT cultures or neutralization of EFEMP1<sup>R345W</sup> in Efemp1<sup>R345W</sup> cultures (Fig. 7G and H).

To determine if interleukins could also contribute to decreasing the activity of MMPs, we tested the MMP-2 activity in WT media after the addition of either IL-1B or IL-6. The MMP-2 activity did not show a significant change after the addition of rmIL-1B or rmIL-6 to WT RPE cultures; however, MMP-2 activity increased after the addition of antibodies for IL-1B or IL-6 in Efemp1<sup>R345W</sup> (Supplementary Material, Fig. S3). This result indicates that interleukins could also contribute to deposit formation, at least in part by inhibiting MMP-2 activity in Efemp1<sup>R345W</sup> cultures. Interleukin expression was shown to be C3a-dependent and not EFEMP1-dependent; thus, interleukins may contribute to the inflammatory process once complement is active.

Working model for basal deposit pathogenesis

Based on the data described above, we have generated a working model of basal deposit formation in EFEMP1-associated MD (Fig. 8). The novel aspects of the model include the central roles of C3a and defective ECM turnover in deposit pathogenesis.

Discussion

A role for the complement system in AMD was suggested by genetic association studies in 2005, but how alterations in the complement system contribute to this disease remain to be defined, in part due to the lack of good models (16–18, 56). The studies reported here, facilitated by the use of RPE cells from a genetic model of MD, provide several new insights into the pathogenesis of MD. First, the data demonstrate that a local complement response by the RPE is responsible for the earliest lesions observed in MDs, basal deposits. Second, this complement response is atypical since it is mediated by C3a, but not by C5a or the MAC. Third, the increased activation of complement in this system occurs in response to abnormal ECM, generated by extracellular aggregates containing EFEMP1<sup>R345W</sup> and reduced ECM turnover due to inhibition of MMP-2. Fourth, increased production of C3a also stimulates the release of cytokines such as IL-6 and IL-1B, which appear to have a role in deposit formation, albeit downstream of C3a. These results demonstrate the central role of the complement system in MD, and have important implications regarding the development of therapies to treat early MD.

A number of investigators have suggested that drusen results from a chronic inflammatory response due to RPE or choriocapillaris injury, but the specific mechanisms that connect RPE pathology, complement activation and drusen formation are...
unknown (3,7,12–15,22,23,57). Use of Efemp1\textsuperscript{R345W} RPE cells made it possible to investigate the specific role of the RPE in early MD pathogenesis. The Efemp1\textsuperscript{R345W} RPE culture system recapitulates the formation of sub-RPE deposits in vitro in only 2 weeks, compared with months in Efemp1\textsuperscript{R345W} and other gene-targeted mice (43,56). Furthermore, the deposits generated by the Efemp1\textsuperscript{R345W} RPE cells share components with basal laminar deposits and drusen (25,38,45,58). So far, this is the most accurate model in reproducing in vitro the in vivo features of basal deposits. Formation of wide-spaced collagen or APOE-rich deposits had been reported for ARPE-19 and human RPE cells, however only after challenge with retinal homogenates or human serum (59,60). The Efemp1\textsuperscript{R345W} RPE cells provide the first model of genetically induced sub-RPE deposits in vitro, and demonstrate a new approach to study the mechanisms responsible for the RPE pathology in inherited and age-related macular degenerations.

In the pathogenesis of MDs, RPE cells are thought to modulate the activation of immune cells by producing complement components and cytokines (13,60–62). Our results show that RPE cells go beyond this and act as immune cells, creating a local immune response in the absence of other cell types. RPE cells from the Efemp1\textsuperscript{R345W}:C3\textsuperscript{−/−} mice do not produce deposits in vitro, confirming the dependence of deposit formation on an intact complement system (45). Our studies provide the first direct indication that complement components produced locally by the RPE are involved in the formation of basal deposits.
The local immune response by the RPE is atypical since it is not based on MAC or C5, but rather a direct action of C3a. Of note, C3 was the only complement component found to be increased in the \textit{Efemp1}\textsubscript{R345W} RPE cultures, and C3a the only complement activation product detected exclusively in deposits. We are investigating which of the three complement pathways (classical, lectin or alternative) is involved with the initiation of complement activation. One possibility is that the initial generation of C3b can be explained by tick-over (63), where the hydrolysis of C3 occurs at physiological level and forms the C3bBbP convertase (64). According to this theory, the C3bBbP complex from the alternative pathway can be deposited on ECM in a stable manner, regardless the complement pathway that was primarily activated (65,66). Alternatively, it is possible that abnormal matrix generated by \textit{Efemp1}\textsubscript{R345W} would favor the deposition of C3bBbP in a stable manner (64). In either case, the C3BbP convertase will generate more C3b creating a positive feedback loop that will result in the increased release of the anaphylatoxin C3a. C3a has been detected in basal deposits and drusen, and reported to alter proteasome function in RPE and to promote choroidal neovascularization through VEGF release in late AMD (7,12,67–70). However, C3a has not been previously associated with early stages of MD pathogenesis. Thus, we propose a new role of C3a as an inflammatory molecule that is also capable of stimulating the

Figure 6. Formation of aggregates in \textit{Efemp1}\textsubscript{R345W} RPE cell cultures. (A) Aggregates stained with Proteostat (Enzo) co-localize with the \textit{Efemp1}\textsubscript{R345W} protein in the deposits. Confocal images, scale bar, 50 µm. (B) Quantification of fluorescent signal obtained from Proteostat and \textit{Efemp1} staining using the Image J software. Data represented as mean ± SD. **P < 0.01. KI, \textit{Efemp1}\textsubscript{R345W}; KICKO, \textit{Efemp1}\textsubscript{R345W:\textit{C3}−/−}.
Figure 7. Efemp1\textsuperscript{R345W} cultures: ECM turnover and interleukin secretion. (Aa) MMP-2 activity decreased in apical and basal media from KI and KIC3KO RPE cultures at 2 weeks (n = 5 per genotype). (Ab) Representative zymography gel showing MMP-2 activity in apical (a) and basal (b) media of WT, KI and KIC3KO RPE cultures. (B) mRNA levels of MMP-2 in WT, KI and KIC3KO RPE cells at 2 weeks (n = 10 WT, 11 KI and 4 KIC3KO). (C) MMP-2 activity decreased by rmEFEMP1\textsuperscript{R345W} in media from WT and by EFEMP1-neutralizing antibody in media from KI after 2 weeks. (D) MMP-2 activity decreased by rmC3a in WT media but not by C3a-neutralizing antibody in KI cultures after 2 weeks. (E and F) IL-6 (pg/ml) and (G and H) IL-1β measured in apical and basal media of WT and KI cultures treated with rmEFEMP1\textsuperscript{R345W}, anti-EFEMP1 (E and G), rmC3a or anti-C3a (F and H). All data represented as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 by ANOVA. KI, Efemp1\textsuperscript{R345W}; KIC3KO, Efemp1\textsuperscript{R345W}:C3\textsuperscript{−/−}; rmC3a, recombinant mouse C3a; rmEFEMP1\textsuperscript{R345W}, recombinant mouse EFEMP1\textsuperscript{R345W}; anti-C3a, neutralizing antibody for C3a; anti-EFEMP1, neutralizing antibody for EFEMP1.
production of abnormal ECM by RPE cells (71,72). This new role of C3a could explain the lack of responses of patients with AMD to treatments targeting C5 (19,73). Furthermore, these results suggest that therapies targeted at C3a in RPE could play an important role in treating AMD.

The primary defect in the EFEMP1-associated MD is the production of mutant EFEMP1R345W protein (36,38,43–45). In contrast to prior studies using cell lines that overexpress EFEMP1R345W, we found that the mutant protein was efficiently produced and secreted by Efemp1R345W RPE cells and acts in a non-cell autonomous fashion to stimulate deposit formation (38,74–76). We also found that the secreted EFEMP1R345W protein forms extracellular protein aggregates that create an abnormal ECM, consistent with the accumulation of the mutant protein in BrM of Efemp1R345W mice and patients (38,45). The protein aggregates may also contribute to making the ECM abnormal via binding other ECM components, and impairing ECM homeostasis (50,58,77,78).

Abnormal ECM may also be a shared feature between inherited and age-related MDs. A study suggested that altered ECM composition in BrM causes impaired RPE function and contributes to the development of AMD (79). The pathology in EFEMP1-associated MD is caused by a single mutation versus the combination of genetic predisposition and life-long environmental influences that are thought to occur in AMD. Despite these differences in etiology, increased complement activation is a common feature in both diseases (45). Thus, EFEMP1-associated MD represents a useful model for studies of disease pathogenesis, and can help to elucidate the mechanisms involved in the inflammatory process of AMD.

We found that both EFEMP1R345W and C3a decrease the activity of the MMP-2, a metalloproteinase required for normal turnover of the ECM in BrM. Loss of MMP-2 favors the accumulation of ECM proteins and is thought to contribute to the development of sub-RPE deposits in vitro and in vivo (59,80). Furthermore, alterations in MMP-2 activity can contribute to perpetuate the inflammatory process (35).

We also found that C3a participates in regulating the expression of the interleukins IL-1B and IL-6 by RPE cells. The pro-inflammatory cytokines are also thought to affect RPE function and to play a role in AMD pathogenesis (71,72). Inhibition of IL-1B and IL-6 repressed the formation of deposits in vitro, but recombinant IL-1B or IL-6 was not sufficient to cause deposit formation without the action of C3a. Thus, interleukins may contribute to the inflammatory process once complement system activation is increased by other stimuli.

In summary, studies using the novel Efemp1R345W RPE cell model system suggest that basal deposit formation is a local inflammatory process, which occurs in response to abnormal ECM (78,81–83). The novel finding that C3a locally produced by RPE cells plays a key role in early MD pathogenesis implies that complement-modulating therapies could be applied locally and addressed specifically against C3a in patients with early macular disease. This could avoid disruption of the complement system systemically, and potentially prevent progression of disease to vision-threatening sequelae such as geographic atrophy or choroidal neovascularization.

Materials and Methods

A detailed description of Materials and Methods can be found in Supplementary Material.

Mice

The guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research were followed. Efemp1R345W/R345W mice, made and characterized previously, were generated from het × het crossings (43). WT littermates generated during het × het crosses of Efemp1R345W mice were used as controls. Mice were also generated from homozygous crossings Efemp1R345W/R345W × C3−/− mice, generated and characterized previously (43,45,84,85). None of these mice contained the rd8 mutation (85).

RPE cell isolation

RPE cells were harvested from 10-week-old mice following CO2-induced euthanasia. Eyes were dissected and RPE cells were collected by trypsin digestion. A very detailed protocol is available in Supplementary Material.

RPE cell cultures

Cells were cultured at 37°C in 5% CO2 under a humidified atmosphere changing media twice a week. Serum was removed at least 72 h before performing any experiment. TER of the RPE cultures were measured using an epithelial voltohmmeter (57,86).

Culture treatments

CM from Efemp1R345W to WT

WT and Efemp1R345W RPE cells were isolated and cultured in parallel in duplicate. Apical and basal supernatants were collected from Efemp1R345W cultures every 24 h and added into the
Fractionated CM from Efemp1R345W to WT

After 2 weeks in culture, cells were WT and 3 of the transwells with media. WT RPE cells were cultured in 1 40µl of orthopaedic chamber and 600µl of orthobasal chamber (MA, USA) under sterile conditions. Volumes were brought to 1 h at room temperature (RT).

A small molecule (<3 kDa) was discarded. Each fraction was added daily to the counterpart WT culture during 2 weeks per duplicate with the last 72 h in the absence of serum.

Treatment with recombinant proteins and neutralizing antibodies

Primary RPE cell cultures were treated with different doses of recombinant proteins or neutralizing antibodies every 48 or 72 h, respectively, for 2 weeks. For treatments with rmC3a or neutralizing antibody for C3a, cells were serum starved after the first 72 h and grown without serum for another 11 days. Recombinant proteins, neutralizing antibodies and inhibitors are listed in Supplementary Material.

Recombinant protein FLAG-EFEMP1-R345W
cDNA from Efemp1R345W RPE cells was subcloned into Gateway compatible pCAG-FLAG-GFP (expression vector containing FLAG) and transfected into CHO cells using Lipofectamine 2000 (Life Technologies, Grand Island, NY, USA). Recombinant protein was purified from supernatants after 72 h using the FLAG-M purification kit (Sigma Aldrich, St Louis, MO, USA).

RNA and protein extraction

After 2 weeks in culture, RPE cells were lysed and RNA and protein were extracted with the DNA/RNA/Protein Mini Kit (Qiagen, Venlo, Netherlands). The quality and quantity of RNA was assessed using the Agilent RNA 6000 Nano Kit Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). All samples had RNA integrity number values between 9 and 10. Supernatants from the upper and bottom chamber were separately removed, and proteins were precipitated with 100% (w/v) TCA and resuspended in a buffer containing 50 mM Tris–HCl pH 8, 100 mM DTT, 10 mM EDTA, 2% SDS and 1 mM PMSF.

Expression of complement components by RPE cells

qRT-PCR was used to analyze mRNA levels. PCR conditions and specific primers can be found in Supplementary Material.

Western blot analysis

Approximately 1 µg of protein was electrophoresed on a 4–12% polyacrylamide gel (Life Technologies) and then transferred to a PVDF membrane using the iBlot System (Life Technologies). Immunoblotting was performed according to the Odyssey System (Li-Cor, Lincoln, NE, USA), using rabbit anti-EFEMP1 antibody at 0.4 µg/ml and incubated overnight at 4°C (SDIX, Newark, DE, USA). Odyssey secondary antibody anti-rabbit was incubated 1 h at room temperature (RT).

Enzyme-linked immunosorbent assay

Apical and basal supernatants from RPE cell cultures were collected after 2 weeks in culture and concentrated to equal volumes through 10 and 3 kDa Amicon filters (Millipore). The fraction over 10 kDa was used to quantify IL-1β, IL-6, EFEMP1, SC5b-9 and C3. The fraction between 3 and 10 kDa was used to quantify C3a and C5a. ELISA kits used to quantify IL-1β, IL-6 and C5a were purchased from R&D Systems (Minneapolis, MN, USA). ELISA kits used to quantify EFEMP1, SC5b-9 and C3a were purchased from MyBioSource (San Diego, CA, USA). C3 was quantified by the ALPCO ELISA kit (Salem, NH, USA).

Characterization of deposits

Deposits were characterized by TEM, SEM and immunofluorescence (87).

(i) TEM: Samples were fixed in ½ Karnovsky’s fixative for 12 h at 4°C. After fixation, samples were rinsed with 0.1 M sodium cacodylate buffer, post-fixed with 2% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1.5 h, en bloc stained with 2% aqueous uranyl acetate for 30 min, then dehydrated with graded ethyl alcohol solutions and resin infiltrated in ethyl alcohol and Spurr’s epoxy resin mixtures (Electron Microscopy Sciences, Hatfield, PA, USA) utilizing an automated EMS Lynx 1 EM tissue processor (Electron Microscopy Sciences). Processed samples were infiltrated in two changes of fresh Spurr’s resin for 24 h and then oriented in Spurr’s resin, and polymerized within silicone molds using an oven set for 60°C for 24 h minimum. The grids were imaged using an FEI Tecnai G2 Spirit transmission electron microscope (FEI, Hillsboro, OR, USA).

(ii) SEM: RPE cells grown on inserts were fixed in cold 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) followed by fixation in 1% glutaraldehyde, washed in PBS and then in dH2O, and dehydrated by serial ethanol, 35, 50, 70, 95, and 100% followed by critical dehydration using the SAMDRI-795 system. After dehydration, inserts were split into two pieces and the top or bottom side was coated with chromium using a Gatan Ion Beam Coater for 10 min. Coated inserts were imaged by an Field Emission Scanning Electron Microscope (JEOL 7401F).

The percentage of deposit formation was defined as the percentage of SEM images positive for deposits divided by the total number of images taken of the whole insert (# images per equal parts insert). A minimum of 10 images was taken per sample. The experimenter was masked to the sample identity.

(iii) Immunostaining: Inserts were rinsed in PBS, fixed for 10 min in cold 4% PFA in PBS followed by fixation in 1% glutaraldehyde for 30 min at RT. The inserts were cut with a razor blade and stored in PBS at 4°C pending sectioning and immunohistochemical analyses. Inserts were placed face down on slides as flatmounts of the bottom side. For staining, sections were blocked with 1% BSA and incubated with primary antibodies (listed in Supplementary Material) overnight at 4°C. Secondary antibodies labeled with Alexa-488 or Alexa-555 (Life Technologies) were incubated for 1 h at RT. Slides were mounted with Fluoromount G (Electron Microscopy Sciences) and visualized by a TCS SP5 II confocal laser scanning microscope (Leica). RPE bottom flatmounts stained in parallel with the samples but incubated with 1% BSA in the absence of primary antibody were used as negative controls.
(iv) Protein aggregation assay: Proteostat protein aggregation dye was used the following manufacturer’s instructions (Enzo Life Sciences).
(v) Quantification of the fluorescent signal: Images were converted to binary format with ImageJ (87). The integrated intensity was measured.

MMP-2 activity
Was measured in media from RPE cultures by zymography. Briefly, 5 µl of equal volume supernatants were loaded onto Novex 10% gelatin gels (Life Technologies). Zymography assays were then performed as per the manufacturer’s instructions. Gels were scanned using the Odyssey system (Li-Cor). MMP-2 was identified by molecular weight. Gelatinase activity was quantified using densitometry and the software ImageStudioLite (Li-Cor).

Statistical analyses
Results are expressed as mean ± SD, with $P < 0.05$ considered statistically significant. Differences between groups were compared using Student’s t-test or ANOVA as appropriate.

To calculate the EC50 and IC50 doses in RPE treated with recombinant proteins or antibodies, respectively, we used the GraphPad Prism software. Non-linear regression (curve fit) was performed using the log (concentration) versus the percentage of deposits calculated as described above.

Supplementary Material
Supplementary Material is available at HMG online.

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