

Anti-fumarase Antibody Promotes the Dropout of Photoreceptor Inner and Outer Segments in Diabetic Macular Oedema

Shin Yoshitake¹, Tomoaki Murakami¹, Kiyoshi Suzuma¹, Tatsuya Yoshitake¹, Akihito Uji¹, Satoshi Morooka¹, Yoko Dodo¹, Masahiro Fujimoto¹, Yang Shan², Patrice E. Fort^{2,3}, Shinji Ito⁴, Akitaka Tsujikawa¹, Nagahisa Yoshimura¹

Affiliation

¹Department of Ophthalmology and Visual Sciences, Kyoto University Graduate School of Medicine, Kyoto, Japan

²Department of Ophthalmology and Visual Sciences, University of Michigan, Kellogg Eye Center, Ann Arbor, Michigan, USA.

³Department of Molecular and Integrative physiology, University of Michigan, Kellogg Eye Center, Ann Arbor, Michigan, USA.

⁴Medical Research Support Center, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Correspondence to Tomoaki Murakami, MD, PhD, Department of Ophthalmology and Visual Sciences, Kyoto University Graduate School of Medicine, 54 Shogoin-Kawaracho, Sakyo, Kyoto 606-8507, Japan. Phone: 81-75-751-3250; fax: 81-75-752-0933; e-mail: mutomo@kuhp.kyoto-u.ac.jp

The word count (abstract); 228

The word count (main text); 3991

Tweet

Biochemical methods and mass spectrometry identified anti-fumarase antibody as a novel serum biomarker of DME and subsequent translational research suggested that this might be a pathological mechanism of autoimmune photoreceptor injuries in DME. (We consider Figure 4a-h as the best summary of this paper.)

Abstract

Aims/hypothesis

In diabetic macular oedema (DME), blood components passing through disrupted blood-retinal barrier cause neuroinflammation, but the mechanism by which autoantibodies induce neuroglial dysfunction is unknown. The aim of this study was to identify a novel autoantibody and to evaluate its pathological effects on clinically relevant photoreceptor injuries.

Methods

Biochemical purification and subsequent peptide fingerprinting were applied to identify autoantigens. The titers of autoantibodies in DME sera were quantified and their associations with clinical parameters were evaluated. Two animal models, i.e., passive transfer of autoantibodies and active immunization, were characterized with respect to autoimmune mechanisms underlying photoreceptor injuries.

Results

After screening serum IgG from DME patients, fumarase, a Krebs cycle enzyme expressed in inner segments, was identified as an autoantigen. Serum levels of anti-fumarase IgG in DME participants were higher than those in diabetic participants without DME ($P<0.001$) and were related to photoreceptor damage and visual dysfunction. Passively transferred fumarase IgG from DME sera in concert with complement impaired the function and structure of rodent photoreceptors. It is consistent with complement activation in damaged photoreceptors of mice immunised with fumarase. Fumarase was recruited to the cell surface by complement and reacted to this autoantibody. Subsequently, combinatorial administration elicited mitochondrial disruption and caspase-3 activation.

Conclusions/interpretation

Our data identify anti-fumarase antibody as a serum biomarker and demonstrate that this might be a pathological mechanism of autoimmune photoreceptor injuries in DME.

Keywords

Anti-fumarase antibody, Autoantibody, Complement, Diabetic macular oedema, Optical coherence tomography, Photoreceptor damage, Serum biomarker

Abbreviations

BRB	blood–retinal barrier
CAR	cancer-associated retinopathy
CSF	central subfield

DME	diabetic macular oedema
DRIL	disorganization of retinal inner layers
ELM	external limiting membrane
ERG	electroretinogram
EZ	ellipsoid zone of the photoreceptors
MAC	membrane attack complex
OCT	optical coherence tomography
ONL	outer nuclear layer
RPE	retinal pigment epithelium

Research in context

What is already known about this subject?

- Diabetes disrupts the blood-retinal barrier (BRB) which sequesters retinal antigens from autoimmunity
- Intraocular cytokines and infiltrated inflammatory cells promote neuroinflammation in diabetic retinopathy
- Neuroglial tissue including photoreceptor cells is impaired in diabetic macular oedema

What is the key question?

- Is humoral autoimmunity elicited and how do autoantibodies against retinal antigens promote neuroglial impairment in diabetic macular oedema?

What are the new findings?

- Anti-fumarase antibody is identified in sera from patients from diabetic macular oedema
- Anti-fumarase antibody is clinically related to visual reduction and photoreceptor damage in diabetic macular oedema
- Anti-fumarase antibody in collaboration with complement promote subcellular injuries in photoreceptors in animal models and cell culture experiments

How might this impact on clinical practice in the foreseeable future?

- Anti-fumarase antibody could be a novel serum biomarker and serve as a potential target for the treatment of diabetic macular oedema

Introduction

Diabetic retinopathy often leads to severe visual loss worldwide [1, 2]. An increasing number of studies have determined that disturbances in both vascular cells and neuroglial components reciprocally promote the pathogenesis of diabetic retinopathy [3-5]. In particular, diabetes mellitus exacerbates blood–retinal barrier (BRB) breakdown and concomitant diabetic macular oedema (DME), which often leads to severe visual disturbances. However, it remains largely unknown how the extravasated blood components damage neuroglial cells.

Advances in optical coherence tomography (OCT) have enabled clinicians to evaluate retinal status [6]. Retinal thickening in the macula is one of the major indicators of DME [7], and high-resolution OCT enables assessment of the status of the ellipsoid zone of the photoreceptors (EZ) as a representative of photoreceptor integrity. Histologically, this region contains high accumulation of mitochondria that support the increased level of metabolism of photoreceptor cells [8]. In eyes with DME, the EZ line is often disrupted, which significantly correlates with visual impairment [9, 10]. However, the molecular or cellular mechanisms behind the photoreceptor damage must be elucidated to establish novel therapeutic strategies for DME [11, 12].

Subretinal spaces are limited by the barrier properties of the external limiting membrane (ELM) and retinal pigment epithelium (RPE) and are known to be isolated in healthy retinas from immunity ("immunoprivileged") [13]. In chorioretinal diseases, impairment of neurovascular units leads to microvascular pathogenicity and neuroinflammation, as seen in neurological diseases [14]. In cancer associated retinopathy (CAR), autoantibodies against proteins expressed in photoreceptor cells, such as recoverin and arrestin, contribute to progressive photoreceptor degeneration [15]. This evidence encouraged us to speculate that BRB breakdown in diabetic eyes might lead to neuroglial damage through various neurotoxic blood components, including immunological agents, which is to some extent supported by publications regarding the recruitment and activation of inflammatory cells in chorioretinal diseases [16, 17].

In our current study, we identified autoantibodies against photoreceptor cells in a subgroup of DME patients and investigated their contribution to photoreceptor damage.

Methods

Patients and Biosampling

Participants with type 2 diabetes were consecutively recruited at the Department of Ophthalmology of Kyoto University Hospital and divided into the following three groups: (1) DM group, participants with type 2 diabetes but not diabetic retinopathy; (2)

DR group, participants with diabetic retinopathy but not centre-involved DME; and (3) DME group, participants with centre-involved DME.

In the discovery cohort from July 2014 to February 2016, we first included participants as shown in the flow chart (**ESM Fig. 1**). These samples were used for the screening by Western blot or immunostaining, immunoprecipitation and subsequent mass spectrometry, enzyme-linked immunosorbent assay (ELISA) to measure the autoantibody titer, and animal or cell culture experiments (**ESM Fig. 1**). In the validation cohort for ELISA, participants were included from March 2016 to December 2017. Cases with similar age, gender, and HbA1c from individual groups were included in ELISA experiments. After we excluded 33 eyes with media opacity affecting visual acuity (VA) according to the medical records, we included 106 eyes in the DME group of both cohorts and analysed the association of anti-fumarase IgG and other clinical parameters (**ESM Fig. 1**). We collected serum samples in 21 cases before and after anti-VEGF treatment according to 3+pro re nata regimen and evaluated the changes in the autoantibody titers. The participants' characteristics in the individual studies are detailed in **ESM Tables 1-10**.

Sera were aliquoted within 1 hour after sampling and immediately stored at -80°C . We conducted this study in accordance with the Declaration of Helsinki. The study was approved by the Kyoto University Graduate School and Faculty of Medicine Ethics Committee and registered at UMIN Japan Clinical Trial Registry (UMIN000014015). All participants provided written informed consent before inclusion in the study. See electronic supplementary material (ESM) Methods.

Enrichment of Photoreceptor Cells

Photoreceptor cells from porcine retinas were prepared by centrifugation in a stepwise Percoll gradient. See ESM Methods.

Cell Culture and Gene Knockout

Human embryonic kidney (HEK) 293 cells and 661W cells were cultured. *Fumarate hydratase (FH)* was knocked out in HEK 293 cells using the CRISPR CAS9 system. See ESM Methods.

Animals

After the approval by the Institutional Review Board of the Kyoto University Graduate School of Medicine, wild-type or Ins2Akita C57BL/6 mice or B10.RIII mice (H2r) were housed in accordance with the Institutional Animal Care and Use Committee

guidelines as well as the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. Eyes were harvested under a lethal dose of anesthesia. We performed subretinal injections of 290 eyes and excluded 14 eyes with media opacities. See ESM Methods.

Polymerase Chain Reaction

The mRNA expression of murine *FH* (accession number: NM_010209.2) in retinas from C57BL/6 mice (wild type or Ins2Akita diabetic) was determined using polymerase chain reaction. See ESM Methods.

Immunoblot Analysis

Porcine retinal lysates were applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Participants' sera were incubated as primary antibodies to detect retinal autoantigens.

We evaluated protein amounts of fumarase in HEK 293 cells or those of cytochrome C, apoptosis-inducing factor (AIF), and β -actin in cytosolic fraction of 661W cells using commercially available antibodies (**ESM Table 11**). The antibodies were used according to the manufacturers' instructions.

See ESM Methods.

Immunoprecipitation and pulldown assay

Cell lysates of porcine photoreceptor cells were incubated with serum IgG conjugated to protein G-coated beads to immunoprecipitate antigens against autoantibodies. See ESM Methods.

Mass Spectrometry

After in-gel digestion of immunoprecipitated proteins from porcine photoreceptor cells, tryptic digests were introduced into a mass spectrometer. See ESM Methods.

Preparation of cytosol fractions

Cytosol fractions were prepared from 661W cells using the subcellular fractionation kit (Mitochondria Isolation Kit for Cultured Cells [Thermo Scientific]). See ESM Methods.

Measurements of the intracellular Ca^{2+} concentration

The intracellular Ca^{2+} concentration was measured using a Fluo 4-AM kit (Calcium kit-Fluo 4; Dojindo Laboratories) in 661W cells. See ESM Methods.

Measurements of mitochondrial membrane potential

After incubation with JC-1 dye (Thermo Scientific), the fluorescence levels were measured in HEK 293 cells according to the manufacturer's protocol. See ESM Methods.

Caspase-3 Activity

Caspase-3 activity in 661W cells were assessed by the colorimetric assay (APOPCYTO™ Caspase-3 Colorimetric Assay Kit, MBL). See ESM Methods.

Flow cytometry

After HEK 293 or 661W cells were incubated with fluorescence-labeled anti-fumarase antibody, the fluorescence intensity was analyzed on FACS Calibur™ (BD Biosciences). See ESM Methods.

Enzyme-linked Immunosorbent Assay

The wells whose bottom was coated with recombinant human fumarase were incubated in diluted serum as the primary antibodies. See ESM Methods.

Antibody Depletion and Subretinal Injection of Serum

Agarose beads conjugated to recombinant human fumarase were incubated with DME sera to prepare sera depleted of anti-fumarase antibodies. These specimens were injected into subretinal spaces in C57BL/6 mice. See ESM Methods.

Purification of Anti-Fumarase Antibodies from DME Sera and Preparation of Complement Components

Anti-fumarase antibodies from DME sera were captured to NHS-activated Sepharose 4 Fast Flow (GE Healthcare) coupled to human recombinant fumarase. See ESM Methods.

Immunisation with fumarase

B10.RIII mice were immunised using fumarase in combination with Complete Freund's adjuvant and Pertussis Toxin. See ESM Methods.

Immunostaining

Retinal sections from mice (C57BL/6 wild-type, C57BL/6 Ins2Akita, or B10.RIII) or

human retinas were incubated with DME sera or antibodies against COX IV, fumarase, recoverin, CD59a, and C5b-9, followed by fluorescence-labeled secondary antibodies (**ESM Table 11**). HEK 293 or 661W cells were also incubated with primary antibodies against fumarase and C5b-9 (**ESM Table 11**).

Electron Microscopy

Ultrathin slices of HEK293 cells were observed by transmission electron microscopy (H-7650; Hitachi, Tokyo, Japan). See ESM Methods.

Cell Viability Assay

The treated HEK 293 or 661W cells were applied to the cell viability/cytotoxicity assay kit (LIVE/DEAD assay; Life technologies) according to the manufacturer's instructions. See ESM Methods.

Electroretinogram

The amplitudes of the mixed cone and rod response were evaluated in C57BL/6 mice. See ESM Methods.

OCT

After retinal sectional images were obtained using spectral domain (SD) OCT in participants with diabetic retinopathy, we evaluated the central subfield (CSF) thickness, EZ status, and the disorganization of retinal inner layers (DRIL). SD-OCT images of C57BL/6 mice were also acquired. See ESM Methods.

Statistics

After confirmation of normality using the Kolmogorov-Smirnov test, the Kruskal–Wallis test of one-way analysis of variance (ANOVA) with Dunnett's multiple comparison, Mann-Whitney U-test, or Wilcoxon signed-rank test was performed to evaluate the nonparametric data. Spearman's rank correlation coefficient was used to show statistical correlations. The paired t-test, Student's t-test, or one-way ANOVA with Bonferroni correction was used for statistical differences in parametric parameters. The sampling distribution in the participants' characteristics was evaluated using Fisher's exact test or the chi-square test. We further applied univariate or multivariate logistic regression analysis and univariate or multivariate linear regression analysis. These statistical analyses were performed using commercial software (PASW Statistics, version 18; SPSS Inc., Chicago, IL), and $p < 0.05$ was considered significant. The values

were shown in the mean±standard deviation (s.d.).

Results

Identification of fumarase as an autoantibody target

During autoantibody screening, western blot analyses showed that serum IgG from DME participants had high levels of immunoreactivity to proteins expressed in porcine photoreceptor cells, suggesting the presence of autoantibodies specific to DME patients (**Fig. 1**). Among several bands, a ~50-kDa band was detected in sera from 19 (70.4%) of 27 DME participants. In addition, immunostaining using serum IgG revealed specific signals in photoreceptor cells, including inner segments, in 23 of 27 DME participants (85.2%) (**Fig. 2**).

To identify the targets of the autoantibodies in DME serum, we performed a combination of immunoprecipitation and mass spectrometry, as described previously [18]. Porcine photoreceptor lysates were immunoprecipitated with serum IgG according to protein G sepharose bead-based procedures. Following SDS-PAGE and silver staining revealed that a unique ~50-kDa band was precipitated with DME serum (**Fig. 3a and b**). After in-gel digestion, peptide mass fingerprinting identified fumarase (predicted molecular weight = 55 kDa) as an autoantigen, which was confirmed by immunoblotting using mouse monoclonal anti-fumarase antibody and recombinant human fumarase (**Fig. 3c and d**).

Fumarase, one of the main metabolic enzymes of the tricarboxylic acid cycle, is transported mainly into the mitochondrial matrix, although some forms are cytosolic [19]. In the retinas of both control and diabetic mice (male heterozygous Ins2Akita mice), fumarase mRNA was amplified by PCR (**ESM Fig. 2**). Immunostaining showed that fumarase is highly expressed in photoreceptor inner segments containing accumulated mitochondria in both human and rodent retinas, although there were no definite differences in its expression between control and diabetic subjects (**Fig. 3e**).

Quantitative analyses of anti-fumarase antibody

ELISA analysis revealed that the titers of total serum IgG against fumarase from DME participants were significantly higher than those from the participants of the DM group and DR group (**Fig. 4a**) [20]. According to the post-hoc comparisons, the titers of anti-fumarase IgG were not different among participants with moderate nonproliferative diabetic retinopathy (NPDR), severe NPDR, and proliferative diabetic retinopathy (PDR) (data not shown) [21]. Furthermore, an independent cohort confirmed that the DME group had higher titers than the DR group ($P<0.001$; **ESM Table 6**). Multivariate

logistic regression analyses indicate that the titer of anti-fumarase IgG may be a serum biomarker of a subgroup of DME among patients with type 2 diabetes or diabetic retinopathy (**ESM Tables 12 and 13**).

In 106 eyes with centre-involved DME, the serum titer of anti-fumarase IgG was modestly correlated to logMAR VA and the EZ disruption on SD-OCT images rather than the DRIL or CSF thickness (**Fig. 4b and c, ESM Table 14**). Interestingly, the titer of this autoantibody was decreased after the anti-VEGF treatment (**Fig. 4d**).

Functional analysis of anti-fumarase antibody

These results encouraged us to investigate the *in vivo* effects of anti-fumarase antibody on photoreceptor damage. DME sera with anti-fumarase antibodies were at randomly selected and injected into the subretinal spaces of C57BL/6 mice (**Fig. 5**). Both the IgG in DME serum and C5b-9, which is a component of the membrane attack complex (MAC) of the complement system, showed a diffuse immunostaining with a punctate appearance mainly in both inner and outer segments and few cellular infiltration 24 hours after the injection. In contrast, these signals were completely diminished in the retinas injected with the serum depleted of anti-fumarase antibodies (**Fig. 5d and e**).

Histological analyses revealed that outer segments and parts of inner segments but not the outer nuclear layer (ONL) disappeared in some areas of the retinas 7 days after the administration with DME serum containing anti-fumarase IgG. In contrast, both inner and outer segments were preserved in retinas received sera depleted of anti-fumarase antibody. It was consistent with SD-OCT findings that the EZ line was not complete and outer retinal thickness was decreased in retinas administrated with DME serum, compared to retinas injected with the serum depleted of fumarase antibodies (**Fig. 5f-i**).

The deposition of MAC and minimal cellular infiltration in photoreceptor outer segments were consistent with the higher titers of IgG1 and/or IgG3 and the absence of CD59a, a membrane-bound inhibitor of the MAC (**Fig. 4e-h and 5c-e**). These results encouraged us to investigate the function of anti-fumarase antibody in concert with the complement cascade among several mechanisms of autoantibody-mediated autoimmunity. We first evaluated photoreceptor damage in mice in which autoantibodies and complement were passively transferred. Histological and electroretinogram (ERG) experiments confirmed that the photoreceptors were morphologically and functionally ablated 7 days after subretinal injection with anti-fumarase IgG and complement, as observed in eyes injected with DME sera, whereas these changes were not observed after injection of fumarase IgG alone or control IgG+complement (**Fig. 6a-c and ESM Fig. 3**). In 6 (42.9%) of 14 B10.RIII

mice (H2r) immunised with fumarase, both HE staining and immunofluorescence delineated structural damage in the photoreceptor inner and outer segments, although cells rarely migrated into the subretinal spaces (**Fig. 6d**). Mouse IgG was partly co-localized with C5b-9 in the inner and outer segments (**Fig. 6e**).

We further planned cell culture experiments to investigate the mechanisms by which this autoantibody and complement induced cellular damage. In vitro experiments revealed that the autoantibody was partially co-localized with fumarase and C5b-9 on the cell surface after incubation with anti-fumarase IgG purified from DME sera and complement, whereas co-staining was not observed in *FH*^{-/-} cells (**Fig. 7a and b, ESM Fig. 4a-c**).

The intracellular Ca²⁺ concentration was transiently increased under control IgG and complement, as in the case of sublytic MAC-induced Ca²⁺ influx in nucleated cells [22]. High levels of Ca²⁺ influx persisted after the treatment with both anti-fumarase antibody and complement compared to no alteration of the Ca²⁺ concentration by anti-fumarase antibody alone (**Fig. 7c**). Ca²⁺ chelators reversed the immunoreactivity of fumarase on the cell surface (**ESM Fig. 4d and e**). Consistent with Ca²⁺ burst-induced mitochondrial disruption [23], the mitochondrial membrane potential (MMP) was decreased and proapoptotic signal transducing molecules, cytochrome C and AIF, leaked into the cytosol under anti-fumarase antibody in combination with complement (**Fig. 7d and e**). Caspase-3, an apoptosis executioner, was also activated in a Ca²⁺-dependent manner (**Fig. 7f**).

TEM revealed multiple processes during cell death, including vacuolation in the cytosol and mitochondria as well as nuclear deformation under anti-fumarase antibody and complement (**Fig. 7g**) [24]. Actually, the number of dead cells increased after the combinatorial administration, which was reversed by *FH* gene-ablation. Furthermore, this cell death partly depended on caspase-3 activation (**Fig. 7h and i**). Pruning of axons and dendrites is mediated by mitochondrial damage and a caspase pathway in developmental or pathological neurons [25, 26], which might allow us to speculate that these *in vitro* experiments support subcellular damage of photoreceptor inner and outer segments.

Discussion

Many publications have shown that, in addition to retinal thickening, foveal photoreceptor damage is related to visual dysfunction in eyes with DME [7, 9, 10], although the cellular and molecular mechanisms are unknown. A disrupted BRB in diabetic retinas allows blood components, including autoantibodies, to pour into the

extravascular space and exacerbate neuroinflammation [3, 4, 27]. In our current study, anti-fumarase autoantibody, which was newly identified in the sera of a subgroup of DME participants, had clinical association with photoreceptor damage and visual impairment. This fumarase autoantibody in concert with complement activation induced the dropout of photoreceptor inner and outer segments. Our results suggest a novel target of autoantibodies in DME sera as well as a novel pathological mechanism for photoreceptor damage, which is clinically evident on SD-OCT images in DME [9, 10]. After screening using immunoblotting, in which the denaturation of proteins provides higher sensitivity for epitopes, we used immunoprecipitation using a nondenaturing buffer (high salt concentration and neutral pH) to mimic the three-dimensional antigen–antibody reaction and to concentrate autoantigens for subsequent mass spectrometry [18]. The second screening excluded many autoantigen candidates, allowing the identification of several putative antigens. Of these, fumarase was both a target for autoantibodies in DME sera and was highly expressed in the inner segment of photoreceptors, which prompted us to focus on qualitative and quantitative analyses of anti-fumarase antibody.

According to the quantitative ELISA data, 32 (49.2%) of 65 participants with DME had a moderate titer of anti-fumarase antibody (above the mean + 2 s.d. in the DM group), whereas western blot detected an ~50-kDa band in 19 (70.4%) of 27 DME serum. The discrepancy might depend on the different biochemical methods to detect the different epitopes. Another possibility might be the presence of additional autoantigens with almost the same molecular weight, such as arrestin and enolase, both of which have been reported as targets for autoantibodies in sera from patients with CAR or age-related macular degeneration (AMD) [28, 29]. Furthermore, Immunoblot revealed that IgG in DME sera had immunoreactivity to several antigens with other molecular weights in photoreceptor cells and immunostaining using serum IgG also showed immunoreactivity to photoreceptor cells in 23 (85.2%) of 27 DME participants. Some cases with the lower titer of anti-fumarase IgG had photoreceptor damage and poor visual function. These results suggest additional autoantigens in photoreceptor cells. Using immunofluorescence, we often observed definite fluorescent signals in the ganglion cell layer and inner nuclear layer. Taken together, systematic analyses of anti-retinal antibodies would promote our understanding of the immunological aspects of neuroinflammation in DME.

The statistical analyses of the clinical data demonstrated that anti-fumarase IgG was increased in a subgroup of DME participants with photoreceptor damage and visual impairment, although whether this autoantibody is the cause or result of photoreceptor

damage remains to be determined. In vitro and in vivo experiments in the current study suggested that this autoantibody might promote photoreceptor damage in DME. Oppositely, whether autoantibody production is induced by the intracellular antigens derived from photoreceptor cells remains ill-defined. We speculate that the disrupted BRB could allow retinal autoantigens to enter the blood or recruit or activate immune cells to migrate to and from the retinas [30], and a future study should elucidate the molecular mechanisms by which vascular permeability contributes to the elicitation of autoimmunity. As another possible explanation, anti-fumarase IgG might represent mitochondria-related systemic complications due to diabetes, which is clinically associated with DME [31]. Antigen-specific regulatory T cells, which lead to immune tolerance, might be modulated by diabetic states [32]. Diabetes induces mitochondrial damage that is at least partly mediated via oxidative stress, which might contribute to the exposure of intracellular autoantigens and immunological tolerance loss by the posttranslational modification of autoantigens [31-34].

Complement MAC was formed in photoreceptor inner and outer segments in the passive transfer model. It encouraged us to speculate that photoreceptor damage is mediated via complement activation at least partly among several autoantibody-related mechanisms [35, 36]. Some autoantibodies by themselves inhibit the function of antigens and concomitantly promote the pathological mechanisms as in the case of myasthenia gravis. By contrast, anti-fumarase IgG did not promote the cellular injuries in the absence of complement. Cell death was not increased in *FH*^{-/-} cells and a few publications documented oncological findings induced by genetic mutations [37, 38]. These results suggest that the functional inhibition by this autoantibody alone did not induce photoreceptor damage. Immunofluorescence in cultured cells might suggest the antibody reacted with the intracellular autoantigens released from the damaged cells and concomitantly contributed to the secondary inflammatory responses [39].

We cannot conclude how the autoantibody for fumarase, a ubiquitously expressed protein, specifically injuries photoreceptor cells in DME; similarly, the mechanism of anti-mitochondrial antibodies in primary biliary cirrhosis has not been determined [40]. Photoreceptor cells highly express fumarase and are the main source of reactive oxygen species under diabetic states, which might promote antigen-antibody reaction in these cells as discussed above [33, 34]. The absence of CD59 might allow complement-mediated autoimmune mechanisms in the photoreceptor inner and outer segments. Additionally, the barrier properties of the RPE and ELM might inhibit the diffusion of autoantibodies or complement components and cellular infiltration from or into the subretinal spaces and confine cellular damage to these layers [13].

Generally, intracellular autoantigens are released during nonapoptotic cell death, and subsequently the deposition of antigen-antibody complex exacerbates the cellular damages via several inflammatory mechanisms. Alternatively, anti-recoverin and anti-enolase antibodies from CAR patients are internalized into cells and induce the cytotoxicity in the absence of complement [41, 42]. Histological findings in animal models led us to investigate complement-mediated mechanisms rather than inflammatory responses. Surprisingly, fumarase was recruited to the cell surface after complement treatment, and was reacted to anti-fumarase antibody derived from DME sera with subsequent MAC formation, as in the case of autoantigens in the cell surface. Such changes depend on calcium signaling, although the downstream executioners remain to be elucidated. This result is consistent with the enigmatic findings that showed that the anti-fumarase antibody reacted with antigens in both photoreceptor inner and outer segments in mice administered with DME sera, although immunostaining with the same sera was limited to the photoreceptor inner segments in untreated retinas. This is a novel molecular mechanism by which this autoantibody reacts to intracellular antigens on the cell surface. Future studies should elucidate whether sublytic MAC formation promotes the elicitation of autoimmune responses to intracellular autoantigens. Such biological changes are to some extent analogous to the autoimmune responses to mitochondrial molecules in a few diseases [43].

Anti-fumarase antibody and complement impaired the structure and function of mitochondria. Generally, overload of calcium signaling damages mitochondria [23, 44], which allowed us to speculate that the persistent Ca^{2+} influx contributed to mitochondrial injuries at least partly in cultured cells incubated with this autoantibody and complement. Mitochondrial disruption is one of the initial changes in neurodegenerative diseases [45]. In several pathological states, mitochondrial damage is one of the main regulators of the synaptic plasticity [26]. Several mechanisms of mitochondrial damage are generally accepted: dysregulated Ca^{2+} levels, reactive oxygen species, loss of the MMP ($\Delta\psi_m$), and efflux of apoptotic or necrotic factors, some of which were shown in this study [26, 31, 45, 46]. The other mechanism is caspase activation. Several publications have reported that the pruning of axons and dendrites is mediated via a caspase cascade [25]. We thus speculated that caspase-3 activation partly contributed to the subcellular damage in the inner and outer segments, which corresponded to the dendrites specialized for photoreception.

VA improvement is the primary endpoint for the efficacy of anti-VEGF drugs for DME, which is supported by the general surrogate marker, decreased macular thickness [12]. Recent publications have documented the restoration of the EZ line on SD-OCT images

after anti-VEGF therapy [47]. Re-establishment of the BRB by this standard treatment might decrease intraocular anti-fumarase IgG or complement components derived from the blood and concomitant photoreceptor damage. The decrease in this autoantibody might be consistent with photoreceptor restoration after the treatment, although further study should elucidate the underlying molecular mechanisms. A translational research study demonstrated the involvement of complement under anti-VEGF treatment in AMD [48]. Future systematic and comparative studies may reveal the cellular and histological injuries induced by individual autoantibodies and/or complement in individual diseases [9, 10, 49, 50].

In conclusion, we identified fumarase as a target of autoantibodies in a subgroup of DME sera and a potential contributor to photoreceptor damage in DME, although whether this autoantibody is the result of this pathogenesis remains to be elucidated. Further characterization of autoantibodies against photoreceptor cells may facilitate the future development of antigen-specific immunomodulatory therapies for photoreceptor damage in DME.

Acknowledgments

Supported by Grant-in-Aid for Scientific Research of Japan Society for the Promotion of Science. Immunostaining of human retinas, which utilized the Core Center for Vision Research funded by P30 EY007003 from the National Eye Institute, was supported by awards from Eversight and the Diabetes Consortium (NIH). Mass spectrometry were performed at the Medical Research Support Center, Graduate School of Medicine, Kyoto University, which was supported by Platform for Drug Discovery, Informatics, and Structural Life Science from the Ministry of Education, Culture, Sports, Science and Technology, Japan. We would like to thank R.H. Rosa Jr. (Texas A&M Health Science Center) for providing discussion and K. Okamoto-Furuta and H. Kohda (Division of Electron Microscopic Study, Center for Anatomical Studies, Graduate School of Medicine, Kyoto University) for technical assistance in electron microscopy.

Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files.

Funding

The study was supported by Grant-in-Aid for Scientific Research of Japan Society for the Promotion of Science (26462637, 17K11423, 18K19610).

Duality of interest

The authors have declared that no conflict of interest exists.

Contribution statement

SY conceived and designed of the study, acquired data, drafted the manuscript, and approved its final version. TM, KS, TY, AU, SM, YD, MF, AT, and NY contributed to the conception and design of the study and acquisition, analysis, and interpretation of data, revised the article's intellectual content, and approved the final version. YS, PEF, and SI contributed to the acquisition and analysis of data, revised the article's intellectual content, and approved the final version. TM is the guarantor of this work.

References

1. Frank RN. (2004) Diabetic retinopathy. *N Engl J Med* 350: 48-58
2. Yau JW, Rogers SL, Kawasaki R et al. (2012) Global prevalence and major risk factors of diabetic retinopathy. *Diabetes Care* 35: 556-564
3. Gardner TW, Antonetti DA, Barber AJ, LaNoue KF, Levison SW. (2002) Diabetic retinopathy: more than meets the eye. *Surv Ophthalmol* 47 Suppl 2: S253-262
4. Antonetti DA, Klein R, Gardner TW. (2012) Diabetic retinopathy. *N Engl J Med* 366: 1227-1239
5. Murakami T, Frey T, Lin C, Antonetti DA. (2012) Protein kinase c beta phosphorylates occludin regulating tight junction trafficking in vascular endothelial growth factor-induced permeability in vivo. *Diabetes* 61: 1573-1583
6. Huang D, Swanson EA, Lin CP et al. (1991) Optical coherence tomography. *Science* 254: 1178-1181
7. Browning DJ, Glassman AR, Aiello LP et al. (2007) Relationship between optical coherence tomography-measured central retinal thickness and visual acuity in diabetic macular edema. *Ophthalmology* 114: 525-536
8. Spaide RF, Curcio CA. (2011) Anatomical correlates to the bands seen in the outer retina by optical coherence tomography: literature review and model. *Retina* 31: 1609-1619
9. Sakamoto A, Nishijima K, Kita M et al. (2009) Association between foveal photoreceptor status and visual acuity after resolution of diabetic macular edema by pars plana vitrectomy. *Graefes Arch Clin Exp Ophthalmol* 247: 1325-1330
10. Murakami T, Nishijima K, Akagi T et al. (2012) Optical coherence tomographic reflectivity of photoreceptors beneath cystoid spaces in diabetic macular edema. *Invest Ophthalmol Vis Sci* 53: 1506-1511
11. Aiello LP, Avery RL, Arrigg PG et al. (1994) Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med* 331: 1480-1487
12. Cunningham ET, Jr., Adamis AP, Altaweel M et al. (2005) A phase II randomized double-masked trial of pegaptanib, an anti-vascular endothelial growth factor aptamer, for diabetic macular edema. *Ophthalmology* 112: 1747-1757
13. Bunt-Milam AH, Saari JC, Klock IB, Garwin GG. (1985) Zonulae adherentes pore size in the external limiting membrane of the rabbit retina. *Invest Ophthalmol Vis Sci* 26: 1377-1380
14. Lo EH, Dalkara T, Moskowitz MA. (2003) Mechanisms, challenges and opportunities in stroke. *Nat Rev Neurosci* 4: 399-415

15. Thirkill CE, Roth AM, Keltner JL. (1987) Cancer-associated retinopathy. *Arch Ophthalmol* 105: 372-375
16. Ishida S, Yamashiro K, Usui T et al. (2003) Leukocytes mediate retinal vascular remodeling during development and vaso-obliteration in disease. *Nat Med* 9: 781-788
17. Ambati J, Anand A, Fernandez S et al. (2003) An animal model of age-related macular degeneration in senescent Ccl-2- or Ccr-2-deficient mice. *Nat Med* 9: 1390-1397
18. Srivastava R, Aslam M, Kalluri SR et al. (2012) Potassium channel KIR4.1 as an immune target in multiple sclerosis. *N Engl J Med* 367: 115-123
19. Tolley E, Craig I. (1975) Presence of two forms of fumarase (fumarate hydratase E.C. 4.2.1.2) in mammalian cells: immunological characterization and genetic analysis in somatic cell hybrids. Confirmation of the assignment of a gene necessary for the enzyme expression to human chromosome 1. *Biochem Genet* 13: 867-883
20. Early Treatment Diabetic Retinopathy Study research group. (1985) Photocoagulation for diabetic macular edema. Early Treatment Diabetic Retinopathy Study report number 1. *Arch Ophthalmol* 103: 1796-1806
21. Wilkinson CP, Ferris FL, 3rd, Klein RE et al. (2003) Proposed international clinical diabetic retinopathy and diabetic macular edema disease severity scales. *Ophthalmology* 110: 1677-1682
22. Triantafilou K, Hughes TR, Triantafilou M, Morgan BP. (2013) The complement membrane attack complex triggers intracellular Ca²⁺ fluxes leading to NLRP3 inflammasome activation. *J Cell Sci* 126: 2903-2913
23. Rizzuto R, De Stefani D, Raffaello A, Mammucari C. (2012) Mitochondria as sensors and regulators of calcium signalling. *Nat Rev Mol Cell Biol* 13: 566-578
24. Elmore S. (2007) Apoptosis: a review of programmed cell death. *Toxicol Pathol* 35: 495-516
25. Williams DW, Kondo S, Krzyzanowska A, Hiromi Y, Truman JW. (2006) Local caspase activity directs engulfment of dendrites during pruning. *Nat Neurosci* 9: 1234-1236
26. Mattson MP, Gleichmann M, Cheng A. (2008) Mitochondria in neuroplasticity and neurological disorders. *Neuron* 60: 748-766
27. Funatsu H, Yamashita H, Noma H et al. (2002) Increased levels of vascular endothelial growth factor and interleukin-6 in the aqueous humor of diabetics with macular edema. *Am J Ophthalmol* 133: 70-77
28. Adamus G, Aptsiauri N, Guy J et al. (1996) The occurrence of serum

autoantibodies against enolase in cancer-associated retinopathy. *Clin Immunol Immunopathol* 78: 120-129

29. Adamus G, Chew EY, Ferris FL, Klein ML. (2014) Prevalence of anti-retinal autoantibodies in different stages of Age-related macular degeneration. *BMC Ophthalmol* 14: 154

30. Shechter R, London A, Schwartz M. (2013) Orchestrated leukocyte recruitment to immune-privileged sites: absolute barriers versus educational gates. *Nat Rev Immunol* 13: 206-218

31. Nishikawa T, Edelstein D, Du XL et al. (2000) Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 404: 787-790

32. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. (2008) Regulatory T cells and immune tolerance. *Cell* 133: 775-787

33. Du Y, Veenstra A, Palczewski K, Kern TS. (2013) Photoreceptor cells are major contributors to diabetes-induced oxidative stress and local inflammation in the retina. *Proc Natl Acad Sci U S A* 110: 16586-16591

34. Doyle HA, Mamula MJ. (2001) Post-translational protein modifications in antigen recognition and autoimmunity. *Trends Immunol* 22: 443-449

35. Saadoun S, Waters P, Bell BA et al. (2010) Intra-cerebral injection of neuromyelitis optica immunoglobulin G and human complement produces neuromyelitis optica lesions in mice. *Brain* 133: 349-361

36. Patrick J, Lindstrom J. (1973) Autoimmune response to acetylcholine receptor. *Science* 180: 871-872

37. Toro JR, Nickerson ML, Wei MH et al. (2003) Mutations in the fumarate hydratase gene cause hereditary leiomyomatosis and renal cell cancer in families in North America. *Am J Hum Genet* 73: 95-106

38. Pollard PJ, Spencer-Dene B, Shukla D et al. (2007) Targeted inactivation of fh1 causes proliferative renal cyst development and activation of the hypoxia pathway. *Cancer Cell* 11: 311-319

39. Hanayama R, Tanaka M, Miyasaka K et al. (2004) Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. *Science* 304: 1147-1150

40. Kaplan MM, Gershwin ME. (2005) Primary biliary cirrhosis. *N Engl J Med* 353: 1261-1273

41. Adamus G, Machnicki M, Seigel GM. (1997) Apoptotic retinal cell death induced by antirecoverin autoantibodies of cancer-associated retinopathy. *Invest Ophthalmol Vis Sci* 38: 283-291

42. Magrys A, Anekonda T, Ren G, Adamus G. (2007) The role of anti-alpha-enolase autoantibodies in pathogenicity of autoimmune-mediated retinopathy. *J Clin Immunol* 27: 181-192
43. Grundtman C, Kreutmayer SB, Almanzar G, Wick MC, Wick G. (2011) Heat shock protein 60 and immune inflammatory responses in atherosclerosis. *Arterioscler Thromb Vasc Biol* 31: 960-968
44. Georgiannakis A, Burgoyne T, Lueck K et al. (2015) Retinal Pigment Epithelial Cells Mitigate the Effects of Complement Attack by Endocytosis of C5b-9. *J Immunol* 195: 3382-3389
45. Swerdlow RH, Parks JK, Cassarino DS et al. (1998) Mitochondria in sporadic amyotrophic lateral sclerosis. *Exp Neurol* 153: 135-142
46. Galluzzi L, Blomgren K, Kroemer G. (2009) Mitochondrial membrane permeabilization in neuronal injury. *Nat Rev Neurosci* 10: 481-494
47. Mori Y, Suzuma K, Uji A et al. (2016) Restoration of foveal photoreceptors after intravitreal ranibizumab injections for diabetic macular edema. *Sci Rep* 6: 39161
48. Keir LS, Firth R, Aponik L et al. (2017) VEGF regulates local inhibitory complement proteins in the eye and kidney. *J Clin Invest* 127: 199-214
49. Sawyer RA, Selhorst JB, Zimmerman LE, Hoyt WF. (1976) Blindness caused by photoreceptor degeneration as a remote effect of cancer. *Am J Ophthalmol* 81: 606-613
50. Ambati J, Atkinson JP, Gelfand BD. (2013) Immunology of age-related macular degeneration. *Nat Rev Immunol* 13: 438-451

Figure legends

Figure 1. Reactivity of nondiabetic or DME Sera with Porcine Retinal Lysates (RE) and Cell Lysates of Enriched Photoreceptor Cells (PC).

Western blotting was used to screen the immunoreactivity of serum IgG from 6 nondiabetic (nonDM) controls (**a**) or 27 DME participants (**b**) to identify proteins expressed in photoreceptor cells. The participants' characteristics are shown in **ESM Table 1**.

Figure 2. Autoantibodies in the Sera from DME Participants.

OCT images revealed the lack of EZ (arrowheads) in parts of photoreceptor cells in an eye with DME (**c and d**), compared with the definite EZ line in an eye with diabetic retinopathy (DR) but not with DME (**a and b**). ELM, external limiting membrane; EZ, ellipsoid zone; RPE, retinal pigment epithelium. Scale bar, 200 μm . (**b and d**) magnified images of **a** and **c**. (**e**) Total retinal lysates (RE) and cell lysates of enriched photoreceptor cells (PC) underwent immunoblotting with serum IgG from these participants. Immunofluorescence showed colocalization of COX IV with serum IgG from the same DME participant at the EZ of photoreceptor inner segments (arrow; **g**; $n = 2$ retinas), compared with no immunoreactivity of serum IgG from the DR participant without DME in photoreceptor cells (**f**; $n = 2$ retinas). Scale bar, 50 μm . GCL, ganglion cell layer; INL, inner nuclear layer, IPL, inner plexiform layer; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segment; RPE, retinal pigment epithelium. The participants' characteristics are documented in **ESM Table 2**.

Figure 3. Identification of Fumarase as a Target of Serum IgG from DME Participants.

SDS-PAGE analysis of porcine photoreceptor cell lysates precipitated with serum IgG of a diabetic retinopathy (DR) participant without (DR serum) or with (DME serum) DME, whose characteristics are shown in **ESM Table 3**, was followed by either silver staining (**a**) or immunoblotting with serum IgG from the same DME participant (**b**). Analysis of the ~50-kDa unique band (arrow in **A**) using mass spectrometry identified fumarase, which was confirmed by reblotting with mouse monoclonal anti-fumarase antibodies (**c**). (**d**) Human recombinant fumarase (FH) was analyzed by immunoblotting with IgG from DR serum or DME serum. (**e**) Immunostaining revealed the immunoreactivity of fumarase in the photoreceptor inner segments in the retinas from nondiabetic subjects and DR patients (The donor's characteristics are documented in **ESM Table 4**). $n = 2$. Scale bar, 50 μm . The representative of 6 nondiabetic subjects or

5 DR participants are shown.

Figure 4. Quantitative Analysis of IgG Reactivity to Recombinant Fumarase in Serum or Vitreous Humor from DME Participants.

(a) ELISA was used to quantify the titers of total anti-fumarase autoantibodies, which revealed higher titers in DME sera than in DM (diabetes mellitus without diabetic retinopathy) or DR (diabetic retinopathy without DME) sera. $*p<0.001$. Kruskal-Wallis test of one-way analysis of variance with Dunnett's multiple comparison. $n = 65$ in individual groups. The participants' characteristics are shown in **ESM Table 5**. (b and c) Post-hoc analyses of 106 DME cases from two datasets after the exclusion of cases with media opacity affecting VA. The titer was modestly associated with logMAR VA (b) or the transverse length of disrupted EZ (c). Spearman's rank correlation coefficient in b and c. The participants' characteristics are documented in **ESM Table 7**. (d) The reduced titers of anti-fumarase IgG after anti-VEGF treatment for DME. $**p=0.001$. Wilcoxon signed-rank test. $n = 21$. The participants' characteristics are reported in **ESM Table 8**. (e-h) The titers of individual IgG subtypes of fumarase-specific antibodies in 10 sera with higher titers of total IgG in a. Most sera contained higher titers of IgG1 and/or IgG3 against fumarase. The participants' characteristics are reported in **ESM Table 9**.

Figure 5. Anti-Fumarase Antibody in DME Serum is Necessary for Complement Activation and Structural Changes in Photoreceptor Inner and Outer Segments in Mice Administrated with DME Sera.

(a) Untreated retinas immunostained with DME serum containing higher titers of anti-fumarase IgG (**ESM Table 10**), which was to be subretinally injected. $n= 3$ retinas. Scale bar, 50 μm . (b) Western blot using the same DME serum with and without the depletion (dep(+)) and dep(-), respectively). CBB, Coomassie Brilliant Blue. $n = 3$ of duplicate ELISA experiments. (c) No or weak immunoreactivity of CD59a, an endogenous inhibitor of MAC, in photoreceptor inner and outer segments of untreated retinas. $n= 3$ retinas. Scale bar, 50 μm . (d-i) Retinal status was analysed 24 hours (d and e) or 7 days (f-i) after subretinal administration of DME serum containing higher titers of anti-fumarase IgG or serum depleted of anti-fumarase antibody. (d and e) Immunofluorescence analysis of IgG from DME serum in the photoreceptor inner and outer segments, compared with fumarase and C5b-9. Representatives of sera from 2 DR or 2 DME participants (**ESM Table 10**) are shown. Hematoxylin and eosin (HE) staining (f and g) and OCT images (h) revealed structural damage in the photoreceptor

inner and outer segments in eyes injected with DME serum, and this damage was partially reversed by depleted serum. Scale bar in **f** and **h** or **g**, 50 or 20 μm , respectively. Representatives of 4 DR or 8 DME sera (**ESM Table 10**) are shown. **(i)** OCT measurement of the outer retinal thickness (from the OPL to the Bruch's membrane). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 16$ in PBS, $n = 5$ in each group. Paired t -test.

Figure 6. Anti-Fumarase Antibody in DME Sera in Combination with Complement Promotes Cellular Injuries in Animal Models.

(a-c) Morphologic and functional analyses of the retinas 7 days after subretinal administration of 4 mg/ml IgG + 50% complement. Hematoxylin and eosin staining **(a)**. IgG_{CON} = control IgG from 4 DR (diabetic retinopathy without DME) sera (**ESM Table 10**; 0.049 AU in 4mg/ml); IgG_{FH} = affinity purified anti-fumarase IgG from 4 DME sera (**ESM Table 10**; 0.417 AU in 4mg/ml). C = complement from 3 nondiabetic subjects (**ESM Table 10**). $n = 10$ retinas. Scale bar, 50 μm . **(b)** The OCT measurements revealed significant decreases in the outer retinal thickness at 7, 14, and 28 days after administration of IgG_{FH}+C ($p < 0.01$ vs. baseline). $n = 15$ retinas. * $p < 0.01$ in the comparison between two groups at each time point. One-way ANOVA with Bonferroni correction. **(c)** Amplitude of the a-wave of ERG in each group. * $p < 0.001$. $n = 10$ retinas. One-way ANOVA with Bonferroni correction. **(d and e)** Active immunisation with fumarase as another autoimmune model. **(d)** HE staining showed that the photoreceptor inner and outer segments were damaged in 6 (42.9%) of 14 mice. Scale bar, 50 μm . **(e)** Immunostaining revealed immunoreactivity to mouse IgG in the inner and outer segments of photoreceptor cells, with which C5b-9 was partly colocalized. Scale bar, 50 or 20 μm (magnified image).

Figure 7. Anti-fumarase Antibody from DME Sera in Concert with Complement Reacts to Autoantigens on the Cell Surface and Promotes Cellular Injuries in Cultured Cells.

(a and b) Immunofluorescence of HEK 293 cells incubated with affinity-purified anti-fumarase IgG from DME sera (1 mg/ml) and complement (5%) for 3 hours. $n=3$. Scale bar, 10 μm . WT = $FH^{+/+}$ HEK 293 cells. KO = $FH^{-/-}$ HEK 293 cells. Scale bar, 10 μm . **(c)** Increased intracellular Ca^{2+} concentration in 661W cells treated with IgG_{FH}+C ($p < 0.001$ vs. IgG_{FH} or IgG_{CON}+C). $n=6$. **(d)** The JC-1 red/green ratio decreased at 30 minutes or later in HEK 293 cells treated with IgG_{FH}+C ($p < 0.001$ vs. IgG_{FH} or IgG_{CON}+C). $n=6$. **(e)** Immunoblot of the cytosolic fraction isolated from 661W cells. $n=8$. **(f)** Colorimetry assay revealed that IgG_{FH}+C increases caspase-3 activity in 661W

cells. $*p<0.001$. $n=10-12$. **(g)** Electron microscope image of HEK 293 cells 24 hours after incubation. $n=3$. Scale bar, 5 μm . **(h)** Dead cells (ethidium homodimer-1 positive cells) after 24 hours of incubation in HEK 293 cells. $*p<0.001$. $n=10$. **(i)** The cell death partly depended on caspase-3 activation in 661W cells. $*p<0.001$. $n=10$. One-way ANOVA with Bonferroni correction in **c, d, f, h, i**.

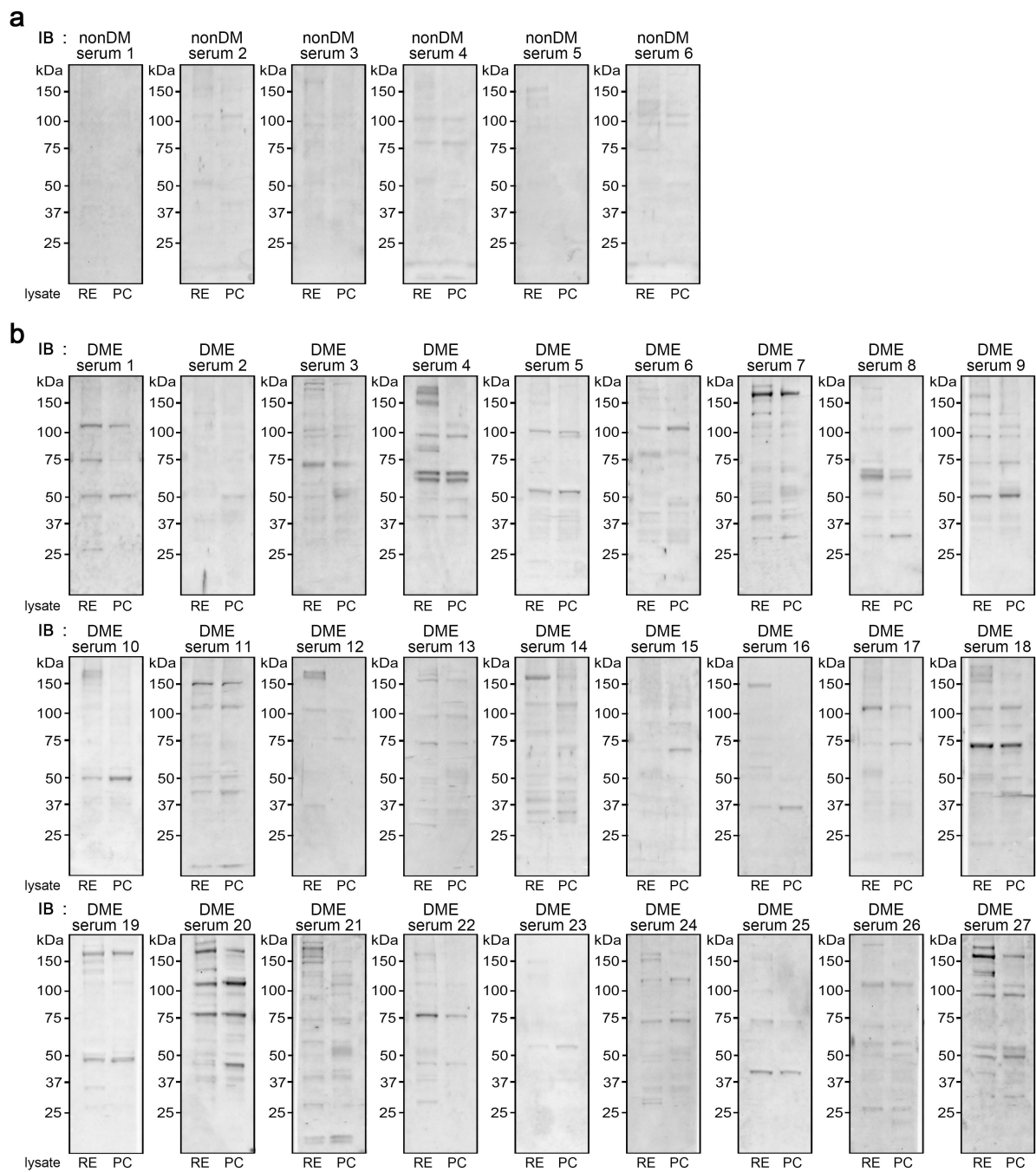


Figure 1

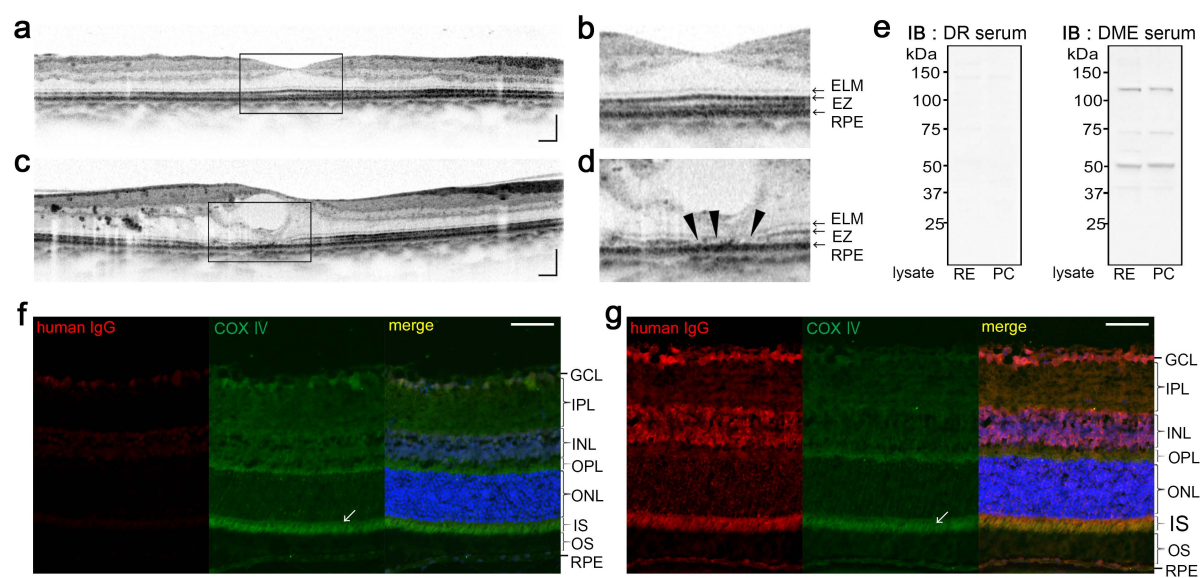


Figure 2

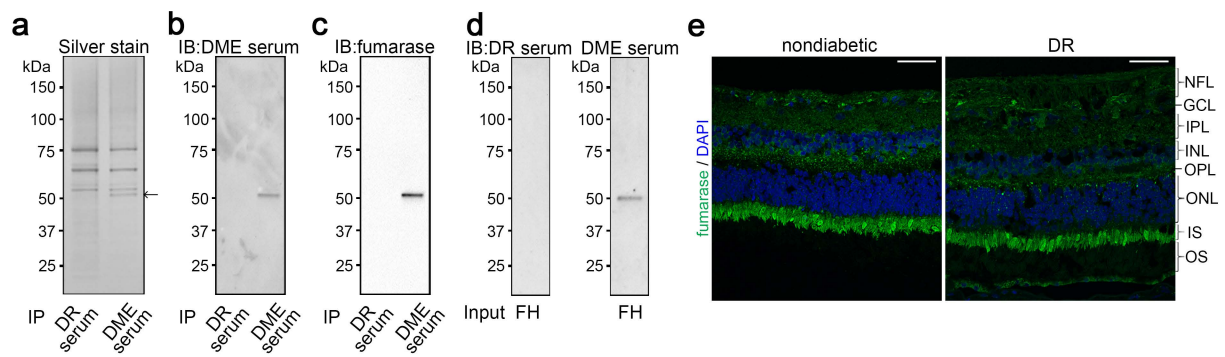


Figure 3

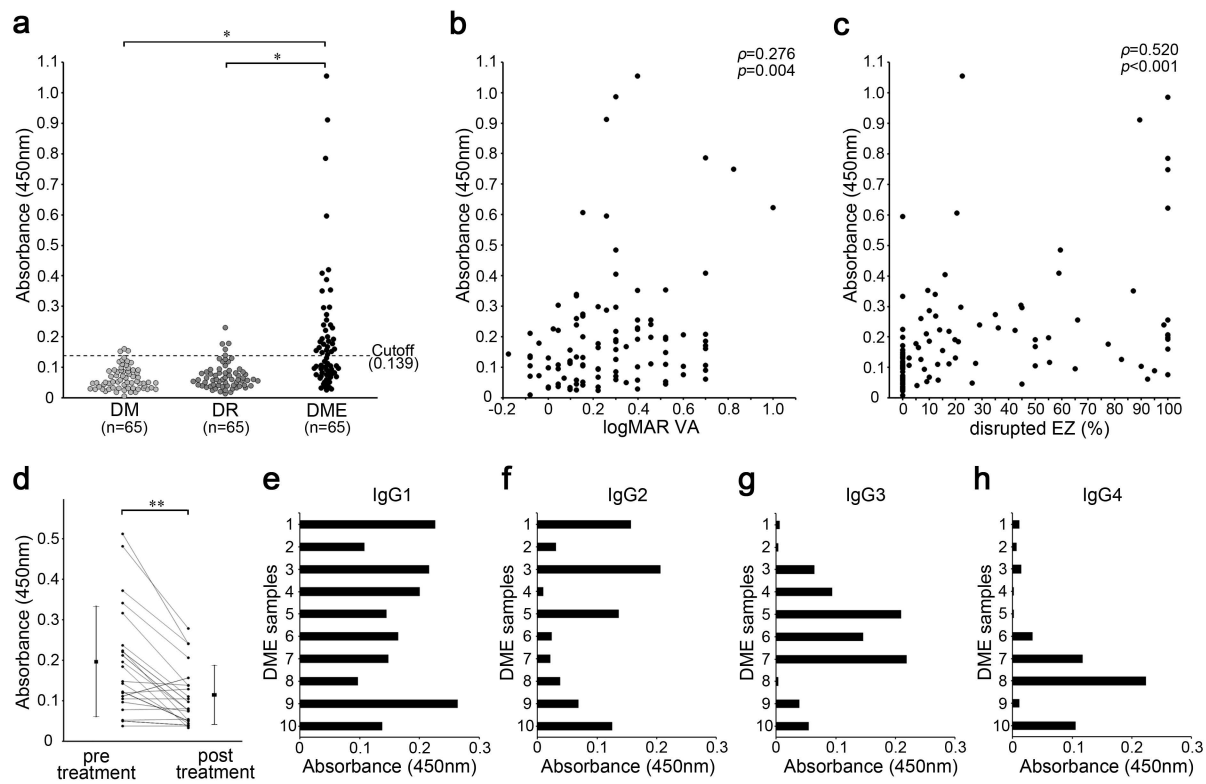


Figure 4

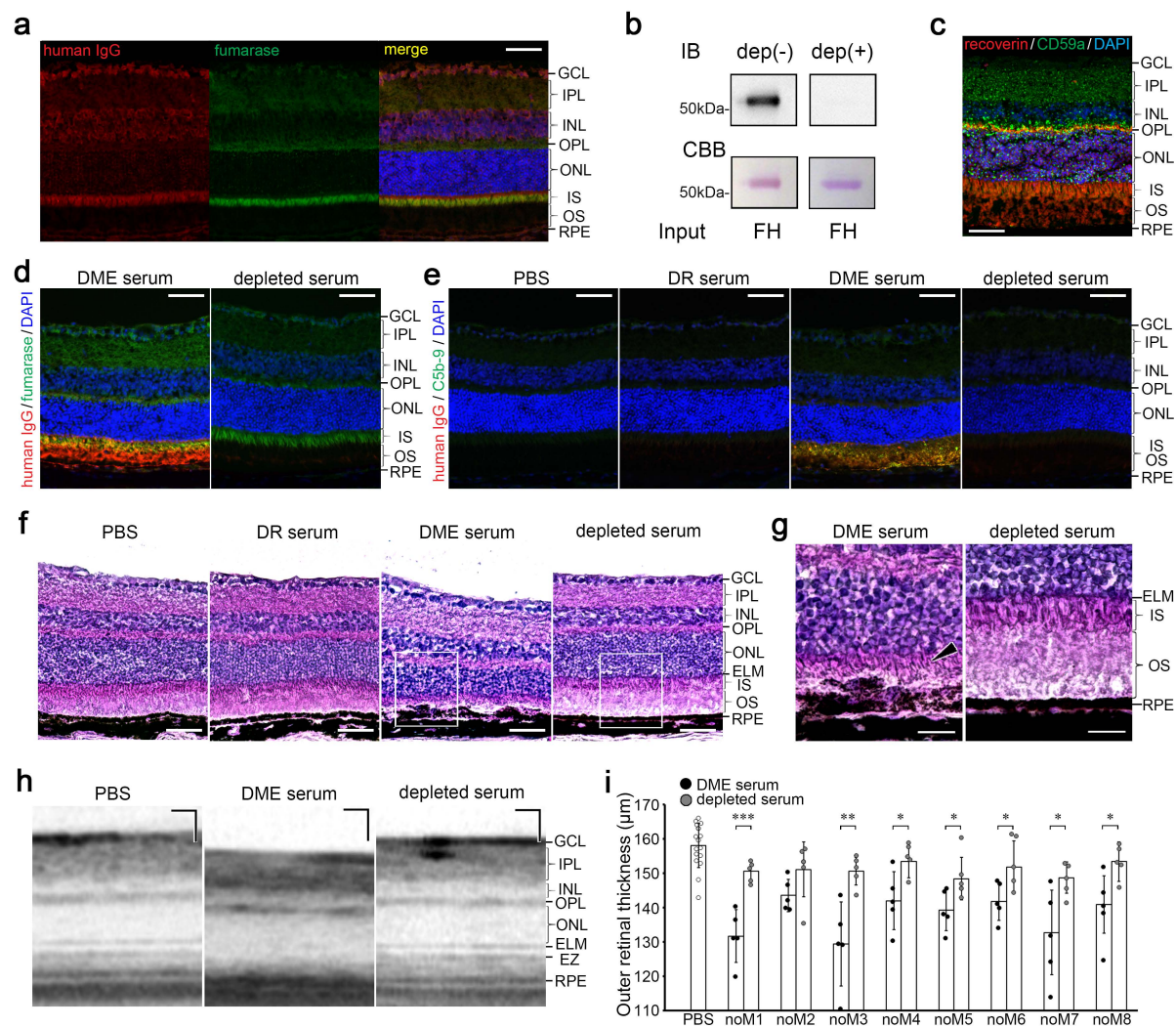


Figure 5

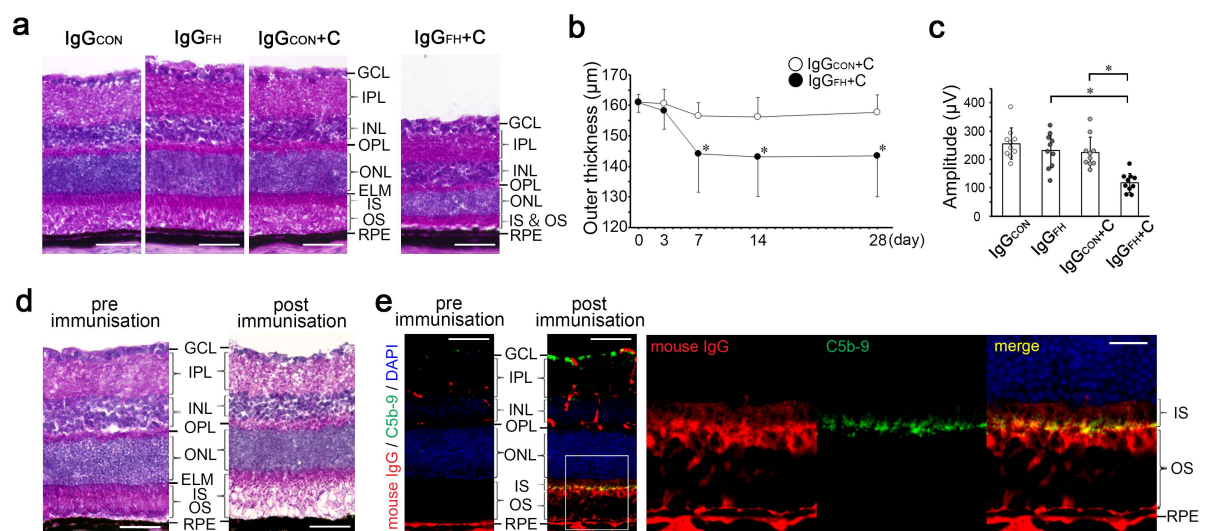


Figure 6

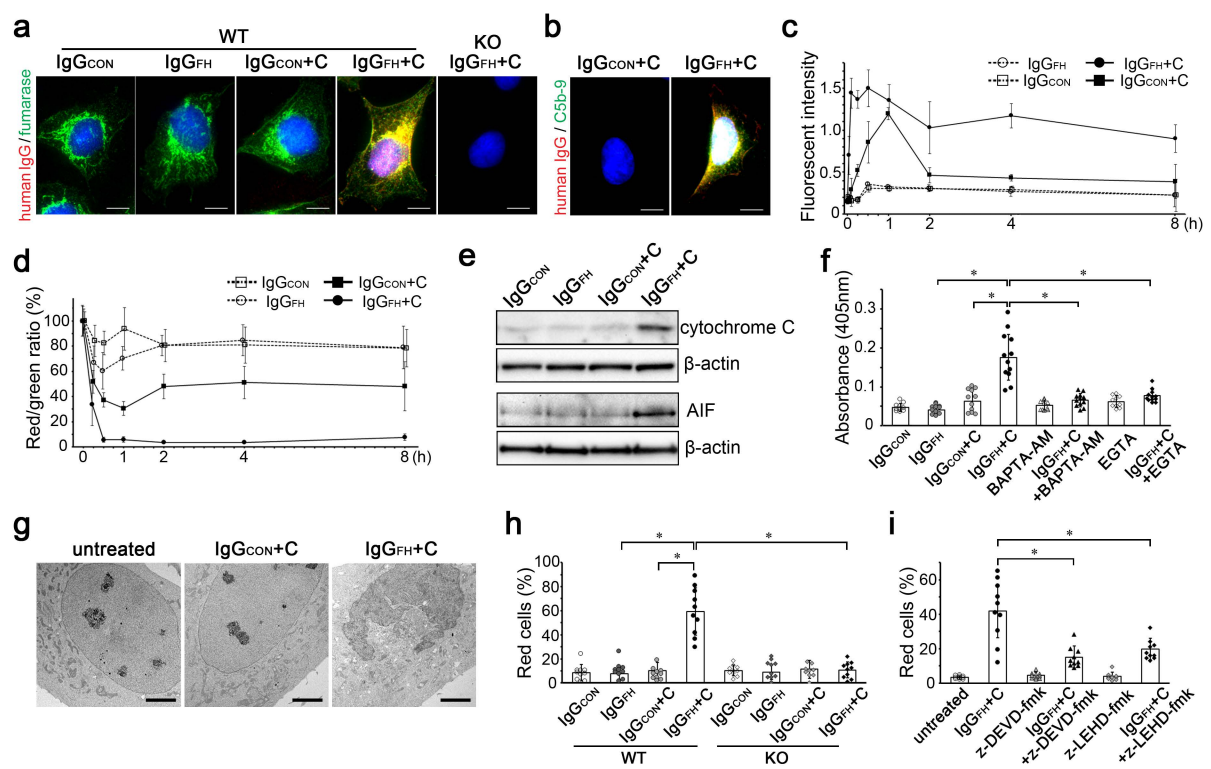


Figure 7

ESM Methods

Patients and Biosampling

Participants were consecutively recruited at the Department of Ophthalmology of Kyoto University Hospital from July 2014 to December 2017. Participants with type 2 diabetes were divided into the following three groups: (1) DM group, participants with type 2 diabetes but not diabetic retinopathy; (2) DR group, patients with diabetic retinopathy but not centre-involved diabetic macular oedema (DME); and (3) DME group, patients with center-involved DME.

The eligibility criteria for nondiabetic subjects were the following: (1) idiopathic macular hole, idiopathic epiretinal membrane, or rhegmatogenous retinal detachment for which vitrectomy was performed; (2) absence of diabetes mellitus; and (3) written informed consent. For diabetic participants, the eligibility criteria were (1) type 2 diabetes; (2) absence of treatment for macular lesions; and (3) written informed consent. The exclusion criteria were (1) other chorioretinal diseases; (2) media opacity affecting fundus examination or spectral domain optical coherence tomography (SD-OCT) imaging; (3) history of ocular surgery other than cataract surgery; or (4) history of cataract surgery or panretinal photocoagulation (PRP) within one year. Given the immunological influences of other diseases, we applied the following exclusion criteria: (1) any infection at sample collection; or (2) any history of autoimmune disease or malignancy.

In the DR group, we consecutively assessed the diabetic retinopathy severity in eyes that met the inclusion and exclusion criteria. If both eyes met the criteria, we selected the eye with higher grade of diabetic retinopathy severity for further study. In the DME group, we evaluated the eyes with centre-involved DME if they were unilaterally affected [1]. When both eyes suffered from centre-involved DME, we selected the eye with more severe damage in the foveal photoreceptors, as shown in the OCT section.

In the discovery cohort from July 2014 to February 2016, we first included 9, 65, 67, and 94 cases in the nondiabetic, DM, DR, and DME groups as shown in the flow chart (**ESM Fig. 1**). These samples were used for the screening by Western blot or immunostaining, immunoprecipitation and subsequent mass spectrometry, enzyme-linked immunosorbent assay (ELISA) to measure the autoantibody titer, and animal or cell culture experiments (**ESM Fig. 1**). In the validation cohort for ELISA, 74 cases of the DR or DME group were included from March 2016 to December 2017. Cases with similar age, gender, and HbA1c from individual groups were included in ELISA experiments to measure the serum titers in two independent cohorts. After we excluded 33 eyes with media opacity affecting visual acuity (VA) according to the medical records, we included 106 eyes in the DME group of both cohorts and analysed the association of anti-fumarase IgG and other clinical parameters (**ESM Fig. 1**). We collected serum samples in 21 cases before and after anti-VEGF treatment (ranibizumab [Lucentis, Novartis Pharma AG, Basel, Switzerland; and Genentech Inc., South San Francisco, CA, USA] and aflibercept [Eylea,

Regeneron Pharmaceuticals, Tarrytown, NY, USA; and Bayer, Berlin, Germany]) for DME according to three monthly injections followed by the PRN phase (3 + PRN regimen) described in the Ranibizumab Monotherapy or Combined with Laser versus Laser Monotherapy for Diabetic Macular Edema (RESTORE) study [2], and evaluated the changes in the autoantibody titers. The participants' characteristics in the individual studies are detailed in **ESM Tables 1-10**.

Sera were all collected, processed and aliquoted within 1 hour after sampling and immediately stored at -80°C . We avoided any freeze-thaw cycle before sample use. We conducted this study in accordance with the Declaration of Helsinki. The study was approved by the Kyoto University Graduate School and Faculty of Medicine Ethics Committee and registered at UMIN Japan Clinical Trial Registry (UMIN000014015). All participants provided written informed consent before inclusion in the study.

Enrichment of Photoreceptor Cells

Photoreceptor cells from porcine retinas were prepared as previously described with slight modifications [3]. Briefly, the photoreceptors were brushed off in Ringer's solution (pH 7.5). The cellular suspension was filtered through a cell strainer (BD Biosciences, San Jose, CA) to eliminate large aggregates of retinal tissues, applied to the top of a stepwise Percoll gradient (0%, 27%, and 50%), and centrifuged for 20 minutes at 10,000 g. Cells at the interface between the 27% and 50% solutions were collected and diluted by mixing with Ringer's solution to reduce the concentration of Percoll. The cells were sedimented by centrifugation at 15,000 g for 5 minutes, lysed with lysis buffers, and stored at -80°C .

Cell Culture and Gene Knockout

Human embryonic kidney (HEK) 293 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD), and 661W cells, a mouse cone photoreceptor cell line, were a kind gift from Dr. Muayyad R. Al-Ubaidi (University of Oklahoma Health Sciences Center, Oklahoma City, OK). They were negative for mycoplasma and were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. *Fumarate hydratase (FH)* was knocked out in HEK 293 cells using the CRISPR CAS9 system. To introduce single-guide RNA (sgRNA) and the CAS9 gene into HEK 293 cells, two complementary oligonucleotides (5'-AAACAGACCGTGAGATCTACGATGC-3', 5'- CACCGCATCGTAGATCTCACGGTCT-3') for sgRNA were annealed and subcloned into cloning sites in the PX330 vector (Addgene plasmid #42230) after digestion with BbsI (New England BioLabs, Beverly, MA). The plasmid was used to transform competent cells (Competent Quick DH5 α , Toyobo, Osaka, Japan), followed by amplification in LB medium and plasmid purification (PureLinkTM HiPure Plasmid Miniprep Kit; Thermo Scientific Inc., Waltham, MA). After transfection using Lipofectamine 3000 (Life Technologies, Inc., Gaithersburg, MD), the cells were

subjected to limiting dilution for the selection of the knockout cells. The mutations were confirmed by the mismatch cleavage assay as well as direct sequencing (**ESM Fig. 4a**). The sequences around the protospacer adjacent motif (PAM) sequence were amplified by PCR (forward primer 5'-CAAGCCAAAATTCCTTCCGGATAG-3'; reverse primer 5'-GCGATGGCTCAAATAATAGCATG-3'). Subsequently, the GeneArt genomic Cleavage Detection Kit (Life Technologies) was used to detect the mismatch of the alleles in the PCR products (Data not shown). The PCR products (forward primer 5'-CCTGAAAGTGAGGAAGAGAATGAG-3'; reverse primer 5'-GCGATGGCTCAAATAATAGCATG-3') containing the mutated sequences were subcloned into the TA cloning vector (pGEMR-T Easy Vector System, Promega, Madison, WI), followed by transformation into competent cells. Eight colonies were picked using blue-white selection, followed by amplification in LB medium and plasmid purification (GenElute™ HP Plasmid Miniprep Kit; Sigma-Aldrich, St. Louis, MO). The mutated sequences were determined by direct sequencing using a 3130xl Genetic Analyzer (Life Technologies). Western blot was applied to confirm the protein expression levels (**ESM Fig. 4b**).

Animals

After the approval by the Institutional Review Board of the Kyoto University Graduate School of Medicine, two- to three-month-old male mice (C57BL/6 wild-type and Ins2Akita diabetic animals) or B10.RIII mice (H2r) were purchased from SLC Japan (Hamamatsu, Japan) and housed in accordance with the Institutional Animal Care and Use Committee guidelines as well as the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. After ketamine/xylazine anesthesia and mydriasis with tropicamide and phenylephrine (0.5% each), patients' sera were administered subretinally or SD-OCT images were obtained. Eyes were harvested once mice were anesthetized with a lethal dose of ketamine/xylazine. We performed subretinal injections of 290 eyes for OCT or electroretinogram (ERG) experiments and excluded 14 eyes with media opacities, i.e., cataract and vitreous opacity, because OCT images or ERG were not available for these eyes.

All protocols were approved by the Institutional Review Board of the Kyoto University Graduate School of Medicine.

Polymerase Chain Reaction

Retinas from 3-month-old control C57BL/6 mice or Ins2Akita diabetic mice were harvested, frozen in liquid nitrogen, and stored at -80°C. Retinas were homogenized in ice-cold Sepasol RNA I Super solution (Nacalai Tesque, Japan). After the addition of chloroform, the solution was centrifuged at 12000 g for 15 minutes. Ethanol precipitation was then used to purify the RNA. After reverse transcription using SuperScript II (Life

Technologies), cDNA was amplified using Taq polymerase (Ex Taq; TaKaRa Bio, Otsu, Japan) as follows: 95°C for 20 seconds, 56°C for 20 seconds, and 72°C for 30 seconds, for 35 cycles. Specific forward and reverse primers (5'-AGCAATGCATATTGCTGCTG-3' and 5'-CGCATACTGGACTTGCTGAA-3', respectively) were used to determine the mRNA expression of murine *FH* (accession number: NM_010209.2) in the retinas. PCR products were subjected to agarose gel electrophoresis and stained with ethidium bromide (Nacalai Tesque).

Immunoblot Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the NuPAGE system (Life Technologies) was performed as described previously [4]. Porcine retinas were harvested, quickly frozen in liquid nitrogen, and stored at -80°C. Porcine photoreceptor cells or total retinas were lysed with Laemmli buffer with protease inhibitors. After centrifugation at 15,000 g for 5 minutes, supernatants were separated on a gradient gel (4%–12%) and transferred to nitrocellulose membranes. To investigate the immunoreactivity of participants' sera against human fumarase, recombinant human fumarase (RayBiotech Inc., Norcross, GA) were applied to these procedures.

After blocked with 5% milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T), the membranes were incubated in the primary antibody, which consisted of serum specimens diluted 1:200 in 1% milk / TBS-T, followed with HRP-labeled anti-human IgG secondary antibodies (**ESM Table 11**). Chemiluminescence signals in the membrane were detected with the enhanced chemiluminescence (ECL) detection system (GE Healthcare, Piscataway, NJ).

In addition, we evaluated protein amounts of fumarase in the immunoprecipitated proteins or those of cytochrome C, apoptosis-inducing factor (AIF), and β -actin in cytosolic fraction isolated from 661W cells using commercially available antibodies (**ESM Table 11**). The antibodies were validated by the manufacturers and were used as per their instructions.

Immunoprecipitation and pulldown assay

Serum IgG were conjugated to protein G-coated beads (Protein G Sepharose 4 Fast Flow; GE Healthcare) via covalent bonds with a dimethyl pimelimidate (DMP) crosslinker (Thermo Scientific). Cell lysates of porcine photoreceptor cells were prepared using immunoprecipitation buffer (50 mmol/l Tris, pH 7.4, 1% Nonidet-P 40, 10 mmol/l EDTA, 150 mmol/l NaCl with protease inhibitors) as described previously [4]. After preclearing with Protein G beads conjugated with IgG from control serum, the lysate was mixed with Protein G beads crosslinked to IgG from DME serum and incubated for 2 hours at 4°C. Immunoprecipitated proteins were eluted with 0.1 mol/l glycine (pH 2.0) at 4°C for 1 hour. After centrifugation, the supernatants were neutralized and subjected to SDS-PAGE as described above. The proteins in the gels were either silver stained or were transferred

to nitrocellulose membrane. Following immunoblotting with the serum IgG from DME participants, the membranes were stripped of bound antibodies and were reblotted with mouse monoclonal anti-fumarase antibodies (**ESM Table 11**).

Mass Spectrometry

Immunoprecipitated proteins from porcine photoreceptor cells were applied to SDS-PAGE. Silver-stained bands corresponding to the immunoblotted signals were excised and in-gel digested with trypsin using an In-Gel Tryptic Digestion Kit (Thermo Scientific) according to the manufacturer's instructions. The excised bands were destained with Silver Destain Reagents, which were provided as part of the Pierce Silver Stain for Mass Spectrometry kit (Thermo Scientific), before undergoing digestion. The molecular weight- and experimental condition-matched controls for individual bands were also excised and simultaneously subjected to the same procedure. Briefly, the proteins in the destained gel pieces were reduced with Tris[2-carboxyethyl]phosphine, alkylated with iodoacetamide, and digested with mass spectrometry-grade TPCK-treated Trypsin Protease (Thermo Scientific). The tryptic digests were eluted from the gels, dried, and resuspended in 0.1% formic acid.

The tryptic digests were separated using nano-flow liquid chromatography (Nano-LC-Ultra 2D-plus equipped with cHiPLC Nanoflex [Eksigent, Dublin, CA]) in a trap-and-elute mode. Peptides were concentrated and desalted on a trap column (200 $\mu\text{m} \times 0.5\text{ mm}$ ChromXP C18-CL 3 μm 120 Å [Eksigent]) for 10 minutes with 0.1% formic acid at a flow rate of 2 $\mu\text{L}/\text{min}$ and redirected online to the analytical column (75 $\mu\text{m} \times 15\text{ cm}$ ChromXP C18-CL 3 μm 120 Å [Eksigent]). Both columns were set to 40°C. The analytical separation was carried out with a binary gradient using solvent A (98% water, 2% acetonitrile, 0.1% formic acid) and solvent B (20% water, 80% acetonitrile, 0.1% formic acid). The gradient program was 2% to 40% solvent B for 50 minutes, 90% solvent B for 5 minutes, and 2% solvent B for 19 minutes, at a flow rate of 300 nL/min. The eluate was directly introduced into a mass spectrometer (TripleTOF 5600+ System coupled to a NanoSpray III source and heated interface [AB SCIEX, Framingham, MA]) and ionized in an electrospray ionization-positive mode. Data acquisition was carried out with an information-dependent acquisition method.

The acquired datasets were analysed by ProteinPilot software, version 4.5 beta (AB SCIEX), with the UniProtKB/Swiss-Prot database (April 2013) appended with known common contaminants (AB SCIEX). The database search was performed with the following parameters: gel-based identification, thorough identification, within the taxonomy *Sus scrofa*; possible biological modifications were taken into consideration. The quality of the database search was confirmed by false discovery rate analysis that used the reversed amino acid sequences as a decoy. The reliabilities of all protein identifications were evaluated by the corresponding Unused ProtScores, which were calculated by the Pro Group algorithm (AB SCIEX). The apparent molecular weights of

the bands in the SDS-PAGE and the differences between the samples and the simultaneously processed controls were also used to confirm the identifications.

Preparation of cytosol fractions

Cytosol fractions were prepared from 661W cells using the subcellular fractionation kit (Mitochondria Isolation Kit for Cultured Cells [Thermo, cat. #89874]) according to the manufacturer's instructions. Briefly, cultured cells were lysed, and subsequently, nuclei and cell debris were pelleted. The supernatant containing both cytosol and mitochondria was fractionated using high speed centrifugation. Cytosol fractions were applied to SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblotting was followed as described above (**ESM Table 11**).

Measurements of the intracellular Ca^{2+} concentration

The intracellular Ca^{2+} concentration was measured using a Fluo 4-AM kit (Calcium kit-Fluo 4; Dojindo Laboratories, Tokyo, Japan). HEK 293 cells or 661W cells were seeded and cultured on 96-well plates. After washing with PBS, cells were incubated for 1 hour in loading buffer (20 mmol/l HEPES pH7.4, 115 mmol/l NaCl, 5.4 mmol/l KCl, 0.8 mmol/l MgCl_2 , 1.8 mmol/l CaCl_2 , 13.8 mmol/l glucose with 4.56 $\mu\text{mol/l}$ Fluo 4-AM), followed by replacement with recording buffer (the same constituents without Fluo 4-AM). After treatment at the indicated time points, the fluorescence level was measured using an ARVO MX plate reader (PerkinElmer).

Measurements of mitochondrial membrane potential

To evaluate the mitochondrial membrane potential ($\Delta\psi_m$) in HEK 293 cells, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1 Dye; Thermo Scientific) was added, and the fluorescence levels were measured according to the manufacturer's protocol. Briefly, after incubation with IgG and complement for specific durations, the cells were treated with JC-1 for 30 minutes, followed by measurements of fluorescence levels using an ARVO MX plate reader (PerkinElmer).

Caspase-3 Activity

661W cells were treated with anti-fumarase antibody and complement in the presence or absence of cell-permeant Ca^{2+} chelators (12.5 $\mu\text{mol/l}$ BAPTA-AM [Dojindo Laboratories, cat#348-05451] or 250 $\mu\text{mol/l}$ EGTA) for 12 hours. Cells were assessed by the colorimetric assay to determine caspase-3 activity (APOPCYTO™ Caspase-3 Colorimetric Assay Kit, MBL, Aichi, Japan) according to the manufacturer's instructions. Briefly, cells were harvested by trypsinisation, followed by neutralisation in fetal bovine serum and washing with PBS. The cell lysates were mixed in reaction buffer with DEVD-p-nitroanilide (NA) and incubated at 37°C for 1 hour. The release of p-NA was measured at 405 nm using an ARVO MX plate reader (PerkinElmer).

Flow cytometry

661W cells were incubated with complement in the presence or absence of cell-permeant Ca^{2+} chelators for 3 hours. Cells were harvested by PBS containing 1 mM EDTA. Cells were incubated with the anti-fumarase antibody or mouse control IgG1 (**ESM Table 11**) to which Alexa Fluor® 488 was conjugated using a fluorescence labelling kit (Zenon™ Alexa Fluor™ 488 Mouse IgG1 Labeling Kit, Thermo, cat. #Z25002). The fluorescence intensity in treated cells was analysed on FACS Calibur™ (BD Biosciences, Mountain View, CA). To evaluate the antigen-antibody reaction, we used flow cytometry with anti-fumarase IgG labelled with Alexa Fluor® 488 and Cy3-conjugated anti-human IgG secondary antibody in $FH^{+/+}$ or $FH^{-/-}$ HEK 293 cells treated with anti-fumarase IgG and complement.

Enzyme-linked Immunosorbent Assay

Recombinant human fumarase (1 µg/ml, RayBiotech) in 100 mM bicarbonate-carbonate buffer (pH 9.6, 100 µl) was pipetted into a 96-well plate Nunc Immunoplate Maxisorp (Thermo Scientific Inc.) and incubated at 4°C overnight to coat the bottom surface of the individual wells with the proteins. After blocking (3% bovine serum albumin [BSA] in TBS-T), the wells were incubated in serum (1:1000 diluted in 3% BSA in TBS-T) for 2 hours, followed by HRP-labeled anti-human or anti-mouse total IgG secondary antibodies for 1 hour, in order to quantify total levels of anti-fumarase IgG. The signals detected using 3,3',5,5'-tetramethylbenzidine (TMB; Nacalai Tesque) were measured at an absorption of 450 nm using an ARVO MX plate reader (PerkinElmer, Norwalk, CT). After serum specimens were allocated at random to individual wells, all experiments were performed in duplicate and the individual plates were calibrated using two different control serum specimens. The investigators were blinded during these procedures and outcome assessment. To measure the titers of individual IgG subtypes of anti-fumarase antibodies, ELISA using HRP-labeled secondary antibodies against individual human IgG subtypes (**ESM Table 11**) was applied to serum IgG from DME patients with higher titers of anti-fumarase antibodies.

Antibody Depletion and Subretinal Injection of Serum

To evaluate the effects of anti-fumarase antibodies in DME serum, we at randomly selected 8 DME sera with higher titers of anti-fumarase IgG, and prepared serum depleted of anti-fumarase antibodies according to a modified method as previously described [5]. Briefly, His-tagged recombinant human fumarase (Life Technologies) was conjugated to Ni-NTA agarose beads (HisPur Ni-NTA Resin; Thermo Scientific) in equilibration buffer (PBS with 10 mM imidazole). After the resin was washed three times in the same buffer, it was incubated with serum for 1 hour to allow the antibodies in the sera to react to the conjugated antigen, followed by filtering using Ultrafree-MC

(Millipore). Western blotting or ELISA was performed to confirm that anti-fumarase antibodies were depleted from the sera (**Fig. 5b**).

To investigate the effects of anti-fumarase antibodies on photoreceptor damage, eyes were allocated at random to individual serum specimens and the subretinal spaces were injected with 0.7 μ l of the following solutions: (1) PBS; (2) DR serum; (3) DME serum with a higher titer of anti-fumarase antibodies; or (4) DME serum depleted of anti-fumarase antibodies. All of these samples were processed via the antibody depletion protocol with or without recombinant fumarase as described above. After anesthesia with ketamine/xylazine, serum specimens were injected into subretinal spaces in the caudal areas (~3 disc diameters from the optic disc) using a 33-gauge needle, as described previously [6]. The investigators were blinded during the manipulation and outcome assessment.

Purification of Anti-Fumarase Antibodies from DME Sera and Preparation of Complement Components

To purify anti-fumarase IgG, DME sera from 4 participants with the higher titers (noM1 - noM4 in **ESM Table 10**) were mixed and applied to a Melon Gel IgG purification kit (Thermo Scientific) as described previously [7]. Further purification of antibodies specific to fumarase was performed according to the modified methods described previously [8]. Human recombinant fumarase (Life Technologies) was coupled to NHS-activated Sepharose 4 Fast Flow (GE Healthcare) in coupling buffer (200 mmol/l NaHCO₃, 500 mmol/l NaCl, pH 8.3), followed by wash buffer (100 mmol/l acetate, 500 mmol/l NaCl, pH 4.0). The IgG was further incubated with the coupled beads for 12 hours at 4 °C. After washing with PBS, the anti-fumarase IgG bound to the beads was eluted under acidic conditions (100 mmol/l glycine, pH 2.0) and immediately neutralized in Tris buffer (pH 9.0). The purified IgG was concentrated using an ultrafiltration membrane (Amicon Ultra 10k, Millipore, Billerica, MA) and applied to further experiments. The control IgG from 4 DR sera (**ESM Table 10**) was purified using the Melon Gel IgG purification kit, followed by concentration by an ultrafiltration membrane.

We prepared the complement components from three seronegative sera of nondiabetic subjects (**ESM Table 10**) as described previously [9]. Briefly, non-hemolyzed blood was allowed to clot and centrifuged at 1000 r.p.m., followed by collection of the serum supernatant. The samples were immediately aliquoted and stored at -80°C.

Immunisation with fumarase

B10.RIII mice were immunised using fumarase according to the modified methods described previously [10]. Briefly, 6- to 8-week-old female B10.RIII mice (H2r) were immunised using 30 μ g of recombinant human fumarase instead of IRBP peptide in combination with Complete Freund's adjuvant (Difco, Detroit, MI, cat.#263810) and

Pertussis Toxin (List Biological Laboratories, Campbell, CA, cat.#179A). Fourteen days after immunisation, sera were prepared from the collected blood and eyes were harvested. The titer of anti-fumarase IgG was measured by ELISA and HE staining and immunostaining were performed according to the protocols in the Methods section.

Immunostaining

Immunostaining of retinas from mice (C57BL/6 wild-type, C57BL/6 Ins2Akita, or B10.RIII) was performed as previously described [11]. Eyes harvested from C57BL/6 mice were fixed in 4% paraformaldehyde in PBS for 1 hour at 4°C. After PBS washes, the eyes were serially dehydrated in 10%, 20%, and 30% sucrose/PBS for 4 hours each and frozen in OCT compound (Sakura Finetek, Torrance, CA). Cryosections at a thickness of 10 µm underwent staining with hematoxylin and eosin or were counterstained with diamidino-2-phenylindole (DAPI).

After being blocked in 10% donkey serum, retinal sections were incubated with primary antibodies at 4°C overnight, followed by fluorescence-labeled secondary antibodies for 1 hour (**ESM Table 11**). Primary mouse monoclonal fumarase or COX IV antibodies were labelled with Alexa Fluor 488 using Zenon Alexa Fluor 488 Mouse IgG1 Labeling Kit (Life Technologies, cat. #Z-25002), followed by the incubation for 1 hour. To evaluate autoantibodies in patients' serum, the sections were incubated with serum specimens diluted 1:100 in 10% donkey serum. Detection was performed using Cy3-conjugated secondary antibodies against human IgG. For the retinal sections from eyes injected with participants' serum into the subretinal spaces, sections were incubated with fluorescent-labeled anti-human IgG antibodies alone (1:5000). The localization of COX IV, fumarase, recoverin, CD59a, and C5b-9 was also evaluated using commercially available primary antibodies (**ESM Table 11**). Retinal images in the rostral areas from the optic disc were obtained with fluorescence microscopy (BZ-9000; Keyence, Osaka, Japan).

For immunostaining of human retinas obtained from Eversight (**ESM Table 4**), the eyeballs were fixed in 10% formalin solution for 24 hours before being transferred in saline. The tissues of the anterior segment and the vitreous were removed, and the prepared eyecups were incubated in PBS solutions containing increasing concentration of sucrose up to 20%. Fragment of the central retina corresponding to a circle of a 1 cm diameter centred onto the macula was then embedded and stored at -80°C. Subsequently, sections 10 µm thick were cut from each group (non-diabetic subject and diabetic retinopathy). Samples from each group were processed in the same time to avoid any variation in the treatment. After permeabilisation with a solution of PBS containing 0.1% Triton X-100 for 15 minutes, the sections were blocked with 3% goat serum and 1% BSA for 1h. The sections were incubated overnight with anti-fumarase antibody (**ESM Table 14**) for 1 hour, followed by Alexa Fluor 488-conjugated anti-mouse secondary antibody. Cell nuclei were counterstained using Hoescht (1:1000). After several washes, the

sections were mounted with Prolong Gold anti-fade mounting media (Life Technologies), and imaged on a Leica TCS SP5 confocal microscope (Leica Microsystems GmbH, Mannheim, Germany).

For the immunocytochemistry, HEK 293 cells or 661W cells were cultured on the Thermanox coverslip (Thermo Scientific), and fixed with 2% PFA for 10 minutes. Cells were incubated with primary antibodies against fumarase and C5b-9 (**ESM Table 11**), followed by the fluorescence-labeled secondary antibodies. HEK 293 cells incubated with anti-fumarase IgG from DME sera was detected using Cy3-conjugated secondary antibodies against human IgG. Fluorescent images were acquired with fluorescence microscopy (BZ-9000; Keyence, Osaka, Japan).

Electron Microscopy

For the structural analyses, HEK 293 cell were cultured in an 8-well chamber slide (Nunc™ Lab-Tek® Chamber Slide™ System, Thermo Scientific) and treated with affinity purified anti-fumarase IgG from DME sera and complement. The cells were rinsed with ice-cold PBS and fixed with 2% glutaraldehyde and 4% paraformaldehyde in PBS overnight. The cells were rinsed with PBS three times and 100 mmol/l phosphate buffer (PB; pH 7.2) three times, followed by a second fixation in 1% osmium in 100 mmol/l PB for 40 minutes. The samples were serially dehydrated in 50% to 100% ethanol and permeated and embedded overnight in Epon resin. After crosslinking at 60 °C for two days, ultrathin slices (70 nm) were placed on a platinum grid and observed by transmission electron microscopy (H-7650; Hitachi, Tokyo, Japan).

Cell Viability Assay

HEK 293 cells on coverslips were treated with DMEM containing 1 mg/ml IgG from the DME or DR sera and 5% human complement for 24 hours according to the modified methods described previously [9]. The treated cells were applied to the cell viability/cytotoxicity assay kit (LIVE/DEAD assay; Life technologies) according to the manufacturer's instructions and examined by fluorescence microscopy (BZ-9000, Keyence). Five fields were obtained in each coverslip using a X20 objective lens, and two independent graders counted ethidium homodimer-1 positive cells (red cells) as injured cells with loss of plasma membrane integrity. The average ratio of red cells to the total cell count was calculated.

Electroretinogram

Visual function was evaluated using ERG in C57BL/6 mice treated with purified IgG and complement, as described previously [12]. Briefly, after overnight dark-adaptation and subsequent anesthetization, ERG was performed using a corneal electrode with a light-emitting diode (Mayo Corp., Inazawa, Japan). The amplified signals were digitized at 10 kHz with a band-pass filter of 0.3 to 500 Hz and analyzed (PowerLab 2/25; AD

instruments, New South Wales, Australia). The amplitudes and latency of the mixed cone and rod response (International Society for Clinical Electrophysiology of Vision [ISCEV] standard; scotopic 3.0) were evaluated.

OCT

After comprehensive examination, including VA measurements, retinal specialists diagnosed diabetic retinopathy according to the fundus findings, and DME was objectively determined by OCT-measured retinal thickness, as described previously [1]. Briefly, retinal sectional images in the macula were obtained using SD-OCT (Spectralis OCT; Heidelberg Engineering, Heidelberg, Germany). The mean retinal thickness in the central subfield (CSF; central 1 mm) of the Early Treatment Diabetic Retinopathy Study (ETDRS) grid was measured on a two-dimensional OCT map constructed by raster scans. DME was diagnosed in eyes with a CSF thickness greater than 320 μm or 305 μm in male or female patients, respectively.

Retinal sections or two-dimensional maps were obtained using the cross-hair or raster scan mode of Spectralis OCT. The CSF thickness was measured as described above, and the photoreceptor damage was also quantified. Briefly, we measured the transverse length of EZ lines in the central 1-mm areas in both the horizontal and vertical sectional images in eyes with centre-involved DME, and the averages were applied to further analyses, as described previously [13].

As another parameter of the disruption of the neuroretina, the disorganization of retinal inner layers (DRIL) was also evaluated as described recently [14]. Briefly, the boundaries between inner retinal layers (ganglion cell layer/inner plexiform layer [GCL/IPL] complex, inner nuclear layer [INL], and outer plexiform layer [OPL]) were often absent in B-scan images in eyes with DME. The horizontal extent in microns for which these boundaries could not be identified within central 1mm was measured as DRIL.

After anesthesia and mydriasis with eye drops, SD-OCT images of C57BL/6 mice were acquired using a Multiline OCT (Heidelberg Engineering, Heidelberg, Germany) 7 days after subretinal injection with serum specimens, as described previously [15]. In vivo retinal sectional images were scanned from rostral areas (~ 3 disc diameters from the optic disc). The retinal thicknesses were quantified using the manufacturer's software. The thicknesses (from inner limiting membrane [ILM] to Bruch's membrane) at 3 disc diameters from the optic disc in the vertical and horizontal sectional images were automatically segmented and measured. The average was applied to further analyses. For the longitudinal quantification, the AutoRescan tool was applied for the automatic follow-up scan placement. In addition, the inner retinal layers (from the ILM to the INL) were quantified according to a previous publication [16]. We also measured the outer retinal layers (from the OPL to Bruch's membrane), which mainly corresponded to photoreceptor cells and RPE.

These OCT parameters were evaluated by two graders in a masked fashion. They

determined the qualitative findings and at the disagreement, a third grader participated. The average of the quantitative parameters was applied for further analyses.

Statistics

After confirmation of normality using the Kolmogorov-Smirnov test, the Kruskal–Wallis test of one-way analysis of variance (ANOVA) with Dunnett’s multiple comparison or Mann-Whitney U-test was performed to evaluate the statistical differences in quantitative data for ELISA and some of the participants’ characteristics, and Spearman’s rank correlation coefficient was used to show statistical correlations. Wilcoxon signed-rank test was employed to evaluate the differences between the autoantibody titers before and after anti-VEGF treatment. The paired t-test was applied to the analyses of OCT measurements after the administration of DME sera with and without the depletion of anti-fumarase antibody. Student’s t-test or one-way ANOVA with Bonferroni correction was used for statistical differences in the participants’ characteristics, the dose escalation studies of OCT measurement, ERG, Ca^{2+} measurement, JC-1 assay, caspase-3 activity assay, and LIVE/DEAD assay. The sampling distribution in the participants’ characteristics was evaluated using Fisher’s exact test or the chi-square test. To validate anti-fumarase antibody as a serum biomarker of centre-involved DME, we applied multivariate logistic regression analyses. We included prescribed glitazone as an independent parameter, because glitazone may be associated with macular oedema [17, 18]. We performed univariate logistic regression analysis and determined the significant parameters (Wald’s chi-square > 2), which were incorporated as independent variables in the subsequent multivariate analysis. We employed multivariate linear regression analysis using the significant parameters ($p < 0.1$ in univariate analyses) as independent parameters to determine the factors that were associated with the transverse length of the disrupted EZ. These statistical analyses were performed using commercial software (PASW Statistics, version 18; SPSS Inc., Chicago, IL), and $p < 0.05$ was considered significant. The values were shown in the mean $\pm$ standard deviation (s.d.). We determined that DME serum contains anti-fumarase antibody when the absorbance was greater than a cutoff value set at two s.d. above the mean value for the sera of the DM group. The numbers of animals for reproducibility in individual experiments are stated in the figure legends.

The sample size was calculated to detect an approximately 100% mean difference in the titers of anti-fumarase antibodies between the DM and DME groups. Based on preliminary data for 10 patients in the individual groups, a two-sample t-test of log-transformed data with a two-sided alpha of 0.01 and a power of 99% led to a required sample size of 65 for each group. Similarly, the first dataset led to the sample size of 74 for the DR or DME group in an additional and independent cohort.

References

1. Chalam KV, Bressler SB, Edwards AR et al. (2012) Retinal Thickness in People with Diabetes and Minimal or No Diabetic Retinopathy: Heidelberg Spectralis Optical Coherence Tomography. *Invest Ophthalmol Vis Sci* 53: 8154-8161
2. Mitchell P, Bandello F, Schmidt-Erfurth U et al. (2011) The RESTORE study: ranibizumab monotherapy or combined with laser versus laser monotherapy for diabetic macular edema. *Ophthalmology*. 118: 615-625
3. Tachibanaki S, Tsushima S, Kawamura S. (2001) Low amplification and fast visual pigment phosphorylation as mechanisms characterizing cone photoresponses. *Proc Natl Acad Sci U S A* 98: 14044-14049
4. Murakami T, Felinski EA, Antonetti DA. (2009) Occludin phosphorylation and ubiquitination regulate tight junction trafficking and vascular endothelial growth factor-induced permeability. *J Biol Chem* 284: 21036-21046
5. Srivastava R, Aslam M, Kalluri SR et al. (2012) Potassium channel KIR4.1 as an immune target in multiple sclerosis. *N Engl J Med* 367: 115-123
6. Matsuda T, Cepko CL. (2004) Electroporation and RNA interference in the rodent retina in vivo and in vitro. *Proc Natl Acad Sci U S A* 101: 16-22
7. Ackerman ME, Crispin M, Yu X et al. (2013) Natural variation in Fc glycosylation of HIV-specific antibodies impacts antiviral activity. *J Clin Invest* 123: 2183-2192
8. Cheung WC, Beausoleil SA, Zhang X et al. (2012) A proteomics approach for the identification and cloning of monoclonal antibodies from serum. *Nat Biotechnol* 30: 447-452
9. Saadoun S, Waters P, Bell BA et al. (2010) Intra-cerebral injection of neuromyelitis optica immunoglobulin G and human complement produces neuromyelitis optica lesions in mice. *Brain* 133: 349-361
10. Agarwal RK, Silver PB, Caspi RR. (2012) Rodent models of experimental autoimmune uveitis. *Methods Mol Biol* 900: 443-469
11. Murakami T, Suzuma K, Takagi H et al. (2006) Time-lapse imaging of vitreoretinal angiogenesis originating from both quiescent and mature vessels in a novel ex vivo system. *Invest Ophthalmol Vis Sci* 47: 5529-5536
12. Ikeda HO, Sasaoka N, Koike M et al. (2014) Novel VCP modulators mitigate major pathologies of rd10, a mouse model of retinitis pigmentosa. *Sci Rep* 4: 5970
13. Murakami T, Nishijima K, Akagi T et al. (2012) Optical coherence tomographic reflectivity of photoreceptors beneath cystoid spaces in diabetic macular edema. *Invest Ophthalmol Vis Sci* 53:1506-11
14. Sun JK, Lin MM, Lammer J et al. (2014) Disorganization of the retinal inner layers as a predictor of visual acuity in eyes with center-involved diabetic macular edema. *JAMA Ophthalmol* 132: 1309-1316
15. Garcia Garrido M, Beck SC, Muhlfriedel R et al. (2014) Towards a quantitative

OCT image analysis. PLoS One 9: e100080

16. Murakami T, Nishijima K, Akagi T et al. (2012) Segmentational analysis of retinal thickness after vitrectomy in diabetic macular edema. Invest Ophthalmol Vis Sci 53: 6668-6674
17. Colucciello M. (2005) Vision loss due to macular edema induced by rosiglitazone treatment of diabetes mellitus. Arch Ophthalmol 123: 1273-1275
18. Bressler SB, Qin H, Beck RW et al. (2012) Factors associated with changes in visual acuity and central subfield thickness at 1 year after treatment for diabetic macular edema with ranibizumab. Arch Ophthalmol 130: 1153-1161

ESM Table 1. Characteristics of the Participants Whose Sera Were Applied for the Screening of Autoantibodies Using Immunoblotting.

Characteristics	Nondiabetic control (n=6)	DME group (n=27)
Age (years)	74.0±8.5	64.3±11.1
Sex (male / female)	2 / 4	17 / 10
Diabetes duration (years)	-	9.1±7.3
Mean arterial blood pressure (mmHg)	98.9±12.4	106.8±13.4
HbA1c (mmol/mol)	-	59.2±14.7
HbA1c (%)	-	7.57±1.34
Systemic hypertension	4	21
Dyslipidaemia	2	10
Cardiovascular diseases	2	7
Renal disease	2	11
Insulin use	-	10
Prescribed glitazones	-	0
Prescribed statins	1	6
Phakic in both eyes	5	21
Prior PRP in either eye	-	16
logMAR VA in the study eye	0.245±0.072	0.449±0.314
Subgroups		
Idiopathic epiretinal membrane	4	-
Idiopathic macular hole	1	-
Rhegmatogenous retinal detachment	1	-
International classification of DR severity in the study eye		
Moderate NPDR	-	10
Severe NPDR	-	8
PDR	-	9
OCT features in the study eye		
CSF thickness (µm)	-	474±172
Transverse length of disrupted EZ (%)	-	50.5±41.2
DRIL (µm)	-	498±370
Cystoid abnormalities	-	22
Subretinal fluid	-	6
Vitreoretinal abnormalities	-	6

ESM Table 2. Characteristics of the Representative Participants of the DR or DME group.

Characteristics	DR patient	DME patient
Age (years)	48	64
Sex	female	female
Diabetes duration (years)	12	19
Mean arterial blood pressure (mmHg)	126.5	105.5
HbA1c (mmol/mol)	41	88
HbA1c (%)	5.9	10.2
Systemic hypertension	+	+
Dyslipidaemia	-	+
Cardiovascular diseases	-	+
Renal disease	-	+
Insulin use	-	+
Prescribed glitazones	-	-
Prescribed statins	-	+
Phakic (LE / RE)	+ / +	+ / +
Prior PRP (LE / RE)	+ / +	- / -
logMAR VA (LE / RE)	0.046 / 0.022	-0.130 / 0.155
International classification of DR severity (LE / RE)	PDR / PDR	PDR / PDR
OCT features		
CSF thickness (µm) (LE / RE)	281 / 284	278 / 423
Transverse length of disrupted EZ (%) (LE / RE)	-	0.0 / 35.0
DRIL (µm) (LE / RE)	-	0 / 885
Cystoid abnormalities (LE / RE)	-	- / +
Subretinal fluid (LE / RE)	-	- / -
Vitreoretinal abnormalities (LE / RE)	-	- / -

LE, left eye; RE, right eye.

ESM Table 3. Characteristics of the Participants Whose Sera Were Used for Immunoprecipitation.

Characteristics	DR patient	DME patient
Age (years)	57	77
Sex	male	male
Diabetes duration (years)	23	17
Mean arterial blood pressure (mmHg)	89.0	102.5
HbA1c (mmol/mol)	33	58
HbA1c (%)	5.2	7.5
Systemic hypertension	-	+
Dyslipidaemia	-	-
Cardiovascular diseases	-	-
Renal disease	-	+
Insulin use	-	+
Prescribed glitazones	-	-
Prescribed statins	-	-
Phakic (LE / RE)	+ / +	- / -
Prior PRP (LE / RE)	- / -	+ / +
logMAR VA (LE / RE)	-0.176 / -0.176	0.398 / 0.398
International classification of DR severity (LE / RE)	moderate NPDR / moderate NPDR	moderate NPDR / moderate NPDR
OCT features		
CSF thickness (µm) (LE / RE)	258 / 271	316 / 375
Transverse length of disrupted EZ (%) (LE / RE)	-	0.0 / 22.5
DRIL (µm) (LE / RE)	-	355 / 205
Cystoid abnormalities (LE / RE)	-	- / +
Subretinal fluid (LE / RE)	-	- / -
Vitreoretinal abnormalities (LE / RE)	-	+ / +

ESM Table 4. Characteristics of the Participants Whose Retinas Were Immunostained with Anti-Fumarase Antibody.

Characteristics	Nondiabetic control	DR patient
Diabetes type	nondiabetic	Type 2 diabetes
Sex	female	female
Age (years)	58	74
Race	Caucasian	Caucasian
Diabetes duration (years)	N/A	>20
Diabetic retinopathy severity	N/A	Advanced NPDR

ESM Table 5. Characteristics of the Participants Whose Sera Were Used for ELISA.

Characteristics	DM group (n=65)	DR group (n=65)	DME group (n=65)	P value
Age (years)	64.0±11.3	63.4±11.5	63.4±11.3	1.000
Sex (male / female)	41 / 24	41 / 24	41 / 24	1.000
Diabetes duration (years)	11.0±8.8	16.8±10.1 **	12.1±7.9*	0.001
Mean arterial blood pressure (mmHg)	99.6±10.1	104.7±14.9	107.1±14.4 [†]	0.029
HbA1c (mmol/mol)	62.5±17.2	63.1±19.5	62.3±18.7	1.000
HbA1c (%)	7.87±1.57	7.92±1.78	7.85±1.71	1.000
Systemic hypertension	39	43	45	0.574
Dyslipidaemia	38	36	30	0.345
Cardiovascular diseases	11	16	20	0.203
Renal disease	30	27	29	0.903
Insulin use	23	39	33	0.020
Prescribed glitazones	5	0	0	0.011
Prescribed statins	27	24	24	0.822
Phakic in both eyes	55	46	44	0.065
Prior PRP in either eye	-	17	26	0.135
logMAR VA in the study eye	-	0.017±0.170	0.346±0.312	<0.001
International classification				-
No apparent retinopathy	65	-	-	
Mild NPDR	-	0	0	
Moderate NPDR	-	29	29	
Severe NPDR	-	15	15	
PDR	-	21	21	
OCT features in the study eye				
CSF thickness (µm)	-	-	475±147	-
Transverse length of disrupted EZ (%)	-	-	39.0±39.4	-
DRIL (µm)	-	-	519±389	-
Cystoid abnormalities	-	-	53	-
Subretinal fluid	-	-	18	-
Vitreoretinal abnormalities	-	-	14	-

ANOVA with Bonferroni correction for age, mean arterial blood pressure, and HbA1c. Kruskal-Wallis test with Dunnett's multiple comparison for diabetes duration. * $p < 0.05$ vs. DR group, ** $p < 0.01$ vs. DM group. [†] $p < 0.05$ vs. DM group. Student t-test for logMAR VA. Fisher's exact test for Prior PRP in either eye. Chi-square tests for other discrete parameters.

ESM Table 6. Characteristics of the Participants Whose Sera Were Used for ELISA in an Independent and Additional Cohort.

Characteristics	DR group (n=74)	DME group (n=74)	<i>P</i> value
Age (years)	65.8±11.1	65.8±9.3	1.000
Sex (male / female)	53 / 21	53 / 21	1.000
Diabetes duration (years)	16.6±10.4	16.6±10.1	1.000
Mean arterial blood pressure (mmHg)	106.5±12.6	106.0±14.8	0.836
HbA1c (mmol/mol)	60.4±13.9	60.4±14.9	1.000
HbA1c (%)	7.68±2.91	7.68±2.83	1.000
Systemic hypertension	50	45	0.493
Dyslipidaemia	40	28	0.069
Cardiovascular diseases	10	15	0.381
Renal disease	24	21	0.721
Insulin use	32	30	0.868
Prescribed glitazones	2	2	1.000
Prescribed statins	21	22	1.000
Phakic in both eyes	54	54	1.000
Prior PRP in either eye	14	28	0.017
logMAR VA in the study eye	0.110±0.310	0.316±0.288	<0.001
International classification			1.000
Mild NPDR	5	5	
Moderate NPDR	35	35	
Severe NPDR	11	11	
PDR	23	23	
OCT features in the study eye			
CSF thickness (µm)	-	443±104	-
Transverse length of disrupted EZ (%)	-	33.5±39.1	-
DRIL (µm)	-	412±303	-
Cystoid abnormalities	-	64	-
Subretinal fluid	-	17	-
Vitreoretinal abnormalities	-	21	-

Student t-test for age, mean arterial blood pressure, HbA1c and logMAR VA. Mann-Whitney for diabetes duration. Fisher's exact test for gender, systemic hypertension, dyslipidemia, lens status, and prior PRP in either eye. Chi-square tests for international classification of diabetic retinopathy severity grades.

ESM Table 7. Association of Titers of Anti-Fumarase IgG with Systemic and Ocular Parameters in 106 Cases with Centre-involved DME.

Characteristics		Titers of anti-fumarase IgG (absorbance [450nm])	Association with titers of anti-fumarase IgG
Age (years)	65.5±9.6	0.200±0.200	$\rho=0.115$, $p=0.069$
Sex (male / female)	74/32	0.200±0.210 / 0.198±0.177	$p=0.947$
Diabetes duration (years)	14.6±9.1	-	$\rho=-0.029$, $p=0.684$
Mean arterial blood pressure (mmHg)	107.3±14.0	-	$\rho=-0.047$, $p=0.635$
HbA1c (mmol/mol)	61.1±16.3	-	$\rho=-0.058$, $p=0.412$
HbA1c (%)	7.74±1.49	-	$\rho=-0.058$, $p=0.412$
Systemic hypertension (presence / absence)	66/40	0.208±0.201 / 0.186±0.199	$p=0.583$
Dyslipidaemia (presence / absence)	44/62	0.165±0.117 / 0.224±0.240	$p=0.096$
Cardiovascular diseases (presence / absence)	23/83	0.272±0.254 / 0.180±0.178	$p=0.047$
Renal disease (presence / absence)	34/72	0.261±0.239 / 0.171±0.173	$p=0.008$
Insulin use (presence / absence)	51/55	0.225±0.225 / 0.176±0.172	$p=0.128$
Prescribed glitazones (presence / absence)	1/105	0.028 / 0.201±0.200	-
Prescribed statins (presence / absence)	35/71	0.165±0.144 / 0.217±0.221	$p=0.293$
Lens status (phakia / pseudophakia)	79/27	0.185±0.176 / 0.240±0.253	$p=0.296$
Prior PRP (presence / absence)	34/72	0.243±0.269 / 0.179±0.155	$p=0.206$
logMAR VA	0.272±0.239	-	-
International classification			N.S.
Mild NPDR	3	0.254±0.138	-
Moderate NPDR	54	0.184±0.173	-
Severe NPDR	22	0.159±0.174	-
PDR	27	0.257±0.261	-
OCT features			
CSF thickness (μm)	461±128	-	$\rho=-0.045$, $p=0.647$
Transverse length of disrupted EZ (%)	28.4±35.9	-	-
DRIL (μm)	380±177	-	$\rho=-0.151$, $p=0.122$
Cystoid abnormalities (presence / absence)	93/13	0.199±0.190 / 0.200±0.268	$p=0.689$
Subretinal fluid (presence / absence)	31/75	0.222±0.200 / 0.190±0.200	$p=0.250$
Vitreoretinal abnormalities (presence / absence)	12/94	0.223±0.274 / 0.197±0.190	$p=0.780$

Spearman's rank correlation coefficient for age, diabetes duration, and HbA1c. N.S., no statistical significance between any groups of DR severity grades using Kruskal-Wallis test with Bonferroni correction. Mann-Whitney U-test for other parameters.

ESM Table 8. Characteristics of 21 eyes of 21 participants who received intravitreal administration of anti-VEGF agents for DME.

Characteristics	
Age (years)	65.1±14.0
Sex (male / female)	13/8
Mean arterial blood pressure (mmHg)	100.7±13.0
Diabetes duration (years)	9.7±6.8
HbA1c (mmol/mol)	59.4±18.7
HbA1c (%)	7.59±1.71
Systemic hypertension	13
Dyslipidaemia	9
Cardiovascular diseases	7
Renal disease	8
Insulin use	8
Prescribed glitazones	0
Prescribed statins	8
Phakic	15
Prior PRP	9
LogMAR VA	0.246±0.208
International classification	
Moderate NPDR	10
Severe NPDR	5
PDR	6
OCT features	
CSF thickness (µm)	456±116
Transverse length of disrupted EZ (%)	32.4±33.0
DRIL (µm)	579±309
Cystoid abnormalities	20
Subretinal fluid	5
Vitreoretinal abnormalities	3
Anti-VEGF agents (Ranibizumab / Aflibercept)	17/4
Time points of blood sampling after treatment (months)	10.7±5.0
Number of injections	6.0±3.0

ESM Table 9. Characteristics of 10 participants in whom the titers of individual IgG subtypes were measured.

Characteristics	
Age (years)	69.3±10.4
Sex (male / female)	6 / 4
Diabetes duration (years)	17.5±10.9
Mean arterial blood pressure (mmHg)	106.3±11.3
HbA1c (mmol/mol)	65.5±17.5
HbA1c (%)	8.14±1.61
Systemic hypertension	8
Dyslipidaemia	3
Cardiovascular diseases	5
Renal disease	8
Insulin use	7
Prescribed glitazones	0
Prescribed statins	4
Phakic in both eyes	9
Prior PRP in either eye	4
LogMAR VA	0.287±0.243
International classification	
Moderate NPDR	3
Severe NPDR	3
PDR	4
OCT features	
CSF thickness (µm)	431±90
Transverse length of disrupted EZ (%)	47.6±29.8
DRIL (µm)	393±368
Cystoid abnormalities	7
Subretinal fluid	3
Vitreoretinal abnormalities	1
Titer of total anti-fumarase IgG (absorbance [450nm])	0.306±0.067

ESM Table 10. Characterization of Nondiabetic, DR, or DME Participants Whose Sera were Used in the In vivo and In Vitro Experiments.

Characteristics	Nondiabetic control (n=3)	DR group (n=4)	DME group (n=8)
Age (years)	65.7±15.0	58.3±7.2	67.3±8.9
Sex (male / female)	0 / 3	3 / 1	4 / 4
Diabetes duration (years)	-	19.3±7.7	16.0±6.7
Mean arterial blood pressure (mmHg)	88.8±15.5	105.8±13.4	103.3±7.4
HbA1c (mmol/mol)	-	51.7±17.3	67.5±17.4
HbA1c (%)	-	6.88±1.35	8.33±1.50
Systemic hypertension	0	2	5
Dyslipidaemia	0	1	2
Cardiovascular diseases	0	0	2
Renal disease	1	0	5
Insulin use	-	1	4
Prescribed glitazones	-	0	0
Prescribed statins	0	0	2
Phakic in both eyes	3	3	8
Prior PRP in either eye	-	1	1
logMAR VA in the study eye	-	-0.127±0.086	0.283±0.270
Subgroups			
Idiopathic epiretinal membrane	2	-	-
Idiopathic macular hole	1	-	-
International classification of DR severity in the study eye			
Moderate NPDR	-	2	3
Severe NPDR	-	0	2
PDR	-	2	3
OCT features in the study eye			
CSF thickness (µm)	-	-	397±85
Transverse length of disrupted EZ (%)	-	-	43.1±34.4
DRIL (µm)	-	-	271±329
Cystoid abnormalities	-	-	5
Subretinal fluid	-	-	3
Vitreoretinal abnormalities	-	-	1

Table 11. Antibodies for each experiment.

Antigen	Species	Source	Catalogue number	Dilution
Primary antibodies for Western blot				
Fumarase	Mouse	Novus Biologicals	NBP1-47753	1:1000
Cytochrome C	Mouse	Biolegend	612504	1:1000
AIF	Rabbit	Cell Signaling	5318	1:1000
β -actin	Mouse	Sigma	A5441	1:5000
Secondary antibodies for Western blot				
Human IgG	Goat	Life Technologies	62-8420	1:5000
Mouse IgG	Sheep	GE Healthcare	NA931	1:5000
Rabbit IgG	Donkey	GE Healthcare	NA934	1:5000
Primary antibodies for flow cytometry				
Fumarase	Mouse	Novus Biologicals	NBP1-47753	1:100
Control IgG	Mouse	Cell signaling	5415	1:100
Secondary antibodies for ELISA				
Human total IgG	Goat	Life Technologies	62-8420	1:5000
Human IgG1	Mouse	Southern Biotech	9054-05	1:1000
Human IgG2	Mouse	Southern Biotech	9070-05	1:1000
Human IgG3	Mouse	Southern Biotech	9210-05	1:1000
Human IgG4	Mouse	Southern Biotech	9200-05	1:1000
Primary antibodies for immunostaining				
Fumarase	Mouse	Novus Biologicals	NBP1-47753	1:100
Fumarase	Mouse	GeneTex	GTX84498	1:100
Recoverin	Rabbit	Millipore	AB5585	1:1000
CD59a	Mouse	GeneTex	GTX54410	1:100
C5b-9	Rabbit	Abcam	ab55811	1:1000
COX IV	Mouse	Abcam	ab33985	1:30
Secondary antibodies for immunostaining				
Human IgG	Donkey	Jackson immunoresearch	709-165-149	1:5000
Mouse IgG	Donkey	Jackson immunoresearch	715-545-151	1:1000
Rabbit IgG	Goat	Life Technologies	A-11034	1:1000
Rabbit IgG	Goat	Life Technologies	A-11012	1:1000

ESM Table 12. Post-hoc Multivariate Logistic Regression Analysis to Determine the Predictor of Centre-involved DME in 195 Diabetic Participants.

Characteristics	Univariate		Multivariate	
	OR (CI 95%)	<i>P</i> value	OR (CI 95%)	<i>P</i> value
Age	0.994 (0.950-1.041)	0.813	-	-
Sex	1.011 (0.373-2.736)	0.983	-	-
Diabetes duration	0.935 (0.883-0.990)	0.021	0.946 (0.892-1.004)	0.066
Mean arterial blood pressure	1.025 (1.000-1.050)	0.046	1.016 (0.982-1.051)	0.361
HbA1c	1.036 (0.783-1.371)	0.803	-	-
Systemic hypertension	0.738 (0.257-2.116)	0.572	-	-
Dyslipidaemia	0.769 (0.296-1.998)	0.590	-	-
Cardiovascular diseases	1.695 (0.862-3.333)	0.126	0.690 (0.220-2.165)	0.525
Renal disease	1.032 (0.567-1.879)	0.919	-	-
Insulin use	1.131 (0.623-2.052)	0.685	-	-
Prescribed glitazones	0.000 (0.000-)	0.999	-	-
Prescribed statins	0.907 (0.490-1.676)	0.755	-	-
Phakic in both eyes	3.332 (0.999-11.112)	0.050	3.511 (1.121-10.999)	0.031
PDR in either eye	1.025 (0.295-3.553)	0.970	-	-
Prior PRP in either eye	9.636 (2.806-33.097)	<0.001	8.005 (2.561-25.020)	<0.001
Titer of anti-fumarase IgG	1.743×10^{11} (7.157×10^6 - 4.247×10^{15})	<0.001	1.379×10^{10} (1.169×10^6 - 1.626×10^{14})	<0.001

Univariate logistic regression or Multivariate logistic analysis using significant parameters (Wald's chi-square > 2) as independent parameters.

ESM Table 13. Multivariate Logistic Regression Analysis to Determine the Predictor of Centre-involved DME in 148 DR Participants in the Independent Cohort.

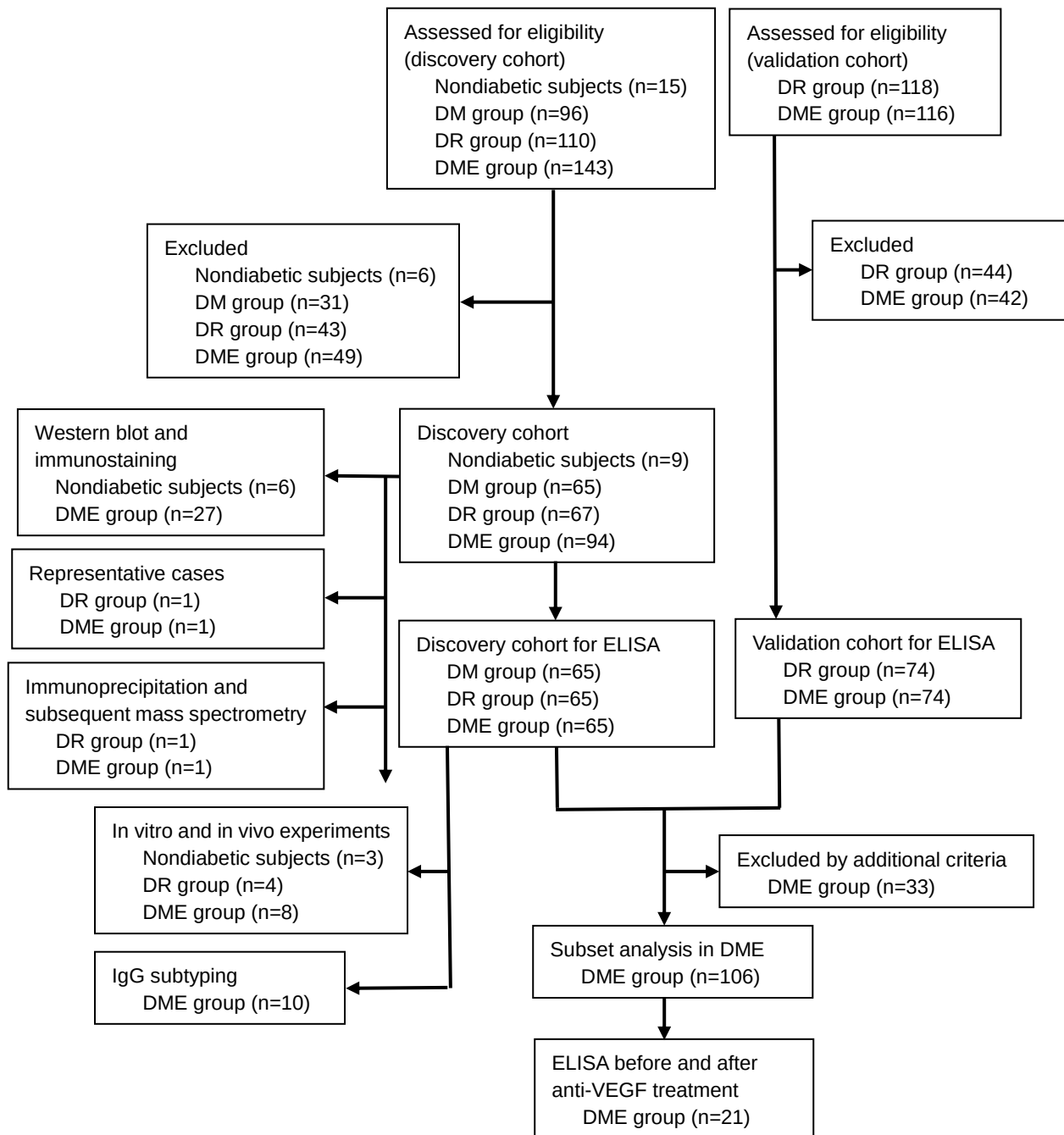
Characteristics	Univariate		Multivariate	
	OR (CI 95%)	<i>P</i> value	OR (CI 95%)	<i>P</i> value
Age	1.000 (0.969-1.032)	1.000	-	-
Sex	1.000 (0.489-2.044)	1.000	-	-
Diabetes duration	0.997 (0.960-1.035)	0.875	-	-
Mean arterial blood pressure	0.997 (0.973-1.022)	0.836	-	-
HbA1c	1.000 (0.767-1.302)	1.000	-	-
Systemic hypertension	0.714 (0.362-1.408)	0.331	-	-
Dyslipidaemia	0.569 (0.294-1.104)	0.096	0.494 (0.224-1.090)	0.081
Cardiovascular diseases	1.627 (0.678-3.903)	0.275	-	-
Renal disease	0.825 (0.409-1.665)	0.592	-	-
Insulin use	0.895 (0.466-1.720)	0.739	-	-
Prescribed glitazones	1.000 (0.137-7.294)	1.000	-	-
Prescribed statins	1.068 (0.525-2.172)	0.856	-	-
Phakic in both eyes	1.000 (0.484-2.066)	1.000	-	-
PDR in either eye	1.000 (0.498-2.006)	1.000	-	-
Prior PRP in either eye	2.609 (1.235-5.510)	0.012	3.043 (1.272-7.283)	0.012
Titer of anti-fumarase IgG	4.749×10^5 (2.230×10^3 - 1.011×10^8)	<0.001	4.267×10^5 (1.606×10^3 - 1.134×10^8)	<0.001

Univariate logistic regression or Multivariate logistic analysis using significant parameters (Wald's chi-square > 2) as independent parameters.

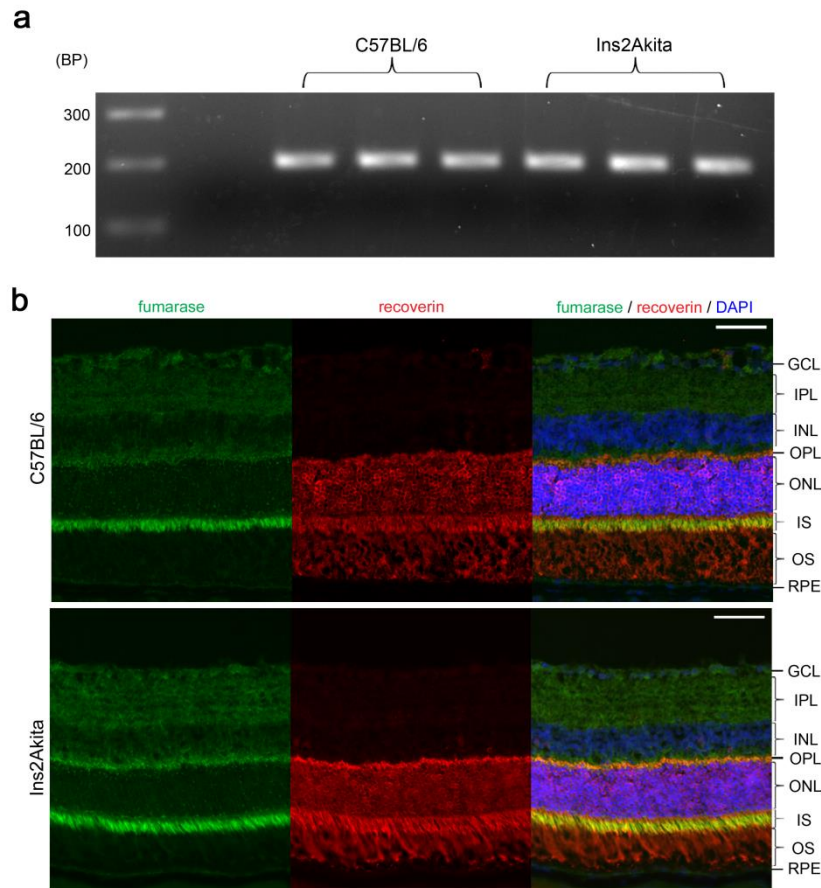
ESM Table 14. Multivariate Linear Regression Analysis to Determine the Predictors of the Transverse Length of Disrupted EZ in 106 Eyes with Centre-involved DME.

Characteristics	univariate	multivariate
Age	$\beta=0.025, P=0.801$	-
Sex (Male)	$\beta=-0.074, P=0.451$	-
Diabetes duration	$\beta=-0.123, P=0.241$	-
Mean arterial blood pressure	$\beta=0.096, P=0.334$	-
HbA1c	$\beta=0.118, P=0.257$	-
Systemic hypertension	$\beta=0.033, P=0.737$	-
Dyslipidaemia	$\beta=-0.102, P=0.299$	-
Cardiovascular diseases	$\beta=0.140, P=0.152$	-
Renal disease	$\beta=0.170, P=0.081$	$\beta=0.048, P=0.574$
Insulin use	$\beta=0.017, P=0.863$	-
Prescribed glitazones	$\beta=-0.078, P=0.429$	-
Prescribed statins	$\beta=-0.047, P=0.636$	-
Phakic	$\beta=0.021, P=0.833$	-
Prior PRP	$\beta=0.175, P=0.074$	$\beta=0.010, P=0.903$
International classification (PDR)	$\beta=0.214, P=0.027$	$\beta=0.089, P=0.294$
OCT features		
CSF thickness	$\beta=0.464, P<0.001$	$\beta=0.402, P<0.001$
DRIL	$\beta=0.133, P=0.173$	-
Cystoid abnormalities	$\beta=0.160, P=0.101$	-
Subretinal fluid	$\beta=0.230, P=0.018$	$\beta=0.161, P=0.057$
Vitreoretinal abnormalities	$\beta=-0.038, P=0.696$	-
Titer of anti-fumarase IgG	$\beta=0.409, P<0.001$	$\beta=0.337, P<0.001$

Multivariate linear regression analysis was employed using significant variables in univariate regression analysis ($P<0.1$) as independent parameters.

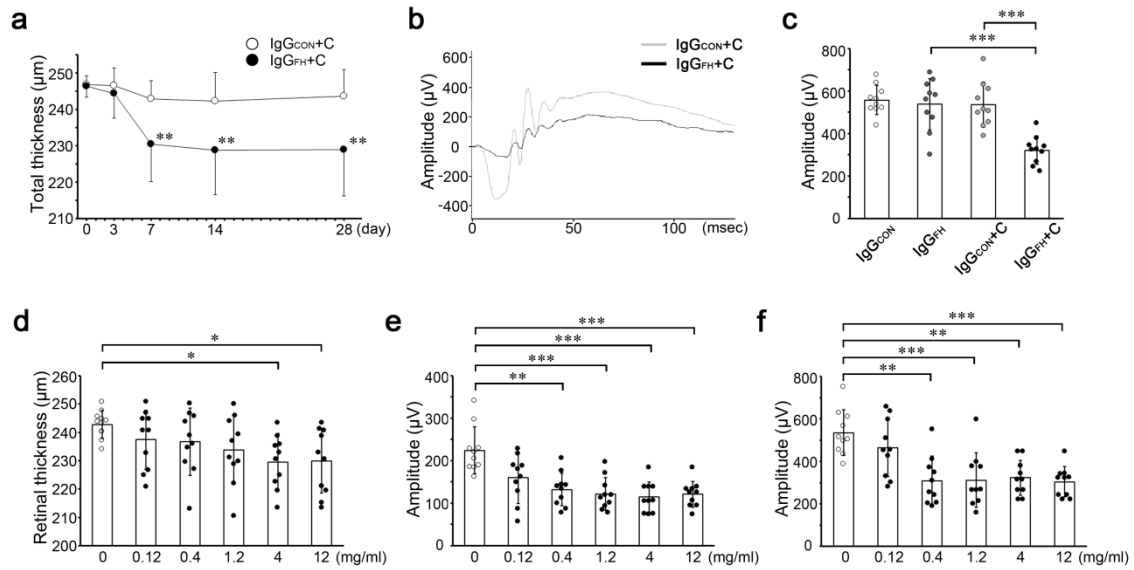


ESM Figure 1. Flow chart of each study using serum specimens.



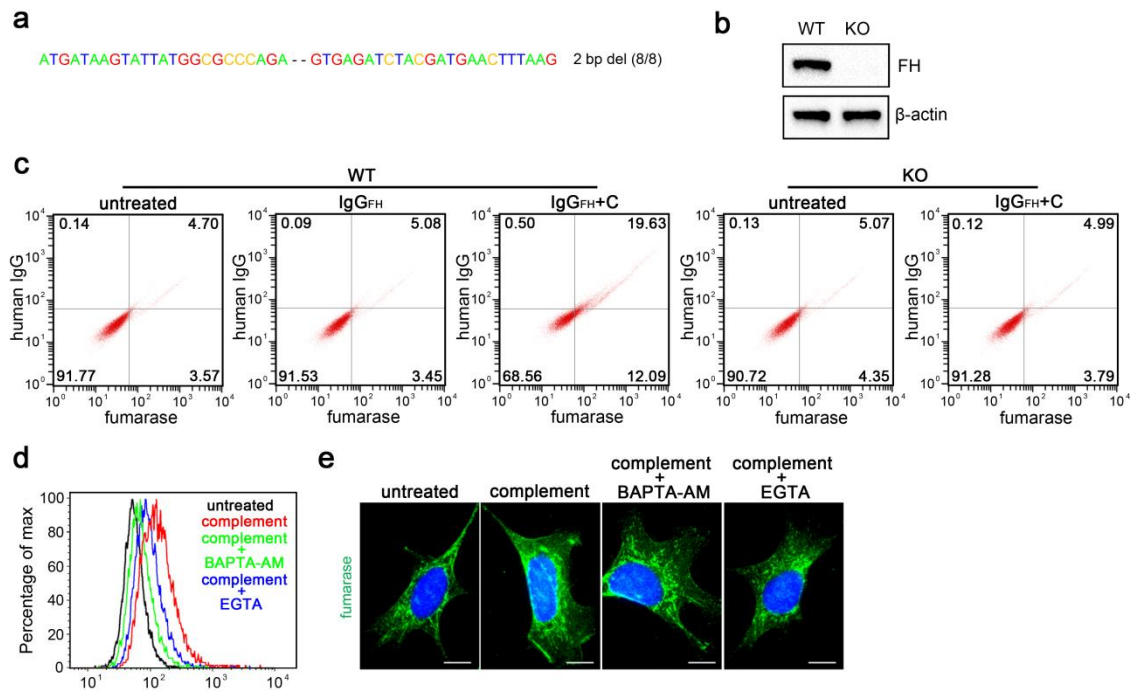
ESM Figure 2. Expression of fumarase in mouse retinas.

(a) Expression of *FH* mRNA was confirmed by PCR in retinas from control (C57BL/6) or diabetic (C57BL/6 Ins2Akita) mice. $n = 3$. (b) Fumarase was expressed in inner segments and counterstained with recoverin, a marker of photoreceptor cells, in the retinas from C57BL/6 and Ins2Akita mice. Scale bar, 50 μm .



ESM Figure 3. Subretinal Injection of Anti-Fumarase IgG and Complement Induces Structural and Functional Damage in the Photoreceptors.

(a) OCT measurements of the total retinal thickness (from the inner limiting membrane to Bruch's membrane), which correspond to **Fig. 6b**. $n=15$ retinas. $**p<0.01$ for the comparison between two groups at each time point. (**b and c**) Representatives of ERG and the quantified amplitude of the b-wave, which corresponds to **Fig. 6c**. $***p<0.001$. $n=10$. IgG_{CON} = IgG from 4 DR sera (**ESM Table 10**); IgG_{FH} = anti-fumarase IgG purified from sera of DME participants (noM1 - noM4 in **ESM Table 10**). C = complement from 3 nondiabetic subjects (**ESM Table 10**). (**d - f**) OCT measurement of retinal thickness (**d**) and the a-wave (**e**) and b-wave (**f**) of ERG after subretinal injection of complement and the indicated IgG_{FH} concentrations. $*p<0.05$, $**p<0.01$, $***p<0.001$. $n=10$. One-way ANOVA with Bonferroni correction in **c-f**.



ESM Figure 4. Antigen-antibody Reaction on the Cell Surface after Treatment with Complement in Cultured Cells.

(a and b) Direct sequencing analysis and Western blot confirmed the frameshift mutations in exon 2 of both alleles of the *FH* gene and concomitant loss of protein expression after CRISPR-CAS9-mediated introduction of *FH* knockout. WT = *FH*^{+/+} HEK 293 cells. KO = *FH*^{-/-} HEK 293 cells. (c) Representative flow cytometry plots confirmed cell surface staining of fumarase and human IgG in HEK 293 cells incubated with IgG_{FH}+C for 3 hours. *n*=4. (d and e) Cell surface staining of fumarase in 661W cells shown in FACS plots (*n*=4) and immunofluorescence (*n*=3). Scale bar, 10 μm.