Fc γ Receptor Upregulation Is Associated With Immune Complex Inflammation in the Mouse Retina and Early Age-Related Macular Degeneration

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PURPOSE. Several lines of evidence suggest the involvement of antibodies and immune complex inflammation in AMD, a blinding disease with a strong inflammatory component. To examine this further, we developed a novel experimental mouse model of retinal inflammation and evaluated whether inflammation associated with immune complex formation was present in eyes of AMD donors.

METHODS. A localized immune complex-mediated reaction was induced in the retina of wild-type (WT), Fc receptor γ chain-deficient ($\gamma^{-/-}$), and C1q-deficient (*C1q*^{-/-}) mice, and donor eyes were obtained after death from donors with early or wet AMD and from healthy control subjects. The presence of immune complexes, Fc γ receptors (Fc γ Rs), and markers of macrophage/microglia activation was investigated by immunohistochemistry.

RESULTS. In WT and *C1q*^{-/-} mice, immune complex deposition in the retina led to a robust inflammatory response with activation of microglia, recruitment of myeloid cells, and increased expression of Fc γ RI through Fc γ RIV and major histocompatibility complex class II. This response was not observed in $\gamma^{-/-}$ mice lacking activating Fc γ Rs. We found that early AMD was associated with deposition of IgG, C1q, and membrane attack complex in the choriocapillaris and with increased numbers of CD45+ cells expressing Fc γ RIIa and Fc γ RIIb. Furthermore, Fc γ RIIa and Fc γ RIIb were observed in eyes of donors with wet AMD.

CONCLUSIONS. Our studies suggest that immune complexes may contribute to AMD pathogenesis through interaction of IgG with Fc γ Rs and might inform about possible adverse effects associated with therapeutic antibodies.

Keywords: immune complex, immunoglobulin, Fc receptor, macular degeneration, inflammation

Age-related macular degeneration is the most common cause of irreversible blindness among the elderly in the developed world.¹ The early stage of the disease is characterized by pigmentary changes to the RPE and the presence of insoluble extracellular deposits (drusen) between the RPE and the Bruch's membrane. In later stages, dysfunction and degeneration of the RPE and subsequent degeneration of the photoreceptor cells occur. Risk factors associated with this blinding disease include age² and lifestyle (i.e., smoking³ and obesity⁴), but the etiology of AMD remains incompletely understood.

Studies^{5,6} suggest that inflammation has a prominent role in AMD pathogenesis. In particular, the Y402H polymorphism in complement factor H has been associated with an increased risk of developing AMD. However, factors other than dysregulation of the alternative complement pathway may contribute to the pathogenesis of AMD. The presence of high titers of circulating retinal-specific autoantibodies,^{7–10} the deposition of IgG in the drusen of patients with AMD,¹¹ and the increased risk of developing AMD associated with polymorphisms in serpin peptidase inhibitor, clade G (C1 inhibitor) 1 (*SERPINC1*)¹²

suggest that antibody-mediated immune responses may also contribute to the pathology of the disease.

Antibodies can mediate inflammation when they bind their cognate antigen and form immune complexes via activation of the classic complement cascade or by binding Fc γ receptors (Fc γ Rs) on effector cells such as macrophages.¹³ In mice, there are four known Fc γ Rs, of which three (Fc γ RI, Fc γ RIII, and Fc γ RIV) are activating and one (Fc γ RII) is inhibitory. In humans, the following six Fc γ Rs have been described: the activating receptors Fc γ RI, Fc γ RIIa, Fc γ RIIc, Fc γ RIIIa, and Fc γ RIIIb and the inhibitory Fc γ RIIb. Cross-linking of activating Fc γ Rs activates cell effector functions such as phagocytosis, oxidative burst, cytokine production, and degranulation, which are inhibited by ligation of Fc γ RII in mice and Fc γ RIIb in humans.¹⁴ Histologic studies^{11,15} have shown the presence of macrophages and IgG in AMD lesions. We hypothesize that macrophage activation in the eye may be partly induced by deposition of IgG and immune complex formation, which could bind Fc γ Rs on retinal macrophages and microglia, contributing to early stages of AMD pathogenesis and/or

progression of disease via activation of recruited macrophages in the late stages of disease.

We show that immune complex deposition in the murine retina leads to robust neuroinflammation characterized by microglial activation and recruitment of CD45⁺ cells from the periphery, which is dependent on the presence of Fc γ Rs. We found that early AMD was associated with C1q, IgG, and membrane attack complex (MAC) deposition and increased expression of Fc γ RIIa and Fc γ RIIb in the choroid. In addition, robust expression of Fc γ RIIa and Fc γ RIIb is detected in the choroid and retinas of patients with wet AMD.

METHODS

Mice

BALB/c mice were obtained from Charles River (Margate, UK) and bred and maintained in local facilities. Fc receptor γ chain-deficient ($\gamma^{-/-}$) C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and backcrossed for 10 generations onto a BALB/c background. The $\gamma^{-/-}$ mice are deficient for the γ subunit of immunoglobulin Fc receptors and lack the ability to express the activating Fc γ Rs Fc γ RI, Fc γ RIII, and Fc γ RIV. C1q-deficient (*C1q^{-/-}*) mice on a BALB/c background were obtained from Aras Kadioglu (Leicester, UK) with permission from Marina Botto (London, UK). All procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and under the authority of a UK Home Office License.

Induction of Immune Complexes in the Retina

Six-week-old BALB/c mice were immunized against ovalbumin (OVA) (Sigma-Aldrich Corp., Poole, UK) by intraperitoneal injection of 100 μ L containing 50 μ g OVA in the presence of Imject Alum (1:1 ratio; Thermo Scientific Pierce, Loughborough, UK). Mice were boosted two times by intraperitoneal injection of 100 μ L containing 100 μ g OVA in saline. Two weeks after the last OVA boost, 10 μ g endotoxin-free OVA (Hyglos, Bernried, Germany) in saline (10 mg/mL) or 1 μ L saline as a control was injected intravitreally using a fine-glass micropipette with a diameter of less than 50 μ m (Sigma-Aldrich Corp.). Eyes were collected 24 hours and 3, 7, and 14 days after intravitreal injection. For investigation of the biological function of Fc γ Rs and C1q, tissue was collected 3 days after intravitreal injection.

Tissue Processing

Serum samples from mice were collected after a terminal dose of tribromoethanol. Following transcardiac perfusion with heparinized saline, eyes were removed and snap frozen in optimum temperature cutting compound embedding medium (Sakura Finetek, Thatcham, UK).

Mouse Immunohistochemistry

Twenty-micrometer sections were dried at 37°C and fixed in 100% ethanol at 4°C for 15 minutes. Five percent BSA (Fisher Scientific, Loughborough, UK) and appropriate 10% animal serum (Vector Laboratories, Peterborough, UK) in PBS were used for blocking. The sections were incubated for 36 hours at 4°C with the following primary antibodies: polyclonal rabbit anti-OVA (Bioscience International, Inc., Saco, ME), FITC-labeled donkey F(ab')₂ fragment anti-mouse IgG (Jackson ImmunoResearch Laboratories, Suffolk, UK), monoclonal rat anti-mouse CD11b (5C6; Serotec, Oxford, UK), monoclonal rat anti-mouse

CD45 (YW62.3; Serotec), monoclonal rat anti-Fc γ RII/III (FCR4G8, CD16/CD32; Serotec), and monoclonal rat anti-mouse major histocompatibility complex (MHC) II (M5/114.15.2; Abcam, Cambridge, UK). Monoclonal rat anti-mouse Fc γ RI (AT152-9), Fc γ RIII (AT154-2), and Fc γ RIV (AT137) were generously provided by Mark Cragg (University of Southampton). AlexaFluor 488-labeled or AlexaFluor 568-labeled donkey anti-rat and goat anti-rabbit secondary antibodies (Invitrogen, Paisley, UK) were incubated for 1 hour at room temperature, and sections were coverslipped using ProLong Gold Antifade Reagent containing 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen).

Quantification of CD11b, CD45, Fc γ RI Through Fc γ RIV, and MHC II on Mouse Sections

Sections were analyzed with a Leica DM5000 microscope (Leica Microsystems, Inc., Buffalo Grove, IL). Nonoverlapping photomicrographs spanning the retinal sections (over a mean length of 3.75 mm per mouse) were collected using the $\times 20$ objective and blinded. Cells were only counted when DAPI-positive nuclei were visible. The numbers of cells were quantified using the Photoshop CS5 count tool (Adobe Systems, Incorporated, Berkshire, UK) and normalized per millimeter of retina.

OVA Antibody ELISA

Serum samples from OVA-immunized mice were serially diluted onto OVA-coated MaxiSorp Nunc plates (10 μ g/mL in PBS; eBioscience, Hatfield, UK), followed by incubation with biotinylated horse anti-mouse IgG (Vector Laboratories) for determination of total OVA-specific IgG levels. Subclasses were determined by IgG1-specific and IgG2a-specific antibodies (Serotec). Binding of OVA-specific antibodies was detected by polystyrene-avidin (Sanquin, Amsterdam, The Netherlands) and visualized using 3,3',5,5'-tetramethylbenzidine/hydrogen peroxide substrate (R&D Systems, Abingdon, UK).

Donor Eyes

Human donor eyes were obtained from the Iowa Lions Eye Bank (Iowa City, IA) following informed consent from the donors' families. The use of human donor eyes conformed to the Declaration of Helsinki. The AMD affection status was determined by medical record review. Early AMD was defined by the presence of pigmentary changes and/or the presence of drusen in the macula.¹⁶ Wet AMD eyes had a clinical diagnosis of choroidal neovascularization secondary to AMD. Only whole globes were used for this study. Maculae were preserved in 4% paraformaldehyde within 8 hours of death.

Human Immunohistochemistry

Eyes were processed immediately on receipt as previously described.¹⁶ Eight-micrometer sections were air dried and blocked with 10% w/v BSA (Fisher Scientific) in PBS containing 1 mM calcium chloride and 1 mM magnesium chloride. The primary antibodies used were the following: FITC-labeled donkey F(ab')₂ fragment anti-human IgG (Jackson ImmunoResearch Laboratories), polyclonal rabbit anti-C1q (Dako, Cambridge, UK), monoclonal mouse anti-MAC-C5b-9 (aE11; Dako), monoclonal mouse anti-human CD45 (HI30; BD Biosciences, Oxford, UK), monoclonal rabbit anti-Fc γ RIIb (EP888Y; Abcam), monoclonal rabbit anti-Fc γ RIIIa (EPR6658; Epitomics—an Abcam Company, Burlingame, CA), monoclonal mouse anti-Fc γ RIII (2h7; Abcam), polyclonal goat anti-Fc γ RI (R&D Systems), and biotinylated *Ulex*

FIGURE 1. Immune complexes form throughout the retinas of sensitized mice following intravitreal injection of OVA. **(A)** Immunization of mice against OVA resulted in high levels of anti-OVA circulating antibodies (total IgG) in mice injected intravitreally with OVA ($n = 8$) or saline ($n = 3$) as detected by ELISA. Serum from nonimmunized mice was used as a control ($n = 4$). **(B)** Immunohistochemical detection of OVA (red) and IgG (green) 24 hours (1 day) after saline injection and 1, 3, 7, and 14 days after OVA injection ($n = 4$ –6 per group). Immune complexes (colocalization, arrows) formed transiently from 1 day after OVA challenge in the GCL, SR, inner plexiform layer, and inner and outer photoreceptor segment layers. INL, inner nuclear layer; ONL, outer nuclear layer; PR, photoreceptors. Scale bars: 50 μ m and 10 μ m (insets).

europaeus agglutinin I (UEA1; Vector Laboratories). AlexaFluor 488-conjugated or AlexaFluor 546-conjugated secondary antibodies (Invitrogen) were used for detection of primary antibodies. Sections were counterstained with DAPI (Molecular Probes, Eugene, OR).

Quantification of CD45, Fc γ RIIa, and Fc γ RIIb on Human Sections

Triple-labeled sections were photographed using the $\times 20$ objective of a fluorescence microscope (BX41; Olympus Corporation, Center Valley, PA) and a digital camera (SPOTRT; Diagnostic Instruments, Inc., Sterling Heights, MD). Nonoverlapping photomicrographs spanning the sections (over a mean length of 511 μ m per donor) were collected and blinded. In the choroid only, cells outside vascular lumens and within 25 μ m of the Bruch's membrane were counted. Cells were only counted when nuclei positively stained with DAPI were visible. The numbers of cells were counted using the Photoshop CS5 count tool (Adobe Systems, Incorporated) and normalized per 100 μ m of the Bruch's membrane.

Statistical Analysis

All statistical tests were performed in GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA). Data sets were tested for normal distribution using the D'Agostino-Pearson omnibus test. All mouse data were logarithmically transformed. Data from the kinetics of immune complex responses in the murine retina were analyzed by two-way ANOVA. Data from Fc

receptor γ chain^{-/-} and *CIq*^{-/-} mice were analyzed by one-way ANOVA. If significant, both tests were followed by the Bonferroni post hoc test. Human data were analyzed by a Mann-Whitney *U* test. All data represent the mean (SEM). $P < 0.05$ was considered significant.

RESULTS

Coimmunolocalization of OVA and IgG in the Mouse Retina Suggests Immune Complex Deposition and Clearance Within 14 Days

To investigate antibody-mediated inflammation in the retina, the reverse Arthus reaction was used in the eyes of wild-type (WT) BALB/c mice (Fig. 1). To induce immune complex formation in the retina, mice were immunized against OVA and then challenged intravitreally with OVA or saline as a control. OVA-immunized mice had high circulating levels of anti-OVA antibodies (Fig. 1A). Coimmunolocalization of IgG and OVA suggested immune complex deposition in the retina following OVA challenge. Intravitreal injection of saline did not result in accumulation of IgG in OVA-immunized mice. Similarly, intravitreal injection of OVA in nonimmunized animals did not result in accumulation of IgG or OVA (data not shown). At 24 hours and 3 days after OVA injection, colocalization of IgG and OVA was found in the inner plexiform layer, ganglion cell layer (GCL), inner and outer photoreceptor segment layers, and subretinal space (SR), herein defined as the space directly adjacent to the RPE. At 7

days after injection, the extent of IgG and OVA colocalization in the GCL, inner and outer photoreceptor segment layers, and SR was reduced, and at 14 days IgG and OVA colocalization was no longer detectable in the retina (Fig. 1B), suggesting clearance of immune complexes.

Immune Complex Formation Leads to Neuroinflammation

Microglia are the tissue-resident macrophages of the retina, and they rapidly respond to disturbances of homeostasis and tissue injury.¹⁷ To investigate whether the presence of immune complexes induced changes in microglia phenotype and/or led to the recruitment of leukocytes from the circulation, immunohistochemistry for the myeloid and general leukocyte markers CD11b and CD45, respectively, was performed (Fig. 2). Microglia cells were identified based on their expression of CD11b and morphology. In the saline-treated controls, microglia expressed low levels of CD11b and were present in the inner plexiform layer and outer plexiform layer (Fig. 2a). Typical resting resident microglia have a ramified morphology characterized by a small cell body and the presence of long thin processes radiating from the cell body.¹⁷ In the saline-treated mice, microglia appeared to have a resting phenotype because some processes were observed (Fig. 2a, inset). Following activation, microglia retract their processes and appear to have a larger cell body and shorter or no processes, acquiring an “amoeboid” morphology.¹⁷ From 24 hours up to 7 days after intravitreal challenge with OVA, CD11b+ microglia in the inner plexiform layer and GCL appeared to have an amoeboid morphology, with less visible processes. CD11b+ cells with a round morphology, characteristic of recruited leukocytes, were found in the GCL. In addition, amoeboid CD11b+ cells appear in the SR 24 hours and 3 days after injection (Fig. 2a). Quantification revealed an increase in total CD11b+ cells per millimeter of retina ($F_{1,6} = 107.3$, $P < 0.0001$), peaking at 3 days after OVA injection with a 2.71-fold increase in total CD11b+ cell numbers compared with saline-injected controls ($P < 0.0001$) (Fig. 2c). CD11b+ cells per millimeter of retina were increased in the SR ($F_{1,6} = 29.95$, $P = 0.0016$) and inner plexiform layer and GCL ($F_{1,6} = 34.95$, $P = 0.001$) following OVA challenge (Fig. 2c). The number of CD11b+ cells with round morphology was increased following OVA challenge ($F_{1,6} = 221.8$, $P < 0.0001$) and peaked 3 days after injection at 27.52 cells per millimeter of retina ($P < 0.0002$) (Fig. 2c). At 14 days after injection, microglia were only seen in the plexiform layers with similar morphology to the saline-treated controls (Fig. 2a, insets). No CD45+ cells were detectable in saline-injected controls (Fig. 2b). At 24 hours following intravitreal injection of OVA, CD45+ cells with a round morphology were observed exclusively in the GCL. From 3 days through 7 days, CD45+ cells with both a microglia-like and round morphology were observed in the inner plexiform layer and GCL (Fig. 2b). Quantification revealed a significant increase of total CD45+ cells per millimeter of retina ($F_{1,6} = 125.9$, $P < 0.0001$) and CD45+ cells per millimeter of retina in the SR ($F_{1,6} = 17.87$, $P = 0.0055$) and inner plexiform layer and GCL ($F_{1,6} = 84.10$, $P < 0.0001$) (Fig. 2d). Moreover, the number of CD45+ cells with a round morphology was also increased ($F_{1,6} = 81.58$, $P = 0.001$), peaking at 7 days at 26.74 cells per millimeter of retina ($P = 0.0354$) (Fig. 2d). At 14 days following OVA challenge, no CD45+ cells were detectable in the retina (Fig. 2b). Eyes from nonimmunized mice injected with OVA were analyzed 3 days after injection. Immunoreactivity of CD11b or CD45 was similar in nonimmunized

mice and immunized mice injected with saline (data not shown).

Immune Complex Formation Leads to Increased Expression of FcγRs and MHC II

Resting microglia express low levels of the IgG receptors FcγRs¹⁸ and do not express detectable levels of MHC II.¹⁹ Microglia have been shown to upregulate FcγRs and MHC II in response to immune complex formation in the brain²⁰; therefore, we next investigated expression of these markers after intravitreal challenge with OVA. Major histocompatibility complex II expression was investigated as a valuable marker of M1 and M2b microglia activation. Cells expressing FcγRI were detected in saline controls but were increased 3 days and 7 days after intravitreal challenge with OVA (Fig. 3a). Very few cells expressing FcγRII/III (Fig. 3b) or FcγRIII (Fig. 3c) were detected in saline controls or 24 hours after OVA injection. The number of cells expressing FcγRII/III or FcγRIII was increased at 3 days and 7 days after intravitreal injection of OVA, and low levels of expression were still detectable after 14 days. Both FcγRIV (Fig. 3d) and MHC II (Fig. 3e) were undetectable in saline controls and 24 hours after injection but increased 3 days and 7 days following intravitreal injection of OVA. At 14 days after injection, no cells expressing these markers were detectable. Quantification of cell numbers revealed increased numbers of cells expressing these markers following intravitreal injection of OVA, with the following values: FcγRI ($F_{1,6} = 60.59$, $P = 0.0002$), FcγRII/III ($F_{1,6} = 47.35$, $P = 0.0005$), FcγRIII ($F_{1,6} = 6.28$, $P = 0.0461$), FcγRIV ($F_{1,6} = 151.3$, $P < 0.0001$), and MHC II ($F_{1,6} = 303.7$, $P < 0.0001$). Immunoreactivity for FcγRs and MHC II was low or undetectable in nonimmunized mice injected intravitreally with OVA (data not shown). These results suggest that immune complex formation leads to microglia activation with upregulated expression of FcγRs and MHC II.

Differential Role of Complement and Fcγ Receptors in the Immune Complex Response in the Retina

Initiation of immune complex-mediated inflammation in peripheral tissues and brain depends on the presence of FcγRs.^{20–22} To investigate the role of FcγRs and the complement system in our model, we induced immune complexes in the retina of $\gamma^{-/-}$ mice (lacking expression of the activating FcγRs FcγRI, FcγRIII, and FcγRIV) and $C1q^{-/-}$ mice and compared the immune complex-mediated inflammatory response with that of WT mice. Immunization with OVA resulted in a similar immune response with comparable levels of anti-OVA IgG1 and IgG2a in all genotypes (Fig. 4a). Furthermore, immune complexes formed abundantly in OVA-sensitized WT, $\gamma^{-/-}$, and $C1q^{-/-}$ mice following intravitreal injection of OVA (Fig. 4b). Changes observed in CD11b+ microglia in the WT mice were similar to those in $C1q^{-/-}$ mice, with increased total CD11b+ cells per millimeter of retina, recruitment of leukocytes, and changes in microglia morphology from ramified to amoeboid morphology. These changes in microglia activation and leukocyte recruitment were not observed in $\gamma^{-/-}$ mice (Fig. 5a). Wild-type mice challenged with OVA showed increased cell numbers expressing FcγRI (Fig. 5b), FcγRII/III (Fig. 5c), FcγRIII (Fig. 5d), FcγRIV (Fig. 5e), and MHC II (Fig. 5f); these changes were also observed in $C1q^{-/-}$ mice. As expected, $\gamma^{-/-}$ mice do not show any expression of FcγRI, FcγRIII, and FcγRIV. Cells positive for FcγRII/III were detected in $\gamma^{-/-}$ mice, likely due to expression of the inhibitory FcγRII.

FIGURE 2. Immune complex formation in the retina leads to activation of microglia and recruitment of leukocytes. (a) CD11b staining 24 hours (1 day) after saline injection and 1, 3, 7, and 14 days after OVA injection. In saline-injected controls, CD11b+ microglia with processes were present in the outer plexiform layer and inner plexiform layer. At 1, 3, and 7 days after intravitreal OVA challenge, microglia with amoeboid-like morphology (*insets*) were seen in the inner plexiform layer and GCL, and CD11b+ round cells (*arrows*) were seen in the GCL and vitreous. Subretinal CD11b+ amoeboid-like cells were observed at 1 day and 3 days after OVA challenge (*arrowheads*). At 14 days after injection, CD11b+ microglia returned to the plexiform layers and had more processes (*inset*), and no CD11b+ round cells were detected. (b) CD45 staining 24 hours (1 day) after saline injection and 1, 3, 7, and 14 days after OVA injection. No CD45+ cells are detected in control retinas. At 1, 3, and 7 days after intravitreal OVA challenge, CD45+ cells with round morphology (*arrows*) or with processes were observed up until 14 days, when almost no CD45+ cells were detectable. (c, d) Quantification of CD11b+ (c) or CD45+ (d) cell number per millimeter of the retina ($n = 4-6$ per group). Error bars denote SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Scale bars: 50 μm .

FIGURE 3. Immune complex formation in the retina results in increased expression of FcγRs and MHC II. (a–e) Immunohistochemistry for FcγRI (a), FcγRII/III (b), FcγRIII (c), FcγRIV (d), and MHC II (e) 24 hours (1 day) after saline injection and 1, 3, 7, and 14 days after OVA injection. Intravitreal challenge with OVA led to upregulation of all FcγRs and MHC II from 3 days to 7 days after OVA challenge. (f) Quantification of cell number per millimeter of the retina expressing each marker showed an increase in cell numbers expressing FcγRII/III, FcγRIII, and FcγRIV peaking at 3 days and FcγRI and MHC II peaking at 7 days ($n = 4-6$ per group). Error bars represent SEM. * $P < 0.05$, *** $P < 0.001$. Scale bars: 50 μm .

FIGURE 4. Immune complexes form in sensitized WT BALB/c, $\gamma^{-/-}$, and $C1q^{-/-}$ mice following intravitreal challenge with OVA. **(a)** Detection of anti-OVA circulating IgG1 and IgG2 antibodies by ELISA showed that sensitized WT ($n=8$), $\gamma^{-/-}$ ($n=4$), and $C1q^{-/-}$ ($n=4$) mice had similar titers of anti-OVA antibodies. **(b)** Immunohistochemical detection of OVA (red) and IgG (green) 3 days after intravitreal challenge with saline in sensitized WT and OVA in WT, $\gamma^{-/-}$, and $C1q^{-/-}$ mice ($n=5-6$). Colocalization of OVA (red) and IgG (green) showed immune complex formation throughout the retina in WT, $\gamma^{-/-}$, and $C1q^{-/-}$ BALB/c mice after OVA challenge (arrows). Scale bars: 50 μ m.

In addition, no expression of MHC II is observed in $\gamma^{-/-}$ mice following immune complex formation.

Deposition of IgG and C1q in Early AMD

The experiments described above demonstrate that immune complex formation in the retina can lead to activation of the retinal microglia and macrophages via Fc γ Rs. To evaluate the possible role of immune complexes in AMD, we analyzed IgG, C1q, and MAC in eyes from healthy donors and patients with early AMD (Fig. 6). Double-labeling for IgG and the endothelial cell marker *U. europaeus* agglutinin I showed that IgG was present in healthy eyes around the choriocapillaris (Fig. 6a) and within the retinal blood vessels (data not shown). In early AMD, IgG staining was similarly present around the choriocapillaris (Fig. 6a) and within the retinal blood vessels (data not shown). Immunohistochemical detection of C1q revealed deposition within the choriocapillaris in early AMD eyes but not in the retina (data not shown) or eyes from age-matched controls (Fig. 6b). To investigate whether IgG and C1q deposition can lead to activation of the complement cascade, double-labeling for IgG and the terminal complement cascade component MAC was performed. MAC deposition was observed in areas surrounding the choriocapillaris in healthy donor eyes. In early AMD, MAC deposition was increased around the choriocapillaris and colocalized with IgG staining (Fig. 6c). No MAC deposition was observed within the retinal layers of healthy or early AMD donors (data not shown). MAC was also detected throughout drusen, while IgG immunoreactivity was only observed on its periphery (Fig. 6c). Areas of stronger-intensity IgG stain in drusen colocalized with areas of more intense stain for MAC (Fig. 6c, inset). No C1q deposits were observed in drusen (Fig. 6b, inset).

Increased Numbers of CD45+/Fc γ RIIb+ and CD45+/Fc γ RIIa+ Leukocytes in Early AMD

To investigate whether the IgG deposits in early AMD were associated with an inflammatory response, we examined expression of CD45 and Fc γ Rs. CD45+ cells were detected in the choroid and retinas of age-matched controls and early AMD donor eyes (Fig. 7). We failed to detect Fc γ RI in the choroid and Fc γ RIII in any of the samples analyzed, possibly due to saturation of the Fc γ RI with monomeric IgG¹³ and/or masking of the epitope by fixation of the tissue. In contrast,

staining for Fc γ RIIb (Fig. 7a) or Fc γ RIIa (Fig. 7b) revealed expression of both these Fc γ Rs in the choroid of patients with early AMD and age-matched controls. To correct for the differential number of leukocytes, we analyzed the number of CD45 and Fc γ RIIb or Fc γ RIIa double-positive cells as a fraction of the total CD45-expressing cells per section (Fig. 7c). An increase in the percentage of CD45+/Fc γ RIIb+ cells (from 39% to 62%, $P=0.0381$) and CD45+/Fc γ RIIa+ cells (from 41% to 71%, $P=0.0037$) was observed in the choroid of early AMD donors. CD45+/Fc γ RIIb+ and CD45+/Fc γ RIIa+ cells were also detected in healthy and early AMD retinas, but no differences were found between the two groups (data not shown). Finally, we investigated the expression of CD45, Fc γ RIIb, and Fc γ RIIa in eyes from patients with wet AMD (Fig. 8). CD45, Fc γ RIIb (Fig. 8a), and Fc γ RIIa (Fig. 8b) expression was extensively present throughout the choroid and retina in the late stage of AMD.

DISCUSSION

Inflammation is widely accepted as an important component in the pathogenesis of AMD.²³ Both clinical and experimental data support the concept that immune complex-mediated inflammation may have a role. In the present study, we have developed a mouse model to investigate the mechanisms underlying immune complex inflammation in the retina, and in this model immune complexes form throughout all retinal layers, resulting in a robust local inflammatory response characterized by activation of microglia, recruitment of leukocytes, and upregulation of Fc γ Rs and MHC II. This inflammatory response is dependent on the presence of activating Fc γ Rs. Immunohistochemical analysis of early AMD donor eyes revealed the presence of IgG, C1q, and MAC deposits and increased expression of Fc γ RIIa and Fc γ RIIb compared with healthy control eyes. Based on these observations, we hypothesize that immune complex inflammation and interaction with Fc γ R-expressing effector cells may have a role in the pathogenesis of AMD.

Immune Complex-Mediated Inflammation in the Retina

It has been reported that from early stages of the disease patients with AMD have high titers of circulating autoantibod-

FIGURE 5. Immune complex-mediated inflammation is dependent on the presence of activating Fc γ Rs. (a–f) Immunohistochemistry for CD11b (a), Fc γ RI (b), Fc γ RII/III (c), Fc γ RIII (d), Fc γ RIV (e), and MHC II (f) 3 days after intravitreal injection of saline in sensitized WT mice and injection of OVA in sensitized WT, $\gamma^{-/-}$, and C1q $^{-/-}$ mice. OVA-immunized mice injected with saline intravitreally showed CD11b+ microglia (a) with a ramified morphology with low expression of Fc γ Rs (b–e) or MHC II (f). Following intravitreal challenge with OVA, an inflammatory response was induced as evidenced by changes in morphology and numbers of CD11b+ microglia (a) and increased expression of Fc γ Rs and MHC II (b–f). These changes were not observed in $\gamma^{-/-}$ mice but occurred in C1q $^{-/-}$ mice. (g) Quantification of cell number per millimeter of the retina expressing each marker in WT, Fc γ R $^{-/-}$, and C1q $^{-/-}$ mice ($n = 5-6$). Error bars denote SEM; * $P < 0.05$, *** $P < 0.001$. Scale bars: 50 μ m.

FIGURE 6. Deposition of IgG and C1q and complement activation are observed in the choriocapillaris of patients with early AMD. (a) Immunohistochemical detection of IgG (green) and *U. europaeus* agglutinin I (red) ($n = 9$ –11 per group) revealed IgG deposition in close proximity to choriocapillaris in both control and early AMD eyes. (b) Immunohistochemical detection of IgG (green) and C1q (red) ($n = 9$ per group) in aged-matched controls and patients with early AMD showed colocalization of IgG and C1q (arrows) in blood vessel lumens, indicating formation of immune complexes but not in drusen (star). (c) Immunohistochemical detection of IgG (green) and MAC (C5b-9, red) in aged-matched controls and patients with early AMD ($n = 9$ per group) revealed MAC and IgG colocalization around the choriocapillaris (arrowheads) and in drusen (star) in early AMD (inset). Note the intense autofluorescence of the RPE due to lipofuscin autofluorescence. CC, choriocapillaris. Scale bars: 50 μ m and 25 μ m (inset).

FIGURE 7. Increased expression of FcγRIIb and FcγRIIa by leukocytes in the choroid is associated with early AMD. (a, b) Immunohistochemical detection of CD45 (red) and FcγRIIb (a) and FcγRIIa (b). Increased numbers of both FcγRIIb+ and FcγRIIa+ cells were observed in the choroids of patients with early AMD compared with healthy age-matched controls. (c) Quantification of FcγRIIb or FcγRIIa cell numbers per 100 μ m of the Bruch's membrane and percentage of CD45 and FcγRIIb or FcγRIIa double-positive cells ($n = 8$ –11 per group). Cells were counted only within 25 μ m of the retina. Error bars denote SEM. * $P = 0.0381$, ** $P = 0.0037$. Scale bars: 50 μ m.

FIGURE 8. FcγRIIb and FcγRIIa are expressed by CD45+ cells in wet AMD choroid and retina. (a, b) Immunohistochemical detection of CD45 (red) and FcγRIIb (green, [a]) or FcγRIIa (green, [b]) in the choroid and retina of eyes from donors with wet AMD ($n = 5$). CNVM, choroidal neovascular membrane; NFL, nerve fiber layer; OPL, outer plexiform layer. Scale bars: 50 μ m and 25 μ m (inset).

ies against retinal antigens.⁸ Most notably, these include antibodies against glial fibrillary acidic protein,²⁴ phospholipids,⁹ and a product of lipid oxidation omega-(2-carboxyethyl) pyrrole.²⁵ However, other retinal targets have been identified.^{8,10} The presence of specific autoantibody signatures in dry and wet AMD in humans⁸ and cynomolgus monkeys with AMD-like pathology²⁶ suggests a relationship between these antibodies and the development of the disease.

The biological effects of immune complex deposition have been well described in nonneuronal tissue, resulting in an inflammatory response characterized by edema, hemorrhage, neutrophil infiltration, and tissue injury.²⁷ A number of investigations have shown that immune complex formation in the anterior ocular chamber leads to increased permeability of the blood-aqueous barrier, but responses in the retina were not studied.²⁸ We have investigated the biological effect of immune complex deposition in the retina and show that immune complex formation in the murine retina results in a robust inflammatory response.

In the brain parenchyma, antibody-mediated inflammation is delayed compared with that in peripheral organs, and no neutrophil infiltration is observed.²⁰ Similarly, immune complex formation in the retina leads to a delayed inflammatory response that peaks at 3 days after antigen challenge and is characterized by activation and migration of microglia/macrophages to the sites of immune complex formation. In resting conditions, microglia have a CD11b^{low}/CD45^{low} phenotype and a ramified morphology and can be distinguished from peripheral myeloid cells based on their CD45 expression.²⁹ Microglia activation and macrophage recruitment from the periphery have been associated with human AMD,^{15,30} as well as with several mouse models of AMD, including the CX3CR1^{-/-} mouse³¹ and laser-induced choroidal neovascularization models.³² Accordingly, in our model 3 days and 7 days after OVA challenge, we show activation of microglia and an increase in the numbers of CD45^{high} cells with a round morphology. Immune complex formation in the retina results in activation of the tissue-resident macrophages, the microglia, as well as recruitment of myeloid cells. We propose that immune complex deposition could contribute to

microglia activation and recruitment of macrophages observed in AMD.

The Role of FcγRs and Complement in Immune Complex-Mediated Inflammation

It is known that activating FcγRs are essential for initiation of immune complex inflammation in nonneural organs because deletion of activating FcγRs (but not the complement proteins C1q, C3, and C4) attenuates the inflammatory response.^{21,22,33} We previously showed that the inflammatory response to immune complex formation in the brain parenchyma similarly depends on FcγR expression.²⁰ Herein, we demonstrate that antibody-mediated inflammation in the retina results in microglia activation and increased numbers of cells expressing CD11b, CD45, FcγRI through FcγRIV, and MHC II. This response was attenuated in $\gamma^{-/-}$ mice but not in $C1q^{-/-}$ mice. The studies performed in our experimental model highlight the importance of FcγRs in mediating antibody responses in the retina.

Fcγ Receptors and AMD

Deposition of IgG has been reported in drusen and in the RPE of patients with AMD.¹¹ We observed an apparent increase in IgG deposition in the lumens of the choriocapillaris in early AMD accompanied by deposition of C1q and MAC, indicating immune complex formation and activation of the complement system. Thinning of the choroid has been demonstrated in aging,³⁴ but it remains controversial whether this is more pronounced in AMD. The predominant view is that RPE cell death leads to removal of trophic factors required for survival of the choroidal blood vessels and hence precedes any changes to the choriocapillaris.³⁵ In contrast, Mullins et al.¹⁶ have shown that loss of endothelial cells in the choriocapillaris is associated with early AMD, before any RPE cell loss is observed. It has also been reported that thinning of the choroid precedes RPE degeneration in late-stage wet AMD,³⁶ indicating that degeneration of the choriocapillaris may occur before any RPE damage. In support of this idea, we found evidence for immune complex formation in the choriocapillaris but not the retina of early AMD donor eyes. We suggest that immune complex inflammation could lead to damage to the choriocapillaris in early AMD and consequent impaired drainage of debris from the retina, contributing to the formation of drusen. Supporting this hypothesis are reports of patients with vasculitis due to systemic immune complex disease such as systemic lupus erythematosus^{37,38} and membranoproliferative glomerulonephritis types I³⁹ and II^{11,40} developing drusen and RPE atrophy.

Immune complex formation can lead to inflammation through activation of effector cells via FcγR cross-linking or through activation of the classic complement cascade.¹³ Genetic studies^{41,42} have put forward overactivation of the complement cascade as an important factor in AMD pathogenesis. In addition, histochemical analysis of AMD donor eyes has shown that deposition of complement proteins such as C3, C5, and MAC is associated with AMD from early through late stages of the disease.^{43,44} We show an increase in C1q and MAC deposition in early AMD, suggesting that the classic complement cascade, via immune complex formation, could also contribute to AMD by amplifying the activation of the complement cascade. In contrast, deleting C1q in our mouse model had no effect on the inflammatory response. It has been proposed that cross-linking of FcγRs in mice can lead to secretion of C5a by effector cells; this could bypass the need for C1q to activate the complement cascade in the $C1q^{-/-}$ mouse.⁴⁵ It is also possible that the disparity between our

mouse model and the human tissue could be explained by species differences.

Immune complex formation can further contribute to inflammation via cross-linking of FcγRs, resulting in generation of proinflammatory mediators such as nitric oxide (NO), TNF- α , IL-6, and CCL2 and upregulation of MHC II.¹³ The ratio of activating versus inhibitory FcγR expression (A-I ratio) determines cellular phenotype following cross-linking with immune complexes, with an increasing A-I ratio favoring cell activation. We show increased numbers of cells expressing all four murine FcγRs following immune complex formation, including the inhibitory receptor FcγRII. However, increased expression of the activating receptors FcγRI, FcγRIII, and Fc receptor IV likely increases the A-I ratio, favoring cell activation. In early AMD donor tissue, we detected increased numbers of CD45+ cells expressing FcγRIIIa and FcγRIIb in the choroid. The human inhibitory FcγRIIb is the only FcγR expressed by B cells and can be expressed by some myeloid cells, including macrophages. This receptor negatively regulates human activating FcγRs. FcγRIIIa is expressed on all myeloid cells but not lymphocytes. Upon cross-linking by complexed IgG, this receptor is a potent activator of effector function, and it has been shown to be essential for a variety of antibody-mediated pathologies, including arthritis and anaphylaxis.⁴⁶ Activation of FcγRIIIa at the choroid could lead to immune activation and increased proinflammatory cytokine production, potentially damaging the blood vessels of the choroid and/or the RPE. Although in our experimental model immune complex formation was mainly observed in the retina (Fig. 1) rather than in the choroid, our results provide proof of principle that immune complex formation in the posterior chamber of the eye results in a local inflammatory response that critically depends on the interaction of effector cells expressing FcγRs. Our data suggest that immune complexes may contribute to AMD pathogenesis by activating cell effector function via both complement activation and FcγR interaction.

Implications for Therapy

Monoclonal antibodies against VEGF are currently used for the treatment of wet AMD, and two antibodies are applied in the clinic, a full-length human IgG1 (bevacizumab) and a Fab fragment (ranibizumab).^{47,48} Although the efficacy of these two agents appears similar, different adverse effect profiles have been reported.^{49,50} We show that FcγRIIIa is extensively present in the retina of wet AMD. Cross-linking of this receptor by bevacizumab (but not ranibizumab, which lacks the Fc fragment) could trigger inflammation⁵¹ and impact the efficacy and safety of therapy. An understanding of antibody interactions with FcγRs in the retina is increasingly important as new therapies emerge for the treatment of choroidal neovascularization such as aflibercept, which contains an Fc portion.⁵²

CONCLUSIONS

We have developed an experimental model showing that immune complexes produce robust neuroinflammation in the retina, largely dependent on the presence of FcγRs. We found evidence for the formation of immune complexes in the choriocapillaris of early AMD with accompanying increased numbers of FcγR+ cells compared with controls, implicating a possible role for antibody-mediated inflammation in AMD. Finally, we observed large numbers of leukocytes expressing FcγRs in the retinas from patients with wet AMD. While these observations provide evidence for a possible role of IgG immune complex formation and associated inflammatory response in the pathogenesis of AMD, further studies are

required to determine the factors that initiate these events. A better understanding of immune complex inflammation in the eye may elucidate some of the mechanisms involved in AMD pathogenesis and, most important, allow for the development of improved therapies.

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