AUTOANTIBODY SIGNATURE FOR AGE-RELATED MACULAR DEGENERATION

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Abstract—Recent findings indicate that immunologic factors, in particular autoantibodies, are involved in the pathogenesis of age-related macular degeneration (AMD). To reveal an autoantibody profile for AMD and identify biomarkers for progression of this disease, we performed an antigen microarray printed with 66 kinds of AMD-associated molecules using serum samples from patients with dry and wet AMD and from age-matched healthy controls. Sera from the AMD groups contained significantly higher levels of IgG and IgM antibodies to 32 ocular antigens and immune molecules, respectively, than sera from the control group and demonstrated the highest IgG reactivity to Helicobacter pylori antigen and highest IgM reactivity to complement C3. Anti–complement C4 IgG (odds ratio, OR = 16.4) and anti-cytomegalovirus IgM (OR = 13.0) were best correlated with the development of dry AMD, and anti–apolipoprotein E IgG (OR = 14.5) and anti–complement factor H IgM (OR = 6.0) were the most reliable biomarkers for progression from dry to wet AMD. Moreover, IgGs purified from sera of patients with AMD contained high reactivity to glutamine synthetase (GS) and inhibited GS activity in a dose-dependent manner. Ocular expression of GS decreased with age in mice, suggesting that the accumulation of glutamate, which has strong neurotoxicity, contributes to retinal degeneration. Our data demonstrate that patient seroreactivities to specific ocular antigens might be used as biomarkers for AMD and that anti-GS IgG could be associated with the pathogenesis of AMD.

Keywords— age-related macular degeneration; anti-retinal antibody; autoantibody; autoimmunity; biomarker; glutamine synthetase; proteomics

1. Introduction

Age-related macular degeneration (AMD) is currently the leading cause of irreversible vision loss in the United States and in other developed countries. It is estimated that AMD will affect almost 3.75 million people worldwide by the year 2020 (Friedman et al. 2004). AMD involves progressive damage to the retina and is classified as either "dry" (atrophic) or "wet" (exudative). The characteristic early findings in dry AMD are drusen, which are yellow, lipid-filled deposits between the retinal pigment epithelium and Bruch's membrane in the macular region. At this early stage, patients usually have no visual disturbance, but retinal atrophy caused by loss of retinal pigment epithelial cells gradually develops and eventually results in loss of central vision. This late stage of dry AMD is called geographic atrophy. Wet AMD is characterized by choroidal neovascularization (CNV), in which fragile, abnormal vessels develop under the retina. These vessels may leak fluid or blood, damaging the neuroretina and/or retinal pigment epithelium and causing rapid damage to the macula. Untreated wet AMD can cause blindness within months; vision loss with dry AMD generally occurs over years (de Jong 2006). The treatments of late phase, wet AMD are primarily aimed at stopping the growth of new blood vessels through anti-angiogenic (anti-VEGF) therapies, but these medications must be continuously administered to patients, and patient compliance can be challenging due to the nature of their administration (intravitreal injection) and cost. Since the early, dry form of AMD can progress to the wet form, it is critical to diagnose the disease early in its development. Simple, accurate tools to identify patients likely to progress to advanced stages of AMD could greatly improve patient care and outcomes, and a further understanding of immune contributions to AMD pathogenesis is needed. In this study, we seek to develop a novel biomarker technique for diagnosis and prognosis of AMD and clarify the pathogenic mechanisms for the disorder.

Risk factors for AMD include advanced age, cigarette smoking, obesity, poor diet and a variety of genetic variants (Lim et al. 2012). The immune system has been strongly implicated in the pathogenesis of AMD (Morohoshi et al. 2009, Camelo 2014, Nussenblatt et al. 2014), and autoimmunity in particular may play a critical role. A large number of autoantibodies that target retinal antigens have been identified, and the complement system, which plays an important role in local inflammation following an antigen-antibody reaction, is activated in patients with AMD (Morohoshi et al. 2009, Camelo 2014, Bora et al. 2015). Whether this autoimmunity directly causes the pathogenesis associated with AMD progression or is a side-effect of retinal damage remains unclear, but anti-retinal autoantibodies can clearly lead to inflammatory damage associated with AMD progression (Camelo 2014), and autoantibody biomarkers may provide much-needed improvements in diagnosis and prognosis for AMD. Previously we used an antigen microarray to perform a comprehensive analysis of autoantibodies in the sera of patients with AMD. We identified numerous specific autoantibodies that were significantly elevated in the sera of AMD patients, and found that several were associated with specific stages of AMD or progression to late-stage disease. Anti-phosphatidylserine (PS) for example, best correlated with AMD development (Morohoshi, Patel, et al. 2012). We further demonstrated that autoantibodies might directly influence the pathogenesis of AMD (Morohoshi, Patel, et al. 2012). To characterize additional biomarkers for the disease and further explore disease

mechanisms, a new microarray was used to screen 66 antigens. These antigens included ocular proteins expressed in the retina and choroid, constituents of drusen, immune molecules such as complement factors, and infectious disease antigens. The pathogenic role of an antibody associated with disease progression was also investigated.

2. Experimental Section

This study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of King's College Hospital (Protocol No. 02-156). All subjects gave their informed consent for inclusion before they participated in the study. The animal protocols used in this work were evaluated and approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University (IACUC ID: 273-2008).

2.1. Serum samples and IgG purification

AMD patients were recruited from the Kings College Hospital, London, UK, and age- and sex-matched normal patients were selected from cataract clinics with local ethics committee approval after obtaining written informed consent. Serum samples were stored at -80°C and were defrosted to room temperature for use in experiments. IgG purification was performed with protein G-Sepharose affinity chromatography (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) according to the manufacturer's instructions. Purity of the filtered IgG was checked by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and concentration of IgGs was measured by enzyme-linked immunosorbent assay (ELISA) (Bethyl Laboratories, Inc., Montgomery, TX, USA) according to the manufacturer's instructions.

2.2. Antigen microarray assay

The antigen microarrays were performed by the University of Texas Southwestern Medical Center Microarray Core Facility as previously described (Li et al. 2007, Morohoshi, Patel, et al. 2012). The 66 antigens used for this assay included ocular proteins expressed in the retina and choroid, constituents of drusen, immune molecules such as complement factors and infectious disease antigens (Table 1). The printing concentration for all antigens was optimized at 1 g/ml. Briefly, antigens diluted in phosphate-buffered saline (PBS) were robotically printed in duplicate and distributed randomly on nitrocellulose-coated 16-pad FASTTM slides (Whatman Schleicher & Scheleicher BioScience, Keene, NH, USA). The slides were incubated for 60 min with serum samples diluted 1:200 with blocking buffer (1% bovine serum albumin, BSA, in PBS) and washed three times for 5 min with washing buffer (PBS with 0.05% v/v Tween 20, pH 7.4). Cy3-labelled anti-human IgG and Cy5-labelled anti-human IgM secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at 1:500 dilution were applied to detect binding of serum autoantibodies to specific antigens and were incubated with the autoantibodies for 60 min at room temperature. The arrays were washed as before and scanned using the Genepix 4000B scanner to generate TIFF images for analysis.

Table 1. Antigens used for the microarray analysis

Antigens	Description	Classification	Company
AGE-BSA	Advanced glycation endproduct-BSA	Advanced glycation endproducts	BioVision, Inc.
CEL-BSA	Ne-(Carboxyethyl) lysine-BSA	Advanced glycation endproducts	CycLex Co.,Ltd.
CML-BSA	Ne-(Carboxymethyl) lysine-BSA	Advanced glycation endproducts	CycLex Co.,Ltd.
Apo B	Apolipoprotein B	Apolipoproteins	EMD Chemicals Inc.
Apo E	Apolipoprotein E	Apolipoproteins	EMD Chemicals Inc.
Apo E4	Apolipoprotein E4	Apolipoproteins	Peprotech, Inc.
Apo J	Apolipoprotein J	Apolipoproteins	AbD Serotec
Annexin V	Annexin V	Apoptosis	Sigma-Aldrich, Co.
Annexin II	Annexin II	Calcium Binding Proteins	Sigma-Aldrich, Co.
Calreticulin	Calreticulin	Calcium Binding Proteins	Abcam, Inc.
Calmodulin	Calmodulin from bovine brain	Calcium Binding Proteins	Sigma-Aldrich, Co.
Factor X	Factor X (Cal)	Coagulation Factors	EMD Chemicals Inc.
CFB	Complement factor B	Complements	EMD Chemicals Inc.
CFH	Complement factor H	Complements	EMD Chemicals Inc.
Complement C3	Complement C3	Complements	EMD Chemicals Inc.
Complement C4	Complement C4	Complements	EMD Chemicals Inc.
Complement C5	Complement C5	Complements	EMD Chemicals Inc.
Complement C8	Complement C8	Complements	EMD Chemicals Inc.
Complement C9	Complement C9	Complements	EMD Chemicals Inc.
beta-Actin	beta-Actin	Extracellular Matrix and Structural Proteins	AbD Serotec
Collagen V	Collagen V	Extracellular Matrix and Structural Proteins	Abcam, Inc.
Collagen VI	Collagen VI	Extracellular Matrix and Structural Proteins	Abcam, Inc.
Decorin	Decorin	Extracellular Matrix and Structural Proteins	R&D Systems, Inc.
Dermatan Sulfate	Dermatan Sulfate	Extracellular Matrix and Structural Proteins	Celsus Laboratories, Inc.
Tubulin alpha	Tubulin alpha	Extracellular Matrix and Structural Proteins	Abcam, Inc.
DHA	Cis-4,7,10,13,16,19-Docosahexaenoi acid	Fatty acid	Sigma-Aldrich, Co.
alpha-1 Antichymotrypsin	alpha-1 Antichymotrypsin	Globulins	EMD Chemicals Inc.
alpha-1 Microglobulin	alpha-1 Microglobulin	Globulins	Cortex Biochem, Inc.
NSE	Neuron-specific enolase	Growth Factors	EMD Chemicals Inc.
PEDF	Pigment epithelium-derived Factor	Growth Factors	Peprotech, Inc.
VEGF	Vascular endothelial growth factor	Growth Factors	Bioclone, Inc.
Hsp 60	Heat Shock Protein 60	Heat Shock Proteins	EMD Chemicals Inc.
Hsp 70	Heat Shock Protein 70	Heat Shock Proteins	Medical & Biological Laboratories, Co., Ltd.
C. pneumoniae	Chlamydia. pneumoniae (TWAR Strain)	Infections	Meridian Life Science, Inc.
CMV	Cytomegalovirus pp150 (UL32), (AD169 Strain)	Infections	Meridian Life Science, Inc.
H. pylori	Helicobacter pylori (Strain 43504)	Infections	Meridian Life Science, Inc.
beta-Lactoqlobulin A	beta-Lactoqlobulin A	Metabolism	Sigma-Aldrich, Co.
CK-BB	Creatine Kinase BB	Metabolism	Meridian Life Science, Inc.
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Metabolism	AbD Serotec
LDH	Lactate dehydrogenas	Metabolism	ProSpec-Tany TechnoGene, Ltd.
PGI	Phosphoglucose Isomerase	Metabolism	Sigma-Aldrich, Co.
SOD1	Superoxide dismutase 1	Metabolism	Abnova, Co.
Triosephosphate Isomerase	Triosephosphate Isomerase	Metabolism	Sigma-Aldrich, Co.
Amyloid beta	Amyloid beta	Neurodegeneration	AbD Serotec, Inc.
Glutamine synthetase	L-Glutamine synthetase	Neurodegeneration	Sigma-Aldrich, Co.
ARPE-19 lysate	ARPE-19 cell lysate	Ocular Proteins	American Type Culture Collection
IRBP	interphotoreceptor retinoid-binding peptide (1-20)	Ocular Proteins	AnaSpec, Inc.
RBP	Retinol binding protein	Ocular Proteins	AbD Serotec
Recoverin	Recoverin	Ocular Proteins	ATGen, Co., Ltd.
Retinal lysate (human)	Human retinal tissue lysate	Ocular Proteins	ProSci, Inc.
Retinal lysate (mouse)	C57BL/6 mouse retinal tissue lysate	Ocular Proteins	ProSci, Inc.
Rhodopsin	Rhodopsin	Ocular Proteins	EMD Chemicals Inc.
RPE	Human retinal pigmental epithelial cell lysate	Ocular Proteins	Sciencell Research Laboratories
Whole eye lysate (human)	Human whole eye tissue lysate	Ocular Proteins	Sciencell Research Laboratories
alpha-1 Antitrypsin	alpha-1 Antitrypsin	Serum and Plasma Proteins	EMD Chemicals Inc.
Amyloid P	Amyloid P	Serum and Plasma Proteins	AbD Serotec
BSA	Bovine Serum Albumin	Serum and Plasma Proteins	BioVision, Inc.
Ceruloplasmin	Ceruloplasmin	Serum and Plasma Proteins	EMD Chemicals Inc.
CRP	C-reactive protein	Serum and Plasma Proteins	AbD Serotec
Haptoglobin	Haptoglobin	Serum and Plasma Proteins	AbD Serotec
HbA1c	Hemoglobin A1c	Serum and Plasma Proteins	Exocell, Inc.
HbA2	Hemoglobin A2	Serum and Plasma Proteins	Sigma-Aldrich, Co.
Prealbumin	Prealbumin	Serum and Plasma Proteins	EMD Chemicals Inc.
Serum Albumin	Serum Albumin	Serum and Plasma Proteins	ProSpec-Tany TechnoGene, Ltd.
Transferrin	Transferrin	Serum and Plasma Proteins	AbD Serotec
Ubiquitin	Ubiquitin	Transcription and Translation Related Proteins	ProSpec-Tany TechnoGene, Ltd.

2.3. Inhibition of glutamine synthetase activity

The assay used to measure glutamine synthetase activity was modified from a previously described method (Seiler, Reid, and Knodgen 1990). Ten l (0.6 units) of L-glutamine synthetase (EC 6.3.1.2, Sigma-Aldrich Co. St. Louis, MO, USA) was pre-incubated with or without 10 l of IgGs, purified from human sera and serially diluted (12.5 – 200 g/ml) for 30 min at 37 °C. The enzyme-serum mixture was then incubated for 15 min at 37 °C with 180 l of a reaction mixture containing 5 mM $MgCl_2$, 20 mM L-glutamate, 100 mM imidazole-HCL buffer (pH 7.4), 50 mM hydroxylamine-HCl and 10 mM ATP. All reactions were stopped with

600 l of an acidic ferric chloride solution containing 0.37 M $FeCl_3$, 0.67 M HCl and 0.2 M trichloroacetic acid. Absorbance of the γ-glutamylhydroxamate was measured at 535 nm.

2.4. Quantitative real-time RT-PCR

Posterior segments of C57BL6 mouse eyes were removed from cornea and iris and RNA was extracted using the AllPrep RNA/Protein Kit (Qiagen, Valencia, CA, USA). cDNA was synthesized with the QuantiTect Reverse Transcription Kit (Qiagen). Relative expression levels of RNAs were determined by using real-time PCR with the QuantiTect SYBR Green RT-PCR kit (Qiagen) and an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Primers corresponding GS, C3, CD45 and beta-actin were purchased from Qiagen. Relative RNA expression was determined by using the comparative method of relative quantification (2^{-Ct}) and normalized to beta-actin levels.

2.5. Western blotting

Protein was extracted from C57BL6 mouse eyes using the AllPrep RNA/Protein Kit (Qiagen). Each protein concentration was measured using the BCA Protein Assay Kit (Thermo Fisher Scientific, Inc., Waltham, MA USA) according to the manufacturer's instructions. Protein was separated using a 4-15 % polyacrylamide SDS gel and transferred to a PVDF membrane (Bio-Rad laboratories, Hercules, CA, USA). After blocking with 5% milk in TBS-Tween (0.1%) for 1 hour, the membrane was incubated overnight at 4 C with rabbit polyclonal anti-GS (Sigma-Aldrich Co.), anti-C3, anti-CD45 (Abcam, Cambridge, MA, USA), anti-mouse IgG or anti-beta-actin (Cell Signaling Technology, Inc., Danvers, MA, USA). The membrane was then incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Inc.) and protein expression was visualized using ECL Chemiluminescence Substrate (Thermo Fisher Scientific Inc.).

2.6. Tissue preparation and immunohistochemistry

Freshly enucleated eyes from 2-month- and 24-month-old C57BL6 mice were incubated with 30% sucrose in PBS overnight at 4°C, embedded in Sakura Tissue-Tek OCT Compound after fixation with 4% paraformaldehyde, and frozen on a liquid nitrogen-cooled duralumin plate. Frozen sections 8 μm thick were cut and air-dried for 30 min, then fixed with 30% methanol in 100% acetone for 10 min at 4°C for immunohistochemistry. Following fixation, young and old mouse eye sections were then blocked with 5% normal donkey serum in 1% BSA + PBS for 30 min at room temperature. The sections were first stained with mouse monoclonal anti-glutamine synthetase IgG (MAB 302, EMD Millipore, Billerica, MA, USA) or goat polyclonal anti-mouse IgG(H+L) (Jackson ImmunoResearch Laboratories, Inc.) overnight at 4 Cº, washed with PBS, and then incubated for 60 min with Alexa Fluor® 488-conjugated donkey anti-mouse or anti-goat IgG (Thermo Fisher Scientific, Inc). The sections were mounted with aqueous mounting medium containing propidium iodide (PI) for nuclear staining

(Vector Laboratories Inc., Burlingame, CA, USA). Finally, the sections were examined using fluorescent microscopy (Olympus America Inc., Center Valley, PA, USA).

2.7. Statistical analysis

Data are presented as mean ± SEM. Two-group comparisons were done using the unpaired Student t test, and for three-group comparisons the parametric one-way analysis of variance was used, followed by the Tukey multiple comparison test. All data were analyzed using GraphPad PRISM version 5.04 (Graphpad, San Diego, CA, USA).

3. Results and Discussion

3.1. Profile of autoantibodies to ocular antigens in patients with AMD

Antigen microarray analysis was performed with serum samples from 35 patients with dry AMD and 20 patients with wet AMD in addition to 20 age-matched, healthy controls to reveal the profile of autoantibodies to 66 molecules known to be strongly associated with the pathogenesis of AMD.

In sera from patients with dry AMD, IgGs to eight antigens, including complement proteins C3 and C4, collagens, lipoproteins and whole eye lysate, were elevated compared to normal controls. Sera from patients with wet AMD contained significantly higher IgG reactivities to 27 antigens compared to normal controls. These antigens included *Helicobacter pylori*, complement C9, C8, C3, apolipoprotein (Apo) E, glutamine synthetase (GS) and ocular molecules such as retinol binding protein (RBP) and rhodopsin. Compared to patients with dry AMD, the sera from patients with wet AMD reacted to a greater extent with 26 antigens, including carboxyethyl lysine (CEL)-BSA, mouse retinal tissue lysate, annexin II, amyloid P and, again, GS (Figure 1).

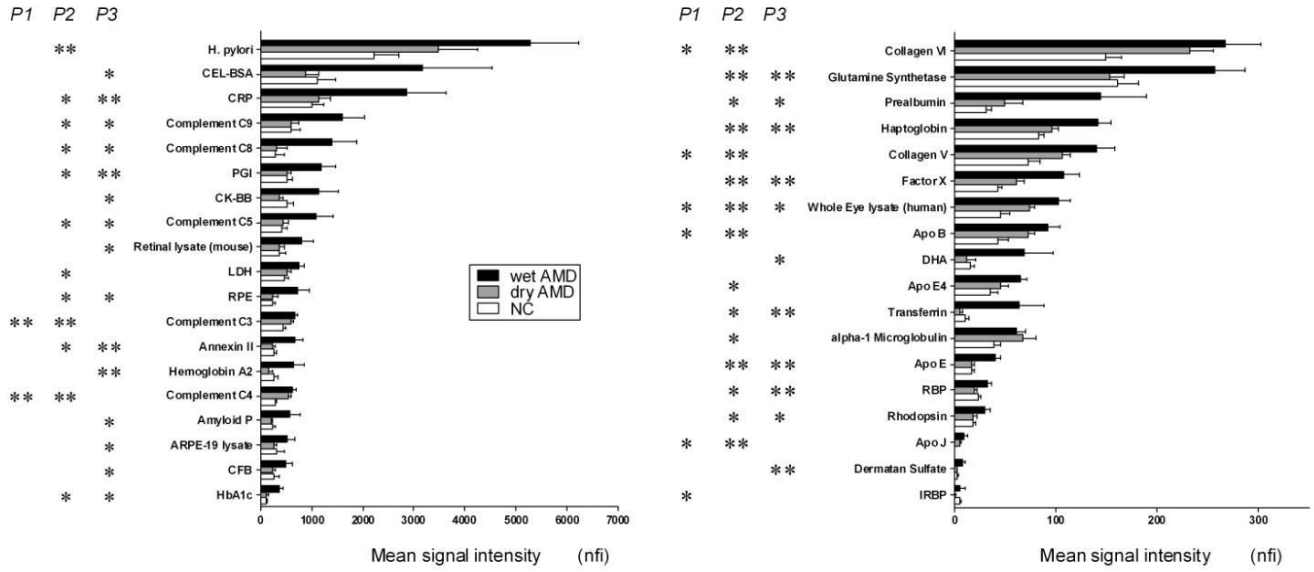

Figure 1. IgG autoantibodies with significantly higher reactivities in sera from AMD patients than in sera from normal controls.

Average seroreactivities for IgG autoantibodies were determined by antigen

microarray. NC (n = 20), dry AMD (n = 35) and wet AMD (n = 20). *P1*, NC vs dry AMD; *P2*, NC vs wet AMD; *P3*, dry AMD vs wet AMD. *, P < 0.05; **, P < 0.01

Likewise, IgM autoantibodies to 11 antigens were expressed at significantly higher levels in the sera of patients with dry AMD than in normal controls. The targeted antigens included cytomegalovirus (CMV), complements C9 and C5, annexin V and superoxide dismutase (SOD) 1. In sera from patients with wet AMD, 29 IgM antibodies were expressed at significantly higher levels compared to normal controls. These included antibodies to complements C3, C9 and C4; complement factors (CF) B; and H; and advanced glycation end product (AGE)-BSA. The autoantibodies increased in patients with wet AMD compared to those with dry AMD included complement C3, CFH, creatine kinase (CK)-BB, mouse and human retinal tissue lysate, retinal pigment epithelium (RPE) lysate, AGE-BSA and annexin II. No autoantibodies were expressed at significantly lower levels in AMD patients than in normal controls (Figure 2).

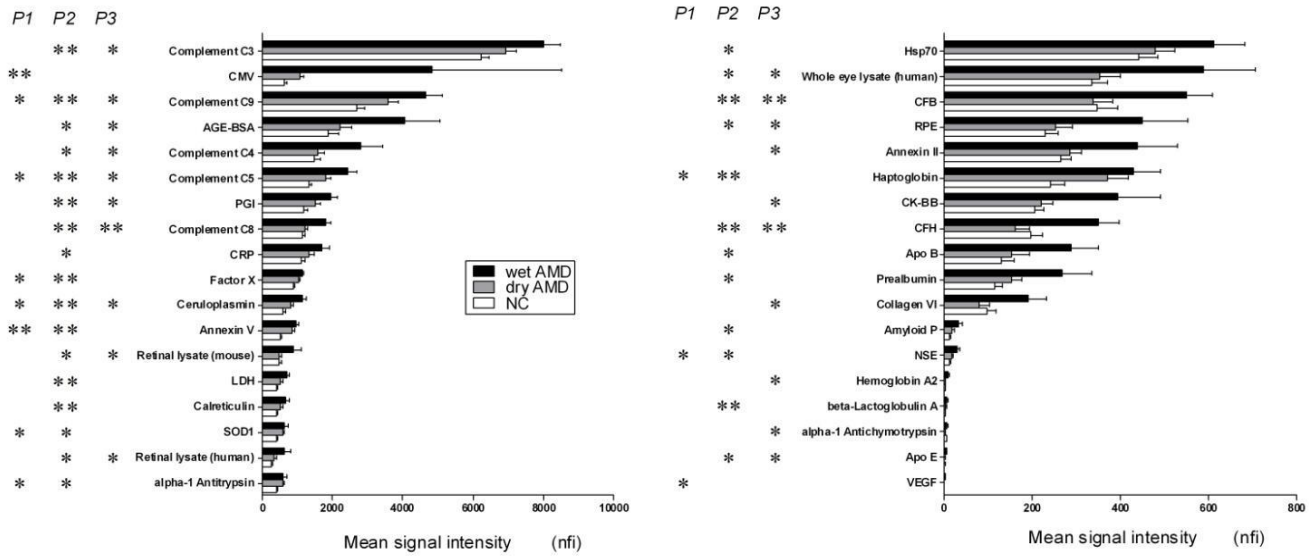

Figure 2. IgM autoantibodies with significantly higher reactivities in sera from AMD patients than in sera from normal controls.

Average seroreactivities for IgM autoantibodies were determined by antigen microarray. NC (n = 20), dry AMD (n = 35) and wet AMD (n = 20). *P1*, NC vs dry AMD; *P2*, NC vs wet AMD; *P3*, dry AMD vs wet AMD. *, P < 0.05; **, P < 0.01

In this study as in many others (Camelo 2014), a likely role for complement activation in AMD pathogenesis is suggested. Sera from AMD patients showed strong reactivities to a variety of complement factors. In other studies, SNPs in the genes C3 and CFH involved in complement activation were strongly associated with AMD (Scholl et al. 2008, Ristau et al. 2014). C3 activates the complement pathway whereas CFH serves

as a regulator of complement activation, and CFH-deficient mice tend to develop ocular features characteristic of AMD compared with wild type controls (Coffey et al. 2007). High expression of anti-CFH IgG might inhibit CFH and lead to complement activation and induction of inflammation. In the absence of CFH, levels of C3 and C4

would likely increase. Elevated expression of anti-C3 IgG and anti-C4 IgG might therefore serve as a protective response for regulating inflammation.

The complete profile of IgG and IgM autoantibody levels for each antigen is provided in Supplementary Table 1. In this table we list the average autoantibody levels and standard deviations for the sera from normal controls, patients with dry AMD and patients with wet AMD levels, in addition to the p values for the comparisons.

3.2 Identification of IgG autoantibody biomarkers for AMD

To identify the IgG biomarkers with the best capacity to predict the stage of AMD, receiver operating characteristic (ROC) curves were used (Table 2). Analysis of the area under the curve (AUC) demonstrated that the anti-complement C4 IgG predicted dry AMD with the greatest accuracy (AUC = 0.8650). IgG reactivity greater than 413.9 was set as the cut-off value, discriminating dry AMD from normal controls. Patients with IgG reactivity greater than 413.9 had a 16-fold greater risk of dry AMD compared to patients with an IgG below the cut-off value. For wet AMD, anti-C4 IgG was also the most accurate predictor (AUC=0.8286). Patients with an anti-C4 IgG greater than 505.5 were 44 times more likely to have wet AMD than those with an IgG less than the cut-off value. Patients in the dry AMD group whose anti-ApoE IgG reactivity was greater than the cut-off value had a 14.5-fold risk for developing wet AMD (AUC = 0.7943). GS, too, could be used to predict dry AMD progression to wet AMD. GS, an important enzyme mainly expressed in retinal Müller cells, catalyzes neurotoxic glutamate to less-toxic glutamine. Patients in the dry AMD group, with an anti-GS IgG reactivity greater than the cut-off value of 235.0, had a 9-fold risk of developing wet AMD (AUC = 0.7353). Normal controls with anti-GS IgG reactivity greater than 223.8 had a 4.5-fold risk of developing wet AMD (Table 2).

Table 2. Odds ratios for IgG autoantibody biomarkers

NC vs dry AMD	Cut-off value	AUC	Sensitivity (%)	Specificity (%)	Odds ratios	95% confidence interval	*P* value
Complement C4	413.9	0.8650	74.29	85.00	16.370	3.869 - 69.273	< 0.0001
Complement C3	386.3	0.7486	88.57	55.00	9.472	2.422 - 37.051	0.0005
Whole eye lysate (human)	74.4	0.6886	57.14	75.00	4.000	1.189 - 13.461	0.0213
Collagen VI	185.2	0.6743	54.29	75.00	3.563	1.061 - 11.959	0.0351
Apo B	132.4	0.6543	31.43	95.00	8.708	1.031 - 73.555	0.0224
NC vs wet AMD	**Cut-off value**	**AUC**	**Sensitivity (%)**	**Specificity (%)**	**Odds ratios**	**95% confidence interval**	***P* value**
Complement C4	505.5	0.8286	80.00	85.00	44.333	4.783 - 410.943	< 0.0001
Apo E	30.7	0.8063	75.00	90.00	27.000	4.566 - 159.663	< 0.0001
Haptoglobin	102.4	0.8350	80.00	80.00	16.000	3.398 - 75.345	0.0001
Factor X	62.1	0.8225	80.00	85.00	22.667	4.374 - 117.468	< 0.0001
Whole eye lysate (human)	81.9	0.8050	65.00	85.00	10.524	2.271 - 48.757	0.0012
Apo B	79.1	0.7663	70.00	75.00	7.000	1.739 - 28.174	0.0044
Complement C3	522.9	0.7575	80.00	80.00	16.000	3.398 - 75.345	0.0001
Collagen VI	239.4	0.7825	55.00	95.00	23.222	2.585 - 208.615	0.0006
Collagen V	120.5	0.7525	55.00	90.00	11.000	1.998 - 60.572	0.0024
Apo E4	51.2	0.7075	70.00	70.00	5.444	1.408 - 21.054	0.0114
H. pylori	3876.0	0.7000	60.00	80.00	6.000	1.458 - 24.686	0.0098
Glutamine Synthetase	223.8	0.6975	60.00	75.00	4.500	1.166 - 17.373	0.0252
Rhodopsin	28.4	0.6900	60.00	85.00	8.500	1.861 - 38.817	0.0033
alpha-1 Microglobulin	38.9	0.6900	75.00	65.00	5.571	1.42 - 21.86	0.0110
dry AMD vs wet AMD	**Cut-off value**	**AUC**	**Sensitivity (%)**	**Specificity (%)**	**Odds ratios**	**95% confidence interval**	***P* value**
Apo E	29.8	0.7943	75.00	82.86	14.500	3.795 - 55.407	< 0.0001
Haptoglobin	96.4	0.7571	85.00	65.71	10.861	2.647 - 44.573	0.0003
Dermatan Sulfate	2.8	0.7457	80.00	60.00	6.000	1.656 - 21.743	0.0042
Glutamine synthetase	235.0	0.7357	60.00	85.71	9.000	2.447 - 33.108	0.0004
RBP	35.1	0.7357	55.00	91.43	13.037	2.982 - 57.004	0.0001
Factor X	65.1	0.7386	80.00	74.29	11.556	3.049 - 43.793	0.0001
Rhodopsin	30.8	0.6900	55.00	80.00	4.889	1.459 - 16.381	0.0078
Complement C8	37.5	0.6814	55.00	88.57	9.472	2.422 - 37.051	0.0005
Annexin II	107.7	0.6729	40.00	94.29	11.000	2.041 - 59.286	0.0015
Hemoglobin A2	843.3	0.6621	35.00	97.14	18.308	2.048 - 163.681	0.0011

NC, normal controls; AMD, age-related macular degeneraion; AUC, the area under the ROC curve; P value, $\chi 2$-test

Interestingly, ROC analysis revealed that the highest cut-off value for the AMD patients compared to normal controls was seen for IgG reactivites to *H. pylori* (Table 2). This bacterium, a well-known cause of gastrointestinal inflammation, is also associated with eye diseases such as glaucoma, uveitis and central serous chorioretinopathy - a type of exudative retinal disease that, like AMD, occurs in the macular region (Mauget-Faysse et al. 2002, Otasevic et al. 2007). In contrast *Miller et al.* (Miller et al. 2004) failed to find an association between infection of *H. pylori* (or *Chlamydia pneumoniae*) and wet AMD, though they did observe that the IgG titer for CMV was correlated with wet AMD. Our microarray analysis revealed that IgG titers for CMV were not significantly different between the dry AMD, wet AMD and normal control groups, but IgM reactivities to CMV were significantly elevated in sera from patients with dry AMD compared to normal controls (AUC = 0.7486, odds ratio = 13.037, Supplementary Table 2). These results suggest that the duration of chronic infection with CMV and *H. pylori* may be involved in the development of AMD. The odds ratios for IgM autoantibodies are provided in Supplementary Table 2.

3.3 Levels *and functional effects of anti-GS IgG*

Since the dry stage of AMD is not typically associated with vision loss, diagnosis and treatment at this stage is particularly important. In this study we were interested not only in diagnostic and prognostic potential of autoantibody biomarkers, but also in possible roles of these molecules in disease progression. Since anti-GS IgG was the most promising biomarker for predicting progression from dry to wet AMD, we chose to examine the expression and function of this autoantibody in greater detail.

To verify the high IgG seroreactivity to GS in sera of patients with AMD, titers for anti-GS IgG were measured using ELISA. Serum samples from wet AMD patients showed significantly higher reactivity to GS compared to normal controls or to patients with dry AMD (Figure 3A, $P < 0.05$), which was consistent with the results of our antigen microarray analysis (Figure 1).

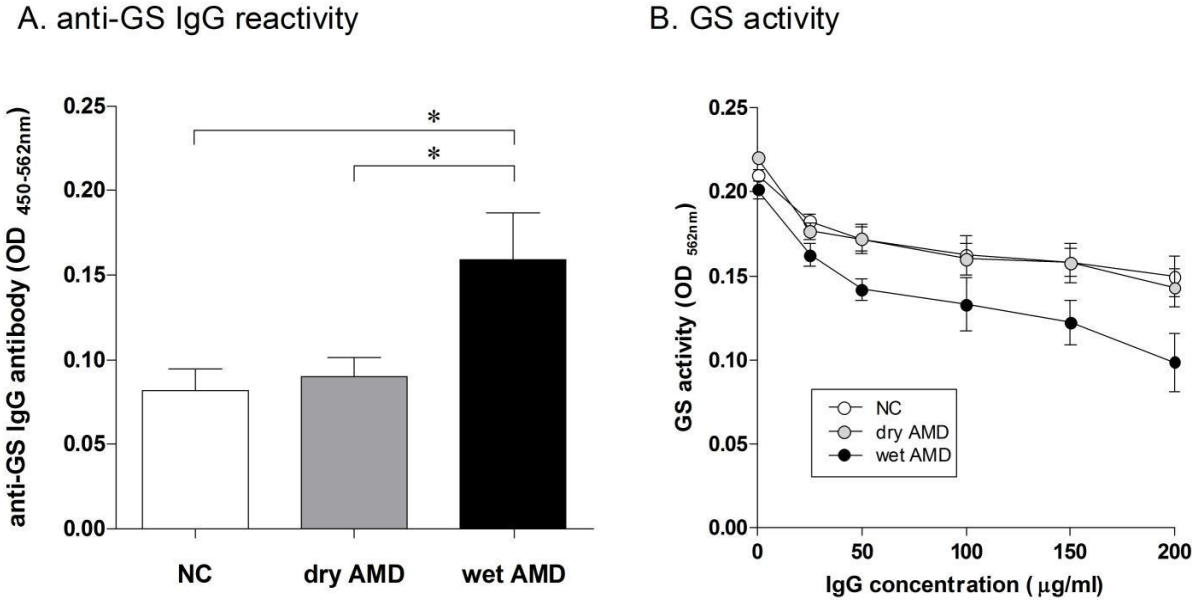

Figure 3. Expression and inhibitory function of anti-GS IgG purified from AMD patients.

(A) IgG was isolated from the sera of normal controls (NC), patients with dry AMD and patients with wet AMD by protein G-Sepharose affinity chromatography. IgG concentrations were measured by ELISA. *, $P < 0.05$.

(B) GS was incubated with increasing concentrations of IgG purified from the sera of normal controls (NC), patients with dry AMD and patients with wet AMD prior to conducting a colorimetric assay for GS activity.

To explore a possible functional role for anti-GS in the pathogenesis of AMD, we examined the effects of patient IgGs on GS activity. IgGs purified from AMD patients were incubated with GS, then the ability of the enzyme to convert glutamate to glutamine was measured. Figure 3B shows that IgG antibodies from wet AMD patients decreased the enzymatic activity of GS in a dose-dependent manner, suggesting that retinal degeneration might occur at least in part due to accumulation of neurotoxic glutamate.

Anti-GS antibodies have not previously been associated with human AMD; however, retinal GS expression is decreased in Ccl2/Cx3cr1-deficient mice that develop AMD-like retinal lesions, including drusen and CNV, compared to wild type mice (Zhou et al. 2011). Marignier and colleagues (Marignier et al. 2010) reported that IgGs

from patients with neuromyelitis optica, who have strong IgG reactivity to the water channel protein aquaporin 4, inhibited GS activity in astrocytes compared to IgGs from normal controls. Moreover, several studies have revealed that GS activity is reduced in the retina of animal models of glaucoma and Alzheimer's disease, which shares common pathogenesis with AMD, including complement activation, accumulation of amyloid beta and oxidative stress (Olabarria et al. 2011, Ohno-Matsui 2011, Gionfriddo et al. 2009). These reports support the involvement of anti-GS IgG in neurodegenerative pathogenesis in AMD.

3.4. GS expression and activity with age in mouse eyes

In humans, GS activity is lower in elderly brains than in young brains, and is particularly low in the frontal lobe of patients with Alzheimer's disease (Smith et al. 1991). This decrease in GS activity is likely due to its susceptibility to oxidation. Interestingly, we have previously identified

other oxidation-sensitive enzymes, namely pyruvate kinase M2 and aldolase C, that are both targeted by antibodies in the serum of patients with AMD (Morohoshi, Ohbayashi, et al. 2012) and are highly oxidized in the brains of patients with Alzheimer's disease (Butterfield et al. 2006). The oxidative modification of these enzymes may cause not only a reduction in the enzyme activity, but also production of autoantibodies targeting the enzymes, since the oxidized form could be regarded as foreign.

Since AMD also is associated with aging and has a strong neurodegenerative component, we investigated levels of GS expression in the eyes of old mice (24 months of age) compared to young mice (2 months of age). We also examined levels of several molecules associated with autoimmune responses and local inflammation. Ocular expression of GS was significantly decreased in the old mice compared to young mice at the mRNA and protein levels, with a distinct decrease in retina-specific expression (Figure 4)

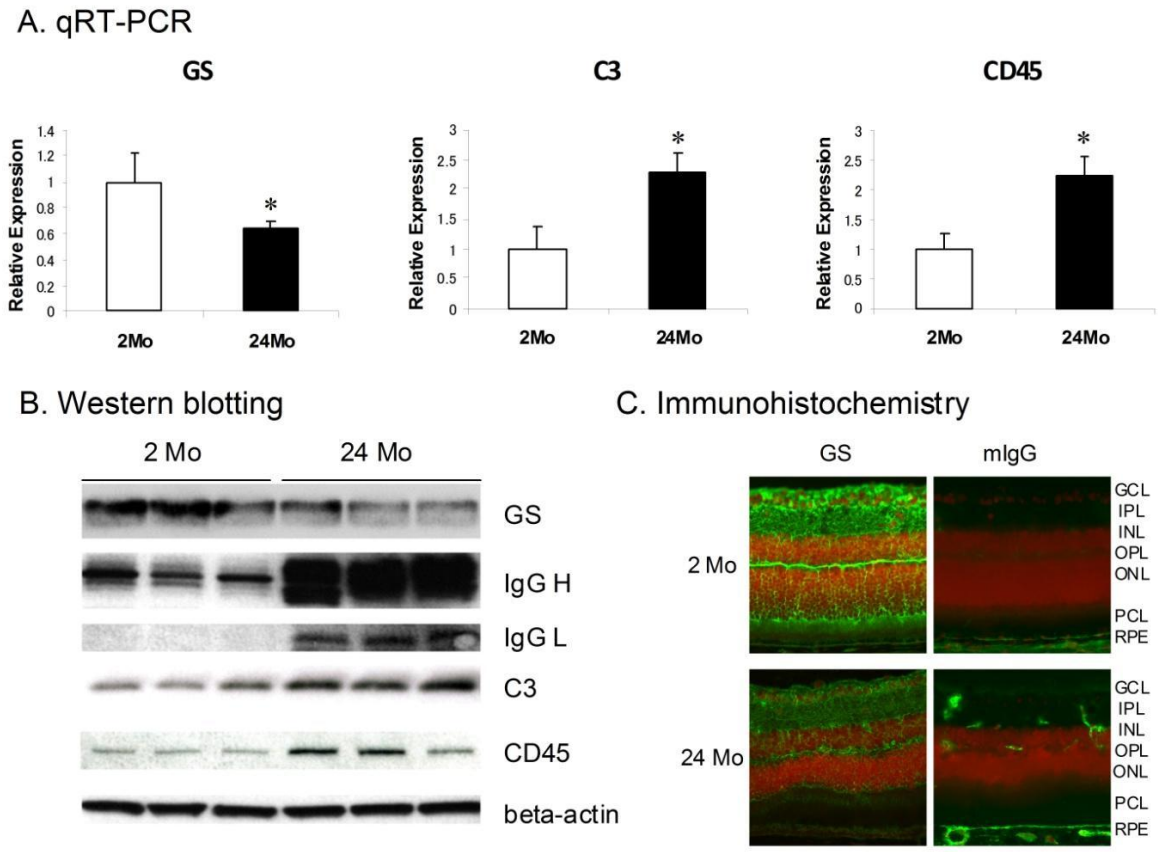

Figure 4. Ocular expression of GS, C3 and CD45 in the eyes of young (2-month-old) and old (24-month-old) mice

(A) Quantitative RT-PCR was used to measure relative mRNA expression of GS, C3 and CD45 in 2-month-old (2Mo) and 24-month-old (24Mo) mice. *, $p<0.05$.

(B) Western analysis was used to visualize relative protein expression of GS, C3, CD45, IgG heavy chain (IgG H), IgG light chain (IgG L) and, for normalization, beta-actin in 2-month-old (2 Mo) and 24-month-old (24 Mo) mice.

(C) Immunohistochemistry of retinal sections was performed using anti-GS (green), murine IgG (mIgG; green) and propidium iodide-counterstained nuclei (red). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PCL, photoreceptor cell layer; RPE, retinal pigment endothelium.

This is in contrast to levels of the complement protein C3, a protein whose SNPs are associated with AMD, and of CD45, a protein expressed by activated B- and T cells. These molecules (both mRNA and protein) are expressed at higher levels in older mice (Figure 4A, 4B). The decrease in GS expression with age likely reduces the amount of GS enzymatic activity, an effect that would be exacerbated by the neutralizing effect of anti-GS IgG, and may promote AMD pathogenesis, particularly in the context of the enhanced inflammation suggested by elevations in C3 and CD45. Interestingly, deposit of IgG antibody was markedly increased in RPE and choroid

compared to retina of aged mice (Figure 4C); this might suggest some inherent immune dysregulation with aging. Further investigation using anti-RPE and anti-choroid antibodies might illuminate the specific origin of drusen and CNV.

4. Conclusions

In this study, novel autoantibodies targeting the retina and drusen were identified in sera of AMD patients using an antigen microarray technique. Among these IgG autoantibodies, anti-complement C4 was best correlated with the development of dry AMD (OR=16.4) and wet AMD (OR=44.3), and anti-Apo E IgG was the most reliable biomarkers for progression from dry to wet AMD (OR=14.5). Furthermore, anti-GS IgG might also directly contribute to the pathogenesis of AMD, since dysregulation of GS activity causes an accumulation of neurotoxic glutamate that could lead to retinal degeneration. It remains to be seen whether these antibodies directly cause AMD or are secondary products of disease progression, however intense IgG deposition in RPE and choroid of aged mice suggests an association of autoimmunity with the development of disease. To better understand the direct pathogenic effects and responses of these autoantibodies, passive transfer of candidate antibodies and active immunization of candidate antigens on animal models are required for future study.

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