Complement Factor C3a Alters Proteasome Function in Human RPE Cells and in an Animal Model of Age-Related RPE Degeneration

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Purpose. Complement activation plays an unequivocal role in the pathogenesis of age-related macular degeneration (AMD). More recent evidence suggests an additional role in AMD for the ubiquitin proteasome pathway (UPP), a protein-degradation nanomachinery present in all types of eukaryotic cells. The purpose of this study was to elaborate on these findings and investigate whether the complement system directly contributes to derangements in the UPP through the activated complement components C3a and C5a.

Methods. In the retinal pigment epithelial cells (RPE) of monocyte chemoattractant protein-1–deficient CCL2$^{-/-}$ mice, a mouse model that may serve as a model for age-related atrophic degeneration of the RPE, proteasome function was investigated by immunohistochemistry of household (β5) and immuno (β5i) subunit expression. Subsequently, proteasome overall activity was determined using the BodipyFl-Ahx$_3$L$_3$VS probe in primary-cultured human retinal pigment epithelial cells (HRPE) cells that were exposed to different stimuli including C3a and C5a, using confocal laser scanning microscopy and flow cytometry. Gene expression and protein levels of proteasome subunits α7, PA28α, β5, and β5i were also studied in RPE cells after exposure to IFN-γ, C3a, and C5a by real-time PCR and Western blotting.

Results. Retinal pigment epithelial cells of CCL2$^{-/-}$ mice showed immunoproteasome upregulation. C3a, but not C5a supplementation, induced a decreased proteasome overall activity in HRPE cells, whereas mRNA and protein levels of household proteasome and immunoproteasome subunits were unaffected.

Conclusions. In HRPE cells, C3a induces decreased proteasome-mediated proteolytic activity, whereas in a mouse model of age-related RPE atrophy, the immunoproteasome was upregulated, indicating a possible role for complement-driven posttranslational alterations in proteasome activity in the cascade of pathologic events that result in AMD.

Keywords: proteasome, retina, age-related macular degeneration, complement, C3a, γ-interferon, ophthalmology, CCL2$^{-/-}$

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness among the elderly worldwide.[1,2] Abnormal complement pathway regulation and retinal pigment epithelium cells (RPE) dysfunction have both been implicated in the early pathogenesis of the disease,[3–5] and specifically in the formation of drusen.[6,7] Approximately 70% of AMD patients are homo- or heterozygous for a specific polymorphism of the gene encoding for the endogenous complement pathway regulator Factor H (CFH), with additional contributions of polymorphisms in the genes encoding for Factors B, C2, and C3.[8–11] The single nucleotide change (1277 T→C, rs1061170) in the CFH gene results in the substitution of histidine for tyrosine at codon 402 of the CFH protein, which subsequently leads to a more than 2-fold increase in risk of AMD in CT heterozygotes (carriers of one single copy of the C allele) and a 3- to 6-fold increase in individuals homozygous for the CC genotype compared with the TT genotype.[12–16] This results in a prolonged state of complement activation, which results in the assembly of the C5b-C9 membrane attack complex and cell lysis, concurrently with liberation of C3a and C5a, two small pro-inflammatory peptide fragments. Most of the complement pathway proteins are present in Bruch's membrane, drusen, and RPE of AMD patients.[17–22] Although the involvement of the complement pathway in the pathogenesis of AMD has unambiguously been established, it is not exactly known how a chronically overactive complement system triggers the development of AMD.[23]

Nonlysosomal proteolysis is essential for cell survival. In eukaryotic cells, the ubiquitin-proteasome pathway (UPP) is the major nonlysosomal proteolytic pathway.[24] Most cytoplasmic and nuclear proteins become ubiquitinated in order to target these proteins for degradation. Once ubiquitinated, these

Table 1. Characterization of the HRPE Donors

Donor	Age	Sex	Postmortem Time, h	Primary Cause of Death
1	13	M	13	Trauma
2	23	M	15	Trauma
3	34	M	13	Trauma
4	37	M	16	Cardiac—endocarditis aorta
5	42	M	14	Respiratory—pulmonary embolism
6	47	F	10	Heart failure
7	49	M	18	Cardiac—heart failure
8	55	M	14	Cardiac—heart failure
9	57	F	10	Malignancy—unknown
10	61	M	7	Respiratory—pulmonary embolism
11	62	M	16	Cardiac—heart failure
12	64	F	9	Malignancy—glioblastoma multiforme
13	65	M	12	Respiratory—respiratory insufficiency
14	68	M	15	Multiorgan failure
15	70	F	5	Respiratory—respiratory insufficiency
16	72	M	6	Malignancy—hepatocarcinoma
17	76	M	8	Heart failure

proteins are recognized by the 19S regulatory particle that together with the 20S catalytic core forms the 26S proteasome. Upon de-ubiquitination and unfolding, the protein enters the cylinder-shaped 20S core particle, which is formed by stacked catalytic subunits that possess hydrolytic activity for the cleavage of the carboxyl end of proteins. There are three catalytic subunits in the standard proteasome: β1 for acidic amino acids, β2 for basic amino acids, and β5 for hydrophobic amino acids. The immunoproteasome is formed upon replacement of the constitutive subunits in the standard proteasome by the inducible subunits, the so-called β1i, β2i, and β5i.[25,26] The immunoproteasome is involved in specific, biological processes including generation of immunogenic peptides for antigen presentation,[27,28] degradation of oxidized proteins,[29] cell signaling,[6,30,31] neuronal maintenance, and synaptic vesicle formation.[32] Therefore, the ratio between proteasomes containing the standard catalytical subunits (β1, β2, and β5) or the corresponding inducible subunits (β1i, β2i, and β5i) can change during inflammation and other stressful situations.

Retinal pigment epithelial cells (RPE) have an active UPP, but relatively limited levels of endogenous ubiquitin, which render these cells more vulnerable to cellular stressors.[33] Abnormalities in the UPP have been implied in the pathogenesis of many ageing diseases, such as Alzheimer's disease,[34] Parkinson's disease,[35] and cataract.[36–38] The aim of the present study was to investigate the involvement of the proteasome in AMD in relation to complement overactivation. The chymotrypsin-like activity (β5) of the proteasome appears to be the rate-limiting activity of the proteasome,[39,40] and it has been shown that ageing affects its functioning in the retina.[41] For this reason, β5 proteasome subunit and its immunoproteasome counterpart β5i were characterized both in cell cultures of complement-activated human retinal pigment epithelial cells (HRPE) and in a mouse model for age-related atrophic degeneration of the RPE.

Materials and Methods

Immunohistochemistry

To characterize β5 and β5i proteasome subunit expression in the RPE of AMD tissue, whole retinal sections (2-μm thick) of 500 day-old monocyte chemoattractant protein-1–deficient CCL2$^{-/-}$ mice ($n = 3$) and wild-type mice ($n = 3$) were stained with mouse antibodies against the β5 and β5i subunits of the 20S proteasome and the β5i subunit of the 20S proteasome (Abcam, Cambridge, UK) and visualized by immunofluorescence. CCL2$^{-/-}$ mice were obtained from The Jackson Laboratory (B6.129S4-*Ccl2*^{tm1Rol}/J, stock no. 004434; Bar Harbor, ME), and these do not contain the rd8 mutation.[42] All the animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Briefly, sections were fixed in 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), equilibrated, and rinsed in 1% PBS four times for 5 minutes, blocked in 10% normal goat serum (Invitrogen, Breda, The Netherlands) for 30 minutes, again rinsed in 1% PBS three times for 5 minutes. Then, sections were incubated in the presence of the primary antibodies for 1 hour at room temperature in a dilution of 1:500 followed by rinsing in PBS and incubation with appropriate secondary goat anti-mouse antibodies, conjugated with Cy3 in a dilution of 1:500 (Jackson, Suffolk, UK), and rinsed in PBS. Sudan Black B staining was performed by incubating sections in a freshly prepared solution of 1% Sudan Black B (Fisher Biotech, Pittsburgh, PA) diluted in 70% ethanol for 10 minutes, followed by brief rinsing in 70% ethanol, and rinsing in distilled water. As controls, whole retinal sections with no antibody treatment nor Sudan Black B staining, as well as whole retinal sections with only Sudan Black B staining were used. Sections were counterstained with 4′-6-diamidino-2-phenylindole (DAPI; Vector, Burlingame, CA).

Culture, Maintenance, and Treatment of HRPE Cells

Donor eyes were obtained from the Euro Cornea Bank (Beverwijk, The Netherlands) after removal of corneal buttons for transplantation. Donor eyes were acquired with consent of the donor or donor family to be used for medical research in accordance with the principles outlined in the Declaration of Helsinki. Characterization of the donors is summarized in Table 1.

For isolation of the HRPE, donor eyes with a post mortem time of less than 15 hours (average postmortem time, 12 hours) were obtained from 17 donors between 13 and 76 years of age. The RPE was isolated from the sclera together with the choroid after dissection of the anterior and posterior segment of the eye. The tissue was subsequently incubated for 1 hour at 37°C in a 6-well plate with 2 mL digestion medium (TrypLE Express; Invitrogen). The RPE and choroid were separated after adding 2.5 mL of F99 medium to the digestion mixture and transferring the tissue to an empty well containing 2.5 mL of F99 medium. Medium containing the RPE cells was then transferred to a cell strainer with 70-μm meshes and centrifuged for 10 minutes (400*g*, 1000 rpm). Supernatant was collected and diluted in 12 mL F99 medium. A suspension of cells in 1 mL F99 medium was transferred to a gelatin-coated 6-well plate.

Growth of cells was monitored and medium was changed every 2 days. After 8 days, the confluent cells were washed with PBS and 0.5 mL TrypLE Express, and 1 mL F99 medium was added. The contents of three wells were then transferred to a fibronectin-coated 75-cm^2 flask, which renders four flasks for passage 1 for each pair of eyes. The medium was replaced by human endothelial serum-free medium upon confluence. In passages 2 through 4, cells were used for experiments upon attaining 100% confluence. The cultured RPE cells exhibited an epithelial cell shape and contained pigment granules in the perinuclear region. The expression of RPE-specific marker genes *CRALBP*, *RPE65*, and *FGFR2* as determined by RT-PCR

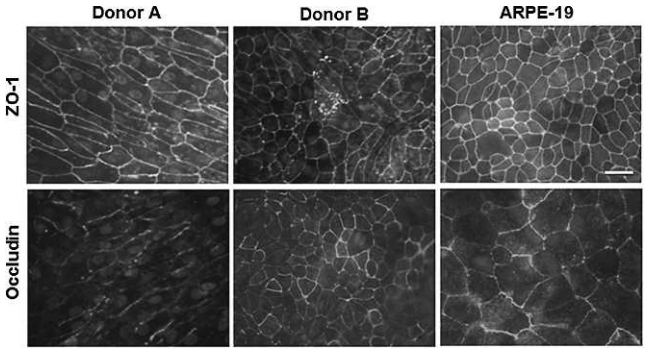

FIGURE 1. Characterization of HRPE cells by immunofluorescence microscopy with antibodies against tight junction protein ZO-1 and occludin. Human donor RPE cells give positive immunostaining for ZO-1 and occludin, which is comparable to staining in the immortalized RPE cell line ARPE-19. Donors A and B are two representative examples. *Scale bar*: 50 μm.

analysis indicated the identity and high differentiation state of the cells as well. When cells were cultured on transwell inserts, a transepithelial resistance was obtained between 36 and 64 Ω-cm^2. Proper polarization of HRPE cells was verified by a positive staining of ZO-1 and occludin protein (Fig. 1).

To investigate the effect of complement factors, inflammation, and oxidative stress, RPE cells were stimulated with C3a (50 ng/mL or 100 ng/ml; R&D Systems, Minneapolis, MN), C5a (50 ng/ml; R&D Systems), IFN-γ (50 U/mL; PBL Biomedical, Piscataway, NJ; and U-CyTech Biosciences, Utrecht, The Netherlands) in serum-free medium for 72 hours.

Genotyping

Ten donors were genotyped for the CFH Y402H polymorphism (1277 T→C, rs1061170) in 2 ng genomic DNA extracted from RPE cells using a standard Taqman assay (Table 2).[43]

Genotype assessment for CFH Y402H polymorphism in the 10 donors used for protein analysis showed six C allele carriers, of which three had the CC genotype, three the CT genotype, and four the TT genotype (Table 2).

Preparation of Photoreceptor Rod Outer Segments

Photoreceptor outer segments (POS) were added to cultured HRPE cells to mimic the situation in vivo where RPE continually phagocytoses POS. Photoreceptor outer segments were isolated from bovine eyes obtained freshly from the slaughterhouse.[44,45] Photoreceptor outer segments were stored suspended in a solution of 10 mM sodium phosphate (pH 7.2), 0.1 M sodium chloride, and 2.5% sucrose at −80°C. Before use, POS were thawed and labeled by addition of 20% volume of 1 mg/mL FITC (Molecular Probes, Invitrogen, Carlsbad, CA) in 0.1 M sodium bicarbonate (pH 9.0), for 1 hour at room temperature in the dark. Photoreceptor outer segments were then washed and resuspended in cell culture media.

Proteasome Activity Measurements by Flow Cytometry of HRPE Cells

Proteasome activity was determined using the probe BodipyFl-Ahx$_3$L$_3$VS (provided by Hermen Overkleeft),[46] which has a similar affinity for all catalytically active subunits of proteasomes in living cells. This probe has a green emission spectrum (1_{ex} = 480 nm, 1_{em} = 530 nm) and can be used for both flow cytometry (FACS) experiments and confocal laser scanning microscopy (CLSM).[46,47]

TABLE 2. Screening for Polymorphisms in Complement Factor-H (CFH Y402H) in Donors Used for Protein Analysis

Donor	Genotype
2	CT
4	TT
3	CT
6	TT
7	TT
8	CC
13	TT
14	CC
16	CC
17	CT

The following cell culture samples were tested for proteasome activity by means of FACS: unstimulated HRPE, POS-fed RPE, C3a-stimulated HRPE, C3a-stimulated POS-fed HRPE, IFN-γ–stimulated HRPE, and HRPE with and without C3a stimulation treated for 1 hour with 500 nM of the proteasome inhibitor epoxomicin (Ep; Sigma-Aldrich, St. Louis, MO). In total, seven human donors were used and divided in a "young age" group (n = 3; 13, 23, and 37 years old) and an "old age" group (n = 4; 47, 55, 57, and 65 years old).

Flow cytometry experiments were performed on a FACS LSRII (Becton Dickinson, Breda, The Netherlands). For uptake experiments, untreated cells, and C3a-treated cells were incubated with 500 nM BodipyFl-Ahx$_3$L$_3$VS for 2 hours. The treated cells were stimulated with 50 ng/mL recombinant human C3a. Cells were washed, trypsinized, and resuspended in medium, and intracellular fluorescence was measured. As a negative control, cells were incubated with 500 nM Ep overnight. Unstained HRPE cells were used to normalize the signal. Two or three parallel wells were used for each experimental condition. Approximately 10,000 of unfixed RPE cells were used for the experiments and a live gate was used to exclude cell fragments, POS particles, and other unwanted debris. The background fluorescence of the system, as assayed without any cells, was subtracted. A logarithmic scale of relative fluorescent intensity was used and signal intensity was calculated by subtracting the geometric mean autofluorescence of control cells from the geometric mean fluorescence of cells incubated with BodipyFl-Ahx$_3$L$_3$VS.

Confocal Laser Scanning Microscopy

To visualize active proteasomes in HRPE cells using a TCS SP2 CLSM (Leica, Rijswijk, The Netherlands), the following cell culture samples were incubated with BodipyFl-Ahx$_3$L$_3$VS: unstimulated HRPE, C3a-stimulated HRPE, IFN-γ–stimulated HRPE, and HRPE with and without C3a stimulation treated for 1 hour with Ep. Two samples of HRPE cells of two donors were studied. Untreated and C3a-treated cells were incubated for 2 hours with the probe and washed with medium before being imaged. Treated HRPE cells were stimulated with 50 ng/mL C3a. As a negative control, cells were incubated with 500 nM Ep overnight before incubation with the activity probe.

Protein Extraction and Western Blot Analysis

For Western blot analysis, protein lysates of 10 donors (three samples per experimental condition) were collected in 100 mL lysis buffer (1% Triton X-100, 50 mM HEPES, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM phenylmethanesulfonyl fluoride, 1X phosphatase inhibitors, and 1X

Table 3. Primer Details

Gene	GenBank	Forward Primer	Reverse Primer	Size, bp	Temperature, °C
RPE65	NM_000329	GATGCCTTGGAAGAAGATGATGGTG	TCCTTGGCATTCAGAATCAGGAGAT	98	79
RLBP1	NM_000326	GAGAAGCTGCTGGAGAATGAGGAAA	TGGGAAGGAATCCTGGAGCATG	144	80
FGFR2	NM_022971	TGATGATGAGGGACTGTTGGCATG	TCGAGAGGTTGGCTGAGGTCCA	108	78
PSME1	NM_006263	CAGCCCCATGTGGGTGATTATC	GCTTCTCGAAGTTCTTCAGGATGAT	139	82
PSMA7	NM_002792	CCTGGAAGGCCAATGCCATAG	TTTGCCACCTGACTGAACCACTTC	149	82
PSMB5	NM_002797	CCATGATCTGTGGCTGGGATAAG	GGTCATAGGAATAGCCCCGATC	144	83
PSMB8	NM_004159	CTGGAGGCGTTGTCAATATGTACC	GCAGCAGGTCACTGACATCTGTAC	81	76
C5AR1	NM_001736	CCCAGGAGACCAGAACATGAACTC	TGACCAAGGCCAGGATGTCTG	143	81
C5L2	NM_018485	GCCAGGACGAAAGTGTGGACAG	CCAGCTATGCCTGAAGCCAGTC	136	81
C3AR	NM_004054	ACCAGACAGGACTCGTGGAGACAT	GCAGAGAAAGACGCCATTGCTAAAC	90	77

Gene nomenclature, GenBank accession code, primer sequences, and predicted size and Tm of the amplified product.

complete protease inhibitors; Roche Biochemicals, Almere, The Netherlands).

Western blot analyses were performed as described previously.[48] Twenty micrograms of protein were separated on a 12.5% SDS-PAGE, transferred to polyvinylidene difluoride membranes and semiquantitatively analyzed. Membranes were incubated for 16 hours at 4°C with a monoclonal antibody against the α7 and α2 subunit of the 20S proteasome and one of the following polyclonal antibodies: anti-β5 subunit of the 20S proteasome, anti-β5i subunit of the 20S proteasome, and anti-11S regulator subunit PA28α (Enzo Life Sciences, Zandhoven, Belgium). All primary antibodies were diluted 1:500 in 3% nonfat dry milk (Bio-Rad, Hercules, CA) in TBS/0.05% Tween-20. Infrared dye-conjugated goat anti-rabbit (for β5, β5i, and PA28α) and goat anti-mouse (for α7 and α2) secondary antibodies (LI-COR Biosciences, Lincoln, NE) were diluted 1:10,000. Immune reactions were quantified by densitometric analysis using Odyssey (LI-COR Biosciences). Anti–β-actin antibody was used to stain a reference sample to normalize sample reactions and allowed for comparison between blots. All Western blot experiments were performed at least twice.

Proteasome Activity Measurements in Cell Extracts

Retinal pigment epithelial cells from a young and old donor were harvested in TSDG buffer (10 mM Tris, pH 7.5, 25 mM KCl, 10 mM NaCl, 1.1 mM $MgCl_2$, 0.1 mM EDTA, and 8% glycerol) and lysed by three freeze/thaw cycles in liquid

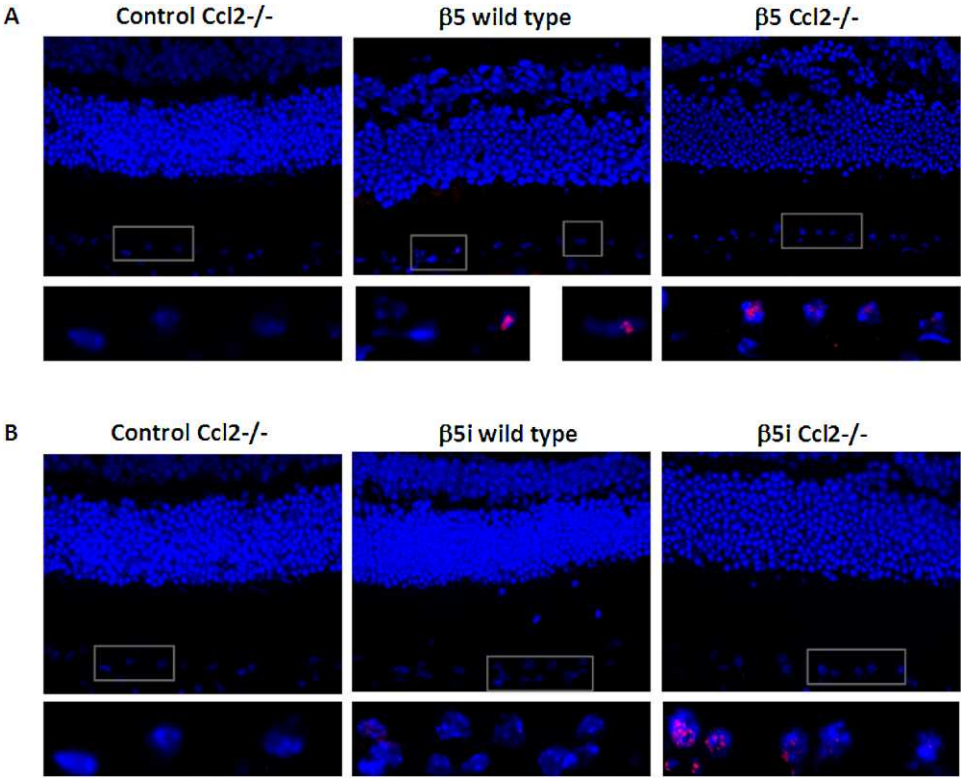

Figure 2. **(A)** Proteasome β5 subunit content is constant in $CCL2^{-/-}$ mice. Whole retina sections from wild-type and $CCL2^{-/-}$ mice were stained with antiproteasome β5 antibody and visualized by immunofluorescence microscopy (in *pink*). Proteasome β5 subunit was present in the nuclei and perinuclear region of the RPE of both wild-type and CCL-$2^{-/-}$ mice. **(B)** Proteasome β5i subunit protein expression is upregulated in $CCL2^{-/-}$ mice. Whole retina sections from wild-type and $CCL2^{-/-}$ mice were stained with antiproteasome β5i antibody and visualized by immunofluorescence microscopy. Proteasome β5i subunit was not present in the RPE of wild-type mice. Immunoproteasome β5i subunit was upregulated in the nuclei and perinuclear regions of the RPE of $CCL2^{-/-}$ mice.

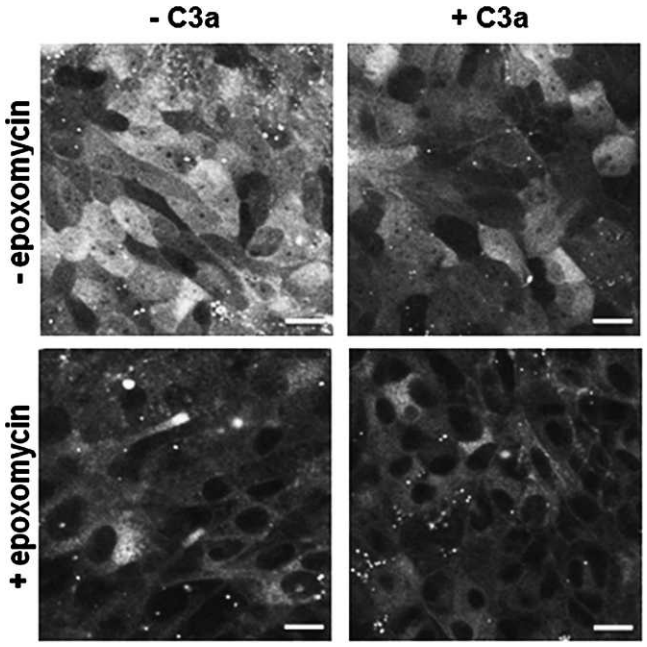

FIGURE 3. Proteasome overall activity is decreased upon C3a stimulation of HRPE cells from old donors. Proteasome overall activity was assessed in the following conditions: unstimulated HRPE, 72-hour C3a stimulation in HRPE, and the same conditions after epoxomicin (Ep) treatment for 1 hour. Proteasome overall activity was visualized by confocal scanning laser microscopy after treatment with the probe BodipyFl-Ahx3L3VS for 2 hours. Epoxomicin, a proteasome inhibitor, is used as a negative control and represents minimal proteasomal activity.

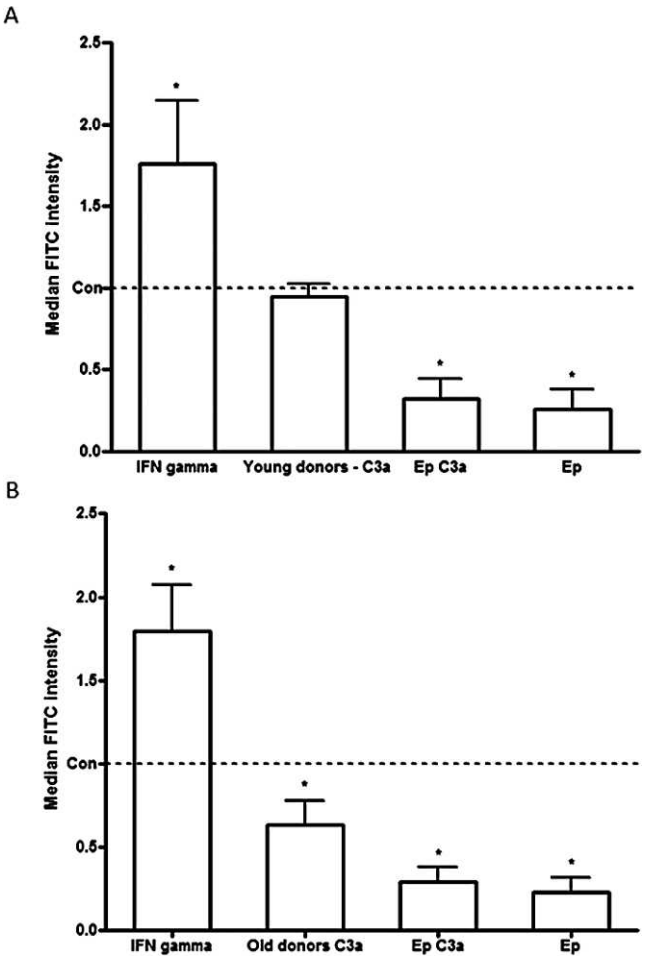

FIGURE 4. Proteasome overall activity is decreased upon C3a stimulation of HRPE cells from old donors. Proteasome overall activity was assessed in the following conditions: unstimulated HRPE, 72-hour C3a stimulation in HRPE of young donors (13–37 years old, $n = 3$) (**A**), 72-hour C3a stimulation in HRPE of old donors (47–65 years old, $n = 4$) (**B**), 72-hour treatment of HRPE with IFN-γ and HRPE with and without C3a stimulation treated for 1 hour with Ep. Proteasome overall activity was measured by FACS assay after treatment with the probe BodipyFl-Ahx3L3VS for 2 hours. Data of HRPE treated with POS are not shown. Interferon gamma is used as a positive control and represents the maximum proteasomal activity; Ep, a proteasome inhibitor, is used as a negative control and represents minimal proteasomal activity. Data are expressed as the median ± SEM. *Significant change ($P < 0.05$).

nitrogen. After centrifugation (15 minutes, 21,000*g*), the protein concentration in the supernatant was determined by a Bradford protein assay (Serva, Heidelberg, Germany). Proteasomes were labeled in the lysate with 0.5 μM Bodipy-Ep probe for 1 hour at 37°C.[49] Six times sample buffer (350 mM Tris/HCl, pH 6.8, 10% SDS, 30% glycerol, and 6% β-mercaptoethanol) was added to 30 μg lysate. The samples were boiled for 3 minutes and loaded on a 12.5% SDS-PAGE gel. Afterwards, fluorescent imaging was performed on a Trio Thyphoon (GE Healthcare, Madison, WI) using the 580 bandpass (BP) 30 filter to detect the Bodipy-Ep probe directly in the gel. Subsequently, the gels were used for Western blot analysis to determine the proteasome levels using α2 subunit levels as a loading control, using the MCP236 antibody (Enzo Life Sciences). Antibody detection was performed using the Odyssey detection system (LI-COR Biosciences).

RNA Isolation and mRNA Quantification

For real-time quantitative PCR (qPCR) experiments, total RNA (6 samples per experimental condition) was isolated according to the manufacturer's instructions (TRIzol; Invitrogen) from the RPE of eight donors that were stimulated as indicated above. The amount of total RNA was approximately 3 μg/sample. A 1-μg aliquot of total RNA was treated with DNase-I (amplification grade; Invitrogen) and reverse transcribed into first strand cDNA (Superscript III and oligo[dT]$^{12-18}$; Invitrogen). The specificity of the primers was confirmed by a nucleotide-nucleotide BLAST (available in the public domain at http://www.ncbi.nlm.nih.gov/blast.cgi; National Center for Biotechnology Information, Bethesda, MD) search. Primer details are given in Table 3. The presence of a single PCR product was verified by both the presence of a single melting temperature peak and detection of a single band of the expected size on a 3% agarose gel. Quantitative PCR was performed (CFX96 system; Bio-Rad). For each primer set, a mastermix was prepared, consisting of 1X SYBR Green mix (iQ SYBR Green Supermix; Bio-Rad) and 2 pM primers with RNase-free water. One microliter of cDNA (diluted 1:20) in 19 μL mastermix was amplified using the following PCR protocol: an activation step at 95°C for 15 minutes, followed by 40 cycles at 95°C for 10 seconds and at 60°C for 45 seconds, followed by 95°C for 1 minute and a melting program (60–95°C). Relative gene expression (R) was calculated by using the equation: $R = E^{-Ct}$, where E is the mean efficiency of all samples for the gene being evaluated and Ct is the cycle threshold for the gene as determined during real-time PCR. The qPCR data were normalized with the expression of the *YWHAZ* gene, as determined by geNorm.[50]

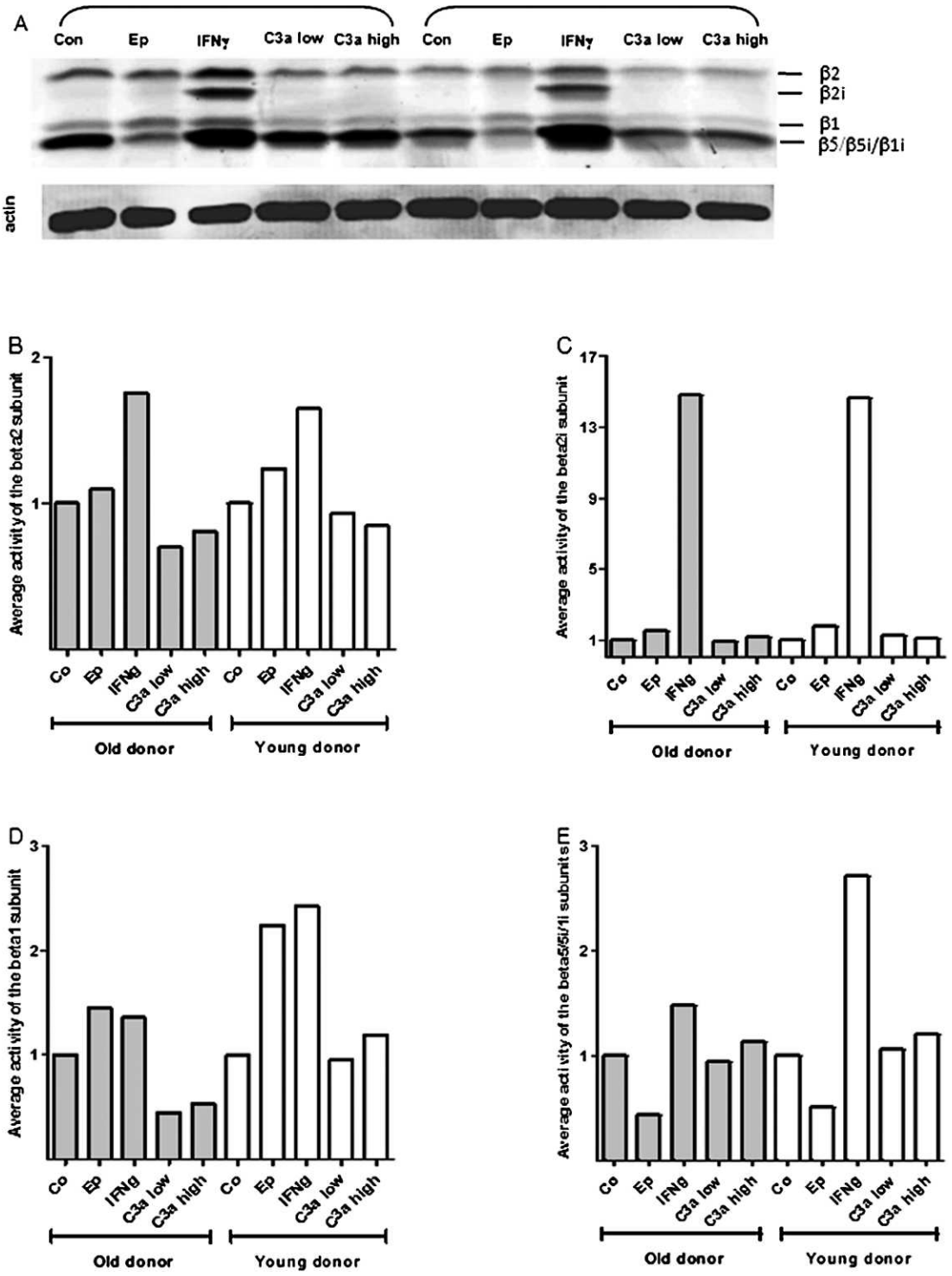

FIGURE 5. Interferon gamma, but not C3a, increases the average activity of proteasome subunits β2, β2i, β1, and β5:β5i:β1i complex both in an old donor (65 years old) and young donor (23 years old). (**A**) After treatment with Ep, IFN-γ, and high and low concentrations of C3a, HEK293 cells were harvested, and proteasomes were labeled with a Bodipy-Ep probe. Detection of different proteasome subunit activities was performed by Western blotting. Quantitative data of the proteasome activity are presented per donor for proteasome subunit β2 (**B**), β2i (**C**), β1 (**D**), and β5:β5i:β1i complex (**E**).

Statistical Analysis

Gene expression data showed a normal distribution. Differences in gene expression levels between groups were calculated by using single ANOVA with $P < 0.05$ indicating significant differences (two-tailed).

For proteasome activity in FACS assays, the total fluorescence intensities from two independent preparations in each group were calculated. Data are presented as mean ± SEM with statistical differences between groups analyzed by standard two-tailed *t*-test using GraphPad Prism (version 5.00

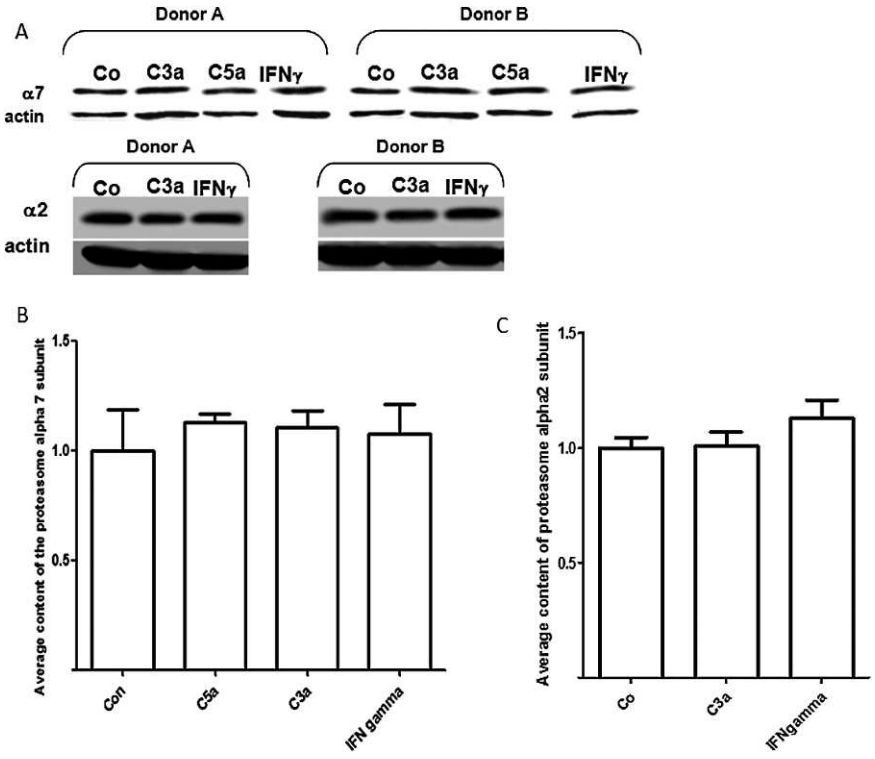

FIGURE 6. The total content of the proteasome α7 and α2 subunit is not increased upon stimulation of HRPE cells with C3a. Human retinal pigment epithelial cells were incubated for 72 hours with or without C3a, C5a (only for α7), or IFN-γ. (**A**) Alpha 7 and α2 levels were assessed by Western blot with actin expression as a loading control. (**B**) Quantitative data of the average content of α7 in the different experimental conditions, corrected for actin, and relative to control samples in HRPE cells without C3a stimulation. (**C**) Quantitative data of the average content of α2 in the different experimental conditions, corrected for actin expression, and relative to control samples in HRPE cells without C3a stimulation. Data are expressed as the mean ± SEM. *Significant change ($P < 0.05$).

for Windows, www.graphpad.com; GraphPad Software, San Diego, CA) and a $P < 0.05$ indicating statistically significant differences.

RESULTS

Increased β5i:β5 Ratio in RPE of CCL2$^{-/-}$ Mice

Immunofluorescence of proteasome subunits β5 and β5i in retinas of CCL2$^{-/-}$ mice ($n = 3$) and wild-type mice ($n = 3$) showed the subunits to be localized in nuclei and perinuclear regions of RPE cells. Retinal pigment epithelium of age-matched wild-type and CCL2$^{-/-}$ mice showed similar levels of β5 staining (Fig. 2A). However, the RPE of CCL2$^{-/-}$ mice also showed high levels of the β5i subunit, while no β5i subunit staining was observed in RPE of wild-type mice (Fig. 2B). This translated in a higher β5i:β5 ratio in the RPE of CCL2$^{-/-}$ mice. These results suggest that proteasome activity may be altered in age-related maculopathy.

Overall Activity of the RPE Proteasome Is Decreased Upon C3a Stimulation in HRPE Cells

Overall proteasome activity was visualized in RPE cells at 72 hours by CLSM imaging and subsequently quantified by FACS assays using the activity probe BodipyFl-Ahx$_3$L$_3$VS. Confocal scanning laser microscope images showed active proteasomes in nuclei and cytoplasm of HRPE cells. In comparison to untreated cells, HRPE cells treated for 72 hours with C3a showed decreased proteasome activity. Cells treated with the proteasome inhibitor, Ep, showed 80% inhibition of proteasome activity ($n = 7$; $P < 0.01$; Fig. 3). C3a-treated HRPE cells of younger donors (13–37 years old, $n = 3$) showed no differences in proteasome activity when compared with untreated HRPE ($P > 0.05$, Fig. 4A), whereas C3a-treated HRPE cells of older donors (47–65 years old, $n = 4$) showed a 37% decrease in proteasome activity when compared to age-matched untreated HRPE cells ($P < 0.05$, Fig. 4B). Treatment of HRPE cells with IFN-γ caused a 76% increase in proteasome activity ($n = 7$, $P < 0.01$). Photoreceptor outer segments–fed cells did not show altered proteasome activity (data not shown). Moreover, the CFH Y402H polymorphism did not affect proteasome activity in untreated and C3a-treated HRPE cells. Overall, these results suggest that complement factor C3a causes decreased proteasome activity in HRPE cells of older individuals.

Specific Proteasome Subunit Activities Are Not Affected by C3a in HRPE Cells

To further characterize whether decreased overall proteasome activity was due to changes in the activities of specific proteasome subunits, we used a Bodipy-Ep probe to label the individual catalytic proteasome subunit activities upon separation by SDS-PAGE (Fig. 5). Retinal pigment epithelial cells from young and old donors were used (ages 23 and 65 years old, respectively). As expected, IFN-γ caused increased activity of immunoproteasomes as indicated by the increase in β2i (14.7-fold higher on average in all donors), and β5:5i:1i levels in the young donor (2.7-fold higher when compared with control). However, treatment with either a high or low concentration of C3a did not induce any changes in β2, β2i, β1, and β5:5i:1i

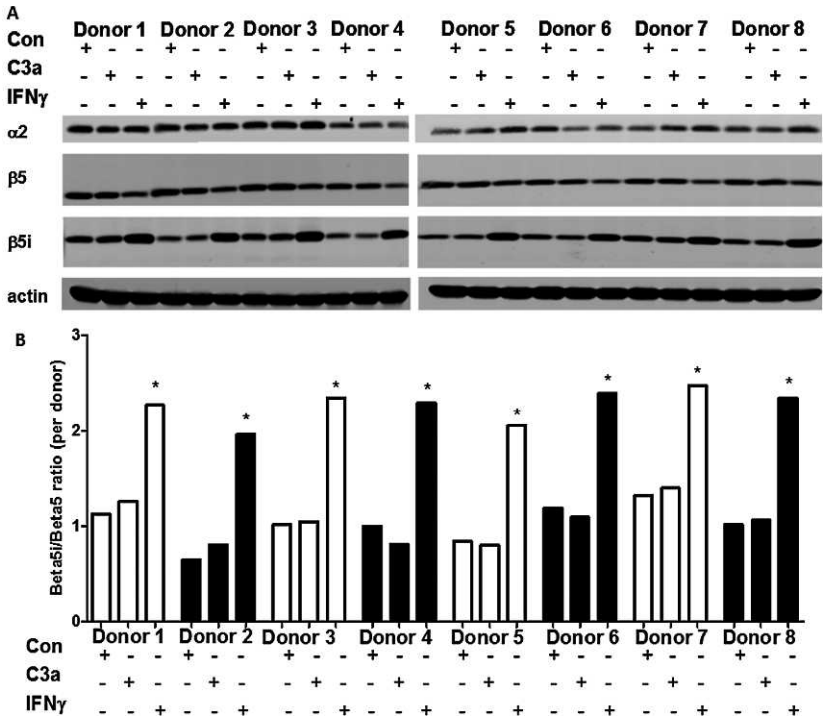

FIGURE 7. Interferon-gamma, but not C3a, increases the average ratio of β5i:β5 in HRPE cells of eight independent donors. Human retinal pigment epithelial cells were incubated for 72 hours with or without C3a and IFN-γ. (**A**) β5 proteasome subunit and β5i proteasome subunit levels were assessed by Western blot with actin as a loading control and α2 subunit as proteasome content control. (**B**) Quantitative data of the average level of the ratio between β5i and β5 per donor and relative to control samples. Data are expressed as the mean ± SEM. *Significant change ($P < 0.05$).

activity levels when compared with control HRPE cells. These results indicate that overall decreased proteasome activity upon C3a stimulation is not due to changes in the activity of specific proteasome subunit complexes, but rather affects proteasome activity posttranslationally.

Expression of Proteasome Is Not Affected by C3a Stimulation in HRPE Cells

To assess whether the observed decrease in proteasome activity upon C3a treatment was due to an alteration in expression of proteasome subunits, we measured total proteasome content (Fig. 6). Levels of the different proteasome subunits were evaluated using the α7 and α2 subunits of the 20S core. Densitometric analysis revealed no statistically significant differences in the content of α7 ($n = 6$) and α2 ($n = 8$) proteasome subunits after treatment with C3a, C5a, or IFN-γ.

Immunoproteasome Expression Is Not Affected by Complement Over Activation in HRPE Cells

Untreated HRPE cells showed protein levels of both the constitutive subunit β5 and the inducible subunit β5i of the proteasome that were similar in C3a-stimulated HRPE cells ($n = 8$, $P > 0.05$, Fig. 7). In IFN-γ–treated HRPE cells, the protein levels for the immuno-subunit β5i were on average 1.8-fold higher than in control HRPE ($n = 8$, $P < 0.05$), whereas those of the constitutive β5 subunit were on average 0.8-fold lower than in untreated HRPE ($n = 8$, $P > 0.05$). This translated in a statistically significant increased average β5i:β5 ratio of 2.3 for IFN-γ–treated HRPE cells ($n = 8$, $P < 0.01$). These results show that inflammatory mediators, but not complement activation, may explain the change in the conformation of the proteasome in RPE cells in mice, as the decreased proteasome overall activity found in the HRPE cells treated with C3a cannot be explained by changes in the content of β5 or β5i. No significant association was found between age, CFH Y402H polymorphism and change in proteasome protein content.

No Alterations in PA28 Levels Upon C3a Stimulation in HRPE Cells

As changes in proteasome activity can also be induced by the proteasome activator PA28, which can replace the 19S cap as an alternative proteasome activator binding to the 20S proteasome core, we determined the total content of the proteasome regulatory complexes by antibody reactions against the α-subunit of PA28, another proteasome regulatory complex.

We did not find any significant change in content of PA28α protein levels due to C3a treatment. A significant 4.6-fold increase was found in IFN-γ–stimulated HRPE cells ($n = 5$, $P < 0.01$, Fig. 8). These data suggest that decreased proteasome overall activity upon C3a stimulation is not due to altered PA28 levels, a mechanism by which IFN-γ and other inflammatory mediators are known to upregulate the level of the immunoproteasome.

C3a Stimulation in HRPE Cells Does Not Change mRNA Expression of Proteasome-Related Genes

We assessed mRNA levels of proteasome-related genes, *PSME1* (PA28), *PSMB5* (β5 subunit), *PSMA7* (α7 subunit), and *PSMB8* (β5i subunit) in untreated HRPE cells and HRPE cells treated with C3a, IFN-γ, and H_2O_2. Expression of the C5a receptors, human C5aR (hC5aR) and human C5L2 (hC5L2), as well as the C3a receptor, human C3aR (hC3aR), was also determined in order to confirm whether complement receptors were expressed in the RPE cells.

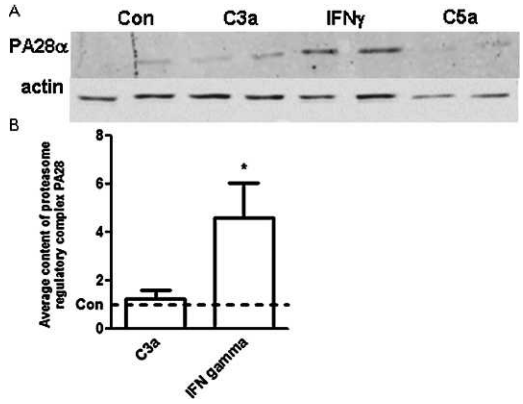

FIGURE 8. The total content of the proteasome regulatory complex PA28α is increased upon stimulation of HRPE cells with IFN-γ, but remains unchanged upon C3a stimulation. Human retinal pigment epithelial cells were incubated with or without C3a, C5a, or IFN-γ. (**A**) PA28α levels were assessed by Western blot with actin used as a loading control. (**B**) Quantitative data of the content of the average content of PA28α, corrected for actin, and relative to control samples in HRPE cells without C3a stimulation. Data are expressed as the mean ± SEM. *Significant change ($P < 0.05$).

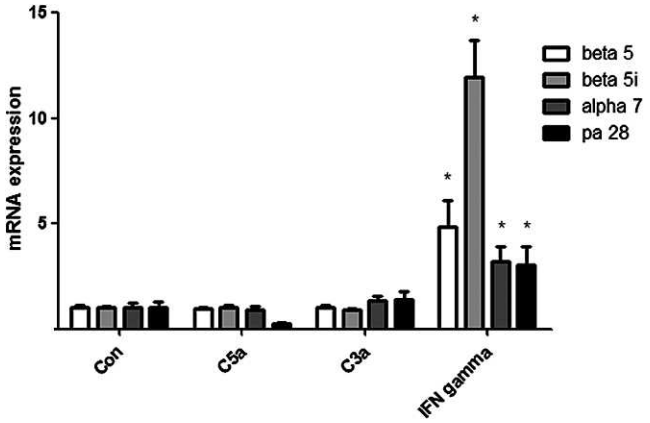

FIGURE 9. Increased mRNA expression of β5 and α7 subunits by C3a treatment in RPE cells. mRNA levels of PA28α, β5, β5i, and α7 subunits of the proteasome in HRPE cells, which were stimulated with IFN-γ, C3a, and C5a for 72 hours. Values represent mRNA expression levels (mean ± SEM) relative to untreated control cells. *Significant change ($P < 0.05$).

Messenger RNA levels of proteasome-related genes did not significantly change after C3a, C5a, and H_2O_2 treatment for 72 hours. However, IFN-γ treatment significantly increased mRNA levels of all genes, 4.9-fold for *PSMB5* (β5 subunit), 11.9-fold for *PSMB8* (β5i subunit), 3.2-fold for *PSMA7* (α7 subunit), and 3.0-fold for *PSME1* (PA28 subunit) ($n = 8$, $P < 0.05$, Fig. 9). Expression of both *hC5aR* and *hC3aR* was detected in the HRPE, whereas *hC5L2* expression was not detected (data not shown).

These results suggest that the complement factor C3a induced changes in overall proteasome activity are not caused by alterations in gene expression.

DISCUSSION

This study shows that in primary cultures of HRPE, C3a leads to decreased proteasome-mediated proteolytic activity, independent of changes in proteasome components at the protein or transcriptional level, while in a mouse model of age-related atrophic degeneration of the RPE, immunoproteasome upregulation was shown by an increased β5i:β5 ratio. Our results support involvement of alterations in proteasome activity in the cascade of pathologic events that result in this disease process.

Interferon gamma had no effect on the total level of proteasomes, but it did cause an increase in proteasome overall and specific activities, increased relative expression of proteasome regulatory complex PA28α, and increased protein and mRNA expression of immunoproteasome subunits. Interferon gamma caused a switch in the predominance of the inducible chymotrypsin-like subunit β5i over its normal counterpart β5. The resulting β5i:β5 ratio increased by 2.27-fold when compared with unstimulated HRPE. This resulted in a high β5i:β5 ratio, which is indicative of more immunoproteasomes. These results are in accordance with previous studies,[51–53] and show that the observed decreased proteasome activity upon stimulation with C3a cannot be explained by similar mechanisms.

Previous studies have shown that β5-driven proteolytic activity is the rate-limiting activity and the primary effector of protein degradation by the proteasome.[39,40] Specific inhibition of its activity has the most significant consequences for key processes involved in cell survival under stressful conditions. Neuronal cells that over-expressed a mutant β5 subunit where the active site threonine had been mutated to an alanine were significantly hypersensitive to oxidative stress.[54] The aged retina is exposed to high oxygen tension, high metabolic activity, presence of photosensitive pigments, all culminating in generation of reactive oxygen species[55] that periodically lead to an imbalance in the cellular redox homeostasis. Additionally, and in agreement with our results, proteasome function in the retina is known to be affected by ageing,[41] with the β5 chymotrypsin–like activity of the proteasome being most affected.[41] Our results suggest that in addition to ageing mechanisms, inflammatory mediators such as IFN-γ and potentially complement over activation may cause alterations in UPP activity, which may render RPE less tolerant to oxidative stress due to impairment in the clearance of oxidatively damaged proteins.

Decreased overall proteasome activity in the RPE may indirectly contribute to the development of age-related maculopathy. A recent study showed that experimental drug-induced proteasome inhibition in the ARPE-19 cell line, a human retinal pigment epithelial cell line with differentiated properties, leads to accumulation of hypoxia-inducible factor 1-alpha and diminished activation of the nuclear factor kappa-light-chain-enhancer of activated B cells pathway (NF-κB). This led in turn to enhanced expression and secretion of pro-angiogenic factors such as VEGF and angiopoietin-2 together with an attenuated expression of monocyte chemotactic protein-1.[56] Pathologic angiogenesis due to upregulation of VEGF is one of the most important causes of visual deterioration in AMD.[57]

Furthermore, C3a-driven reduced proteasome activity in the RPE could lead to an abnormal regulation of key signaling pathways. For instance, the UPP plays a crucial role in the regulation of pathways that respond to light damage[58,59] and to melatonin production.[60] Hence, complement-driven proteasome inhibition could impact the circadian cycles of melatonin production and the subsistence of the retina to light-induced damage.

Proteasome inhibition has been shown to increase lipofuscin accumulation and in turn, lipofuscin inhibits the proteasome system due to proteasomal binding to the lipofuscin surface motifs.[61] Lipofuscin, a highly oxidized aggregate, consists of covalently cross-linked proteins, lipids, and sugar

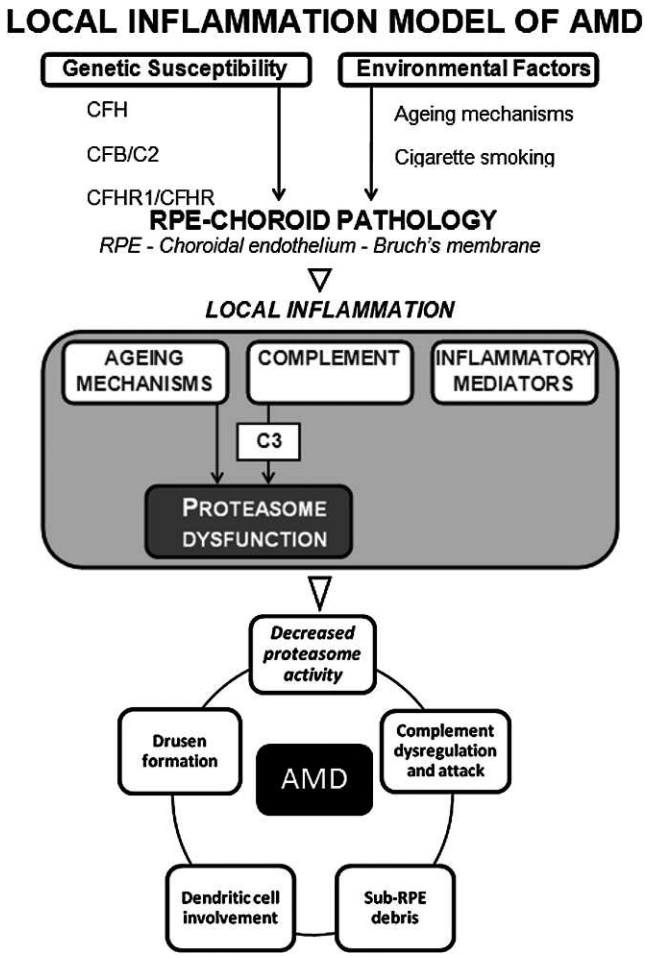

Figure 10. Inflammation model of macular degeneration.[6] Age-related macular degeneration may be triggered by one or more environmental risk factors coupled with a genetic susceptibility profile conferred by variants in the CFH, CFB/C2, and/or C3 gene triad. Late in life, such features culminate in the development of pathologic changes in the RPE–choroid complex, which in turn generate a chronic, local inflammatory response characterized by complement over activation, and inflammatory-mediated events. According to our results, release of anaphylatoxin C3a, a complement activation fragment, together with age-related changes, triggers decreased proteasome overall activity. Collectively, complement activation, inflammation-mediated events, and possibly proteasome dysfunction result in the photoreceptor degeneration and the loss of central vision that defines the clinical entity of AMD.

residues and is one of the major lifespan-limiting factors in post mitotic ageing cells.[61] Lipofuscin accumulation in the RPE has been reported in ageing and has been implied in many ocular disorders such as AMD, autosomal-recessive Stargardt macular degeneration, and Best's vitelliform macular dystrophy. Potential noxious effects of lipofuscin include photochemical blue light damage, inhibition of lysosomal digestion and proteins, detergent-like disruption of membranes, RPE apoptosis, and DNA damage. Lipofuscinogenesis translates in increased autofluorescence of the retinal fundus. However, it is still unclear whether proteasome inhibition would have the same effects in the RPE because unlike in many cell types in which lipofuscin originates internally (autophagy), lipofuscin derives primarily from phagocytosed photoreceptor outer segments in RPE.[55,62–68] Nonetheless, a C3a-induced decrease in overall proteasome activity may contribute to lipofuscinogenesis in age-related maculopathy and AMD.

Besides our studies in in vitro HRPE models, we also studied proteasome function in a mouse model for age-related atrophic degeneration of the RPE. Ambati et al. observed developing features of age-related maculopathy and AMD such as accumulation of lipofuscin, drusen beneath the RPE, photoreceptor atrophy, and choroidal neovascularization.[69] Deposition of complement C3 and C5 intermediates within the RPE and the choroid apparently precedes the accumulation of lipofuscin and deposits in Bruch's membrane. This chain of events is supposedly similar to the processes occurring in human eyes affected by AMD. Complement activation was not present in age-matched wild-type mice.[69] The use of $CCL2^{-/-}$ mice as a mouse model for AMD has been disputed in recent years. Studies have shown that dysfunction of the chemokine ligand–receptor pair CCL2–CCR2 may lead to deregulated retinal para-inflammation mechanisms and retinal lesion development with aging, eventually leading to the development of dry AMD-like lesions in these mice.[69,70] Other studies, however, have failed to show retinal lesions in these mice. Luhmann et al. reported increased subretinal macrophage accumulation, but no retinal degeneration in aged $CCL2^{-/-}$ mice.[71] Vessey et al. showed inner retinal (amacrine cell) dysfunction in 9-month-old CCL2/$CX3CR1^{GFP/GFP}$ mice.[72] In a recent publication, Chen et al. confirmed results of previous studies in which deficiency of CCL2 led to an identifiable dry AMD-like phenotype. Lesions appeared in these age-dependent $CCL2^{-/-}CX3CR1^{GFP/GFP}$ mice not expressing the rd8 mutation, characterized by localized RPE and photoreceptor atrophy similarly to the human geographic atrophy dry type of AMD.[70] In line with these

results, we performed experiments with CCL2$^{-/-}$ mice in order to establish an indirect link between age, complement activation, and age-related atrophic degeneration of the RPE. Retinas of CCL2$^{-/-}$ mice and age-matched wild-type mice were stained for β5 and β5i proteasome subunits. Retinas of CCL2$^{-/-}$ mice showed an increased β5i:β5 ratio. This was related to a higher content of β5i in the RPE of the knockout mouse model when compared with the age-matched control. The increase in the β5i:β5 ratio observed in CCL2$^{-/-}$ mice could be one of the rescue mechanisms against retinal toxicity and oxidative stress.

Our study presents some limitations. It remains inconclusive why retinas of CCL2$^{-/-}$ mice show an increased β5i:β5 ratio. A link with the in vitro experiments with HRPE cells cannot be established. We could not prove, in vitro, that the induction of β5i was specific for complement. The retinas and RPE of CCL2$^{-/-}$ mice might be exposed to other inflammatory mediators that trigger the observed change in proteasome conformation. Another limitation of our study deals with estimating subunit composition from Western immunoblots in RPE homogenates. Unassembled subunits cannot be distinguished from those that are part of the functional complex. Such mechanism of subunit assembly may be altered by C3a stimulation. Another limitation of our method is the inability to differentiate proteasomes in different cellular compartments. C3a-associated changes in subcellular localization would not be detected using our method. Specific functions, for instance the degradation of misfolded proteins by proteasomes docked outside the endoplasmic reticulum, could be affected should the subcellular content of proteasomes be altered.

In conclusion, our results suggest a link between complement activation and proteasome activity in HRPE cells, which may have implications in the development of age-related maculopathy and AMD (Fig. 10). This alteration in UPP activity is not caused by changes in the proteasome composition itself, and probably occurs at a posttranslational level since it is not due to changes in gene expression or changes in the activity of specific proteasome subunits.

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Investigative Ophthalmology & Visual Science

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