Effect of Anti-C5a Therapy in a Murine Model of Early/Intermediate Dry Age-Related Macular Degeneration

Christopher B. Toomey,1–3 Michael Landowski,1 Mikael Klingeborn,1 Una Kelly,1 John Deans,1 Holly Dong,4 Ons Harrabi,4 Thomas Van Blarcom,4 Yik Andy Yeung,4 Ruslan Grishanin,4 John C. Lin,4 Daniel R. Saban,1,5 and Catherine Bowes Rickman1,2

1Department of Ophthalmology, Duke University Medical Center, Durham, North Carolina, United States
2Department of Cell Biology, Duke University Medical Center, Durham, North Carolina, United States
3Shiley Eye Institute, Department of Ophthalmology, University of California-San Diego, San Diego, California, United States
4Rinat, Pfizer Inc, South San Francisco, California, United States
5Department of Immunology, Duke University Medical Center, Durham, North Carolina, United States

Correspondence: Catherine Bowes Rickman, Duke University Medical Center, Box 3802, AERI Rm 5010, Durham, NC 27710, USA; bowes007@duke.edu.

CBT and ML contributed equally to the work presented here and should therefore be regarded as equivalent authors.

Submitted: October 11, 2017
Accepted: December 25, 2017


PURPOSE. A large body of evidence supports a central role for complement activation in the pathobiology of age-related macular degeneration (AMD), including plasma complement component 5a (C5a). Interestingly, C5a is a chemotactic agent for monocytes, a cell type also shown to contribute to AMD. However, the role monocytes play in the pathogenesis of “dry” AMD and the pharmacologic potential of targeting C5a to regulate these cells are unclear. We addressed these questions via C5a blockade in a unique model of early/intermediate dry AMD and large panel flow cytometry to immunophenotype monocytic involvement.

METHODS. Heterozygous complement factor H (Cfh+/−) mice aged to 90 weeks were fed a high-fat, cholesterol-enriched diet (Cfh+/−/HFC) for 8 weeks and were given weekly intraperitoneal injections of 50 mg/kg anti-C5a (4C9, Pfizer). Flow cytometry, retinal pigmented epithelium (RPE) flat mounts, and electroretinograms were used to characterize anti-C5a treatment.

RESULTS. Aged Cfh+/− mice developed RPE damage, sub-RPE basal laminar deposits, and attenuation of visual function and immune cell recruitment to the choroid that was accompanied by expression of inflammatory and extracellular matrix remodeling genes following 8 weeks of HFC diet. Concomitant systemic administration of an anti-C5a antibody successfully inhibited local recruitment of mononuclear phagocytes to the choroid–RPE interface but did not ameliorate these AMD-like pathologies in this mouse model.

CONCLUSIONS. These results show that immunotherapy targeting C5a is not sufficient to block the development of the AMD-like pathologies observed in Cfh+/−/HFC mice and suggest that other complement components or molecules/mechanisms may be driving “early” and “intermediate” AMD pathologies.

Keywords: complement, age related macular degeneration, immunotherapy, monocytosis

Age-related macular degeneration (AMD) is a leading cause of legal blindness in elderly persons in developed countries and has limited therapeutic options.1–4 Multiple studies have linked activation of the complement cascade, in particular the alternative complement pathway (alternative pathway), with AMD development and progression.5 Many components of the alternative pathway are found in the protein- and lipid-rich deposits occurring between the retinal pigmented epithelium (RPE) and Bruch’s membrane (BrM), which are known as drusen and define AMD pathobiology.6–9 In addition, polymorphisms in complement factor H (CFH), a key negative regulator of the alternative pathway, are responsible for a significant portion of the genetic attributable risk of AMD.10 These lines of evidence support the hypothesis that over-activation of the alternative pathway predisposes the posterior eye to AMD development and progression. In fact, intravitreal injections of lampalizumab, an inhibitor for the positive regulator of the alternative pathway, Factor D, showed promise as a therapy for late “dry” AMD or geographic atrophy but failed to meet the primary endpoint during its first of two phase III clinical trials.11 However, it is unknown whether there are still residual activated complement components either from incomplete blockade of the alternative pathway or from activation of the classical pathway that may explain the recent failure of lampalizumab as an AMD therapy.

Early/intermediate dry AMD is characterized by impaired dark adaptation reflecting rod photoreceptor dysfunction, RPE pigmenitary changes, and the presence of intermediate-sized (63–124 μm) drusen.12 We have recently developed a novel murine model of early/intermediate dry AMD based on multiple risk factors associated with AMD: advanced age, alternative pathway dysregulation, and environmental stress.13 Male heterozygous complement factor H knockout (Cfh−/−) mice aged to 90 weeks and fed an 8-week high-fat, cholesterol-enriched (HFC) diet develop attenuated rod-mediated visual function, increased RPE damage, and increased sub-RPE basal laminar deposits, while age-matched homozygous Cfh knockout (Cfh−/−) mice fed a HFC diet develop only increased sub-
RPE basal laminar deposits. Since aged \( C^b/\sim \) mice lack an intact complement system we hypothesize that the decreased visual function and increased RPE damage observed in the \( C^b/\sim \) mice are due to HFC-induced complement activation. Supporting this hypothesis, we observed an increase in plasma complement component 5a (C5a) in aged \( C^b/\sim \) mice on a HFC diet (\( C^b/\sim \)-HFC) compared to aged \( C^b/\sim \) mice on a HFC diet (\( C^b/\sim \)-HFC).

C5a is a critical chemotactic protein responsible for the recruitment, activation, and maintenance of immune cells. Consequently, we observed an increase in extravascular recruitment of mononuclear phagocytes (MNPs) to the RPE/choroid in aged \( C^b/\sim \)-HFC compared to \( C^b/\sim \)-HFC mice, suggesting a potential role of C5a in the development of AMD-like pathologies seen in this dry AMD model. C5a is increased in the plasma of AMD patients and is a constituent of drusen. C5a was shown to increase the expression of proteins in tissues implicated in AMD such as intercellular adhesion molecule-1 (ICAM1), interleukin-17 (IL-17), interleukin-18 (IL-18), interleukin-22 (IL-22), and vascular endothelial growth factor a (VEGF-A). Intraocular injections of antibodies or receptor trap targeting VEGF-A such as bevacizumab, aflibercept, and ranibizumab are current standard treatments for patients with "wet" AMD, a late manifestation of AMD characterized by choroidal neovascularization (CNV). Similarly, antibodies and an aptamer targeting C5a have decreased the lesion size in the acute laser-induced CNV mouse model of wet AMD and show promise as a potential therapy for wet AMD.

In this study, we tested the role of C5a in the development of early/intermediate AMD-like pathology because therapies targeting C5a have been used successfully to reduce damage in a wet AMD mouse model. Aged \( C^b/\sim \) mice fed a HFC diet were treated with weekly systemic injections of an anti-C5a antibody (4C9, Pfizer, San Francisco, CA, USA). Although anti-C5a therapy has a significant effect in an acute model of retinal degeneration and neovascularization, it did not appear to protect \( C^b/\sim \)-HFC mice from the development of early/intermediate dry AMD-like features, including reduced electroretinogram (ERG) responses, increased RPE damage, increased sub-RPE basal laminar deposits, and increased inflammatory and extracellular matrix (ECM) gene expression. Since C5a acts as a chemotactant and C5a receptor 1 (C5aR1) expression is confined to the choroid in the eye, we quantified immune cell populations in the RPE/choroid after anti-C5a therapy and found reduced MNP recruitment in treated aged \( C^b/\sim \)-HFC mice. These results establish that the blockade of C5a is not sufficient to protect mice from the development of early/intermediate dry AMD-like pathologies in aged \( C^b/\sim \)-HFC despite blocking the recruitment of MNPs to the RPE/choroid.

This observation is in contrast to previous studies as well as our findings that C5a blockade appears to be an efficacious mono- or combinatorial therapy with anti-VEGF for models of wet AMD.

**Materials and Methods**

**Mice**

Mice were housed and maintained in accordance with the Institutional Animal Care and Use Committee at Duke University in adherence with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. \( C^b/\sim \) mice were generated as previously described. We confirmed that none of the mice carried the rd8 mutation. Aged male \( C^b/\sim \) mice (\( n = 67, 91-110 \) weeks) were maintained on a normal rodent chow diet (normal diet [ND], Isopurina 5001; Prolab, Dewitt, NY, USA), and a subset of cage- and littermate mice were switched to a HFC diet (\( n = 38; \) TD 88051; Envigo, Madison, WI, USA) for 8 weeks. Mice were randomly assigned to treatment groups with an even distribution by age.

For studies using the laser-induced CNV or the sodium iodate (NaIO\(_3\)) models, male C57BL/6j aged 8 to 10 weeks were obtained from The Jackson Laboratory (Sacramento, CA, USA). A total of 6 to 12 mice were used per dosing group (\( n = 78 \) total/group). The care and use of mice for both of these studies adhered to Pfizer’s Institutional Animal Care and Use Committee guidelines.

**C5a and VEGF Antibodies**

The anti-C5a antibody, 4C9, was isolated from a phage displayed single chain antibody variable fragment (scFv) library derived from human donors and was provided as a gift from Laird Bloom of Pfizer, Inc. It binds to human and mouse C5a with low nanomolar affinity and blocks binding of C5a to the C5a receptor (C5aR). The affinity of this antibody toward C5a was increased using a yeast surface display system; the resulting higher-affinity clone was subsequently used in the study described here. In brief, the antibody was cloned as a scFv into a yeast display vector and then CDRH2, CDRH3, CDRL1, and CDRL3 of the antibody were individually mutated using look-through mutagenesis. S. cerevisiae BJ5465 harboring the library was subjected to three rounds of fluorescence-activated cell sorting (FACS) with gating strategies designed to isolate higher-affinity clones. DNA encoding the enriched clones was randomly combined and subjected to three additional rounds of FACS followed by individual screening. A higher-affinity clone was identified, expressed as a chimeric monoclonal antibody (human VH and VL domains fused to mouse IgG1 heavy chain and kappa light chain constant regions) in HEK293F cells, purified using standard techniques, binding with human and mouse C5a determined by surface plasmogen resonance (SPR) (Biacore, GE Healthcare, Piscataway, NJ, USA), and then used in this study.

Mouse anti-VEGF-A antibody was constructed as murine IgG1 based on the published sequences of the G6-31 antibody (patent CA2533297A1) that was previously shown to bind mouse and human VEGF with high affinity. In brief, DNA encoding both mouse and human VEGF with high affinity. Briefly, 8- to 10-week-old C57BL/6j mice were injected into the intraperitoneal (IP) space with 3, 10, 20, 30, or 60 mg/kg anti-C5a or 60 mg/kg isotype control (day 1). The next day, mice were intravenously injected with 25 mg/kg NaIO\(_3\) or PBS control (day 0). On day 2, mice were dark adapted for 24 hours, and on day 3, ERGs were performed as described below at flash intensities of 1E-4, 1E-3, 1E-1, and 2.5 (cd/s/m\(^2\)).

**NaIO\(_3\) Treatment and Anti-C5a Dose Response (4C9)**

NaIO\(_3\) treatment was performed as previously described in order to determine the appropriate dose of anti-C5a therapy needed to observe a therapeutic effect in the posterior eye. Briefly, 8- to 10-week-old C57BL/6j mice were injected into the intraperitoneal (IP) space with 3, 10, 20, 30, or 60 mg/kg anti-C5a or 60 mg/kg isotype control (day –1). The next day, mice were intravenously injected with 25 mg/kg NaIO\(_3\) or PBS control (day 0). On day 2, mice were dark adapted for 24 hours, and on day 3, ERGs were performed as described below at flash intensities of 1E-4, 1E-3, 1E-1, and 2.5 (cd/s/m\(^2\)).

**Antibody C5a Treatment of \( C^b/\sim \) Mice and Laser CNV Model**

\( C^b/\sim \) mice were treated with 30 mg/kg anti-C5a via a weekly IP injection. Injections began at the onset of HFC diet and were continued during the 8 weeks of diet.
For the laser CNV model, 30 mg/kg anti-C5a was administered with or without anti-VEGF at 3, 5, or 10 mg/kg 1 day prior to CNV induction and at days 5 and 11 post laser CNV.

**Laser-Induced CNV Model**
Experimental CNV was induced bilaterally in C57BL/6J mice at 8 to 10 weeks of age. Animals were anesthetized with IP injection of ketamine (60 mg/kg) and xylazine (9 mg/kg). Animals were pretreated with 1% atropine (atropine sulfate, 1 drop) and tropicamide (1%, 1 drop) eye drops. A coverslip was placed over the cornea with a large drop of Gonisol. Three 532-nm diode laser pulses with a spot size of 50 μm, duration of 0.1 second, and power of 110 mW (OcuLight TX; IRIDEX, Mountain View, CA, USA) were applied between retinal vessels at 12, 4, and 8 o’clock, two disc diameters from the optic nerve, with the use of a slit-lamp and ophthalmologic microscope (SL980/5X; IRIDEX). Formation of a bubble at the time of laser application, which indicates rupture of BrM, was an important factor in obtaining laser-induced CNV. Only laser burns in which a bubble was produced were included in the study. Any lesions that did not yield the characteristic white “bubble” formation or any hemorrhaging lesions automatically resulted in removal of the animal from the study. A total of 6 to 12 mice were used per dosing group.

**Analysis of CNV by Scanning Laser Angiography**
Angiography was performed at day 12 post laser treatment with a scanning laser ophthalmoscope (SLO) using the Spectralis Heidelberg Retina Tomograph (HRT)/SLO (Heidelberg Engineering, Dossenheim, Germany), which was used as a digital confocal SLO (cSLO) since it is equipped with four laser wavelengths (488, 514, 788, and 820 nm) and filters for fluorescein and indocyanine green (ICG) fluorescence. To adapt the commercial system to the optics of the mouse eye, we used a 55-diopter lens as a front objective (Linos Optics, Milford, MA, USA) instead of the 40-mm focal lens used for a human eye. Pupils were dilated with topical 1% atropine. Mice were anesthetized by IP injection of ketamine (60 mg/kg) and xylazine (9 mg/kg) and kept on a heated pad during the procedure. Plasma labeling by sodium fluorescein (Akorn, Inc., Lake Forest, IL, USA) was used for choroidal vessel imaging. Five hundred microliters of 2 mg/ml sterile sodium fluorescein solution was injected IP 8 minutes prior to cSLO imaging. The number of enlarged, multinucleated (nuclei ≥ 5) cells per central field view was counted.

**Quantitative CNV Lesion Analysis**
In order to analyze the fluorescein fundus angiography, image analysis was performed with open-source software, ImageJ (National Institutes of Health, Bethesda, MD, USA). A masked grader performed quantification of the CNV lesion size. In brief, for the quantification, raw images of angiography were imported into ImageJ and the CNV lesion was demarcated using the freehand selection tool, avoiding large vessels. The volume and brightness of the lesions were quantitated with background optical density from the neighboring to CNV region subtracted as arbitrary units. Analysis of variance and post hoc t-test were used to assess the statistical significance between the treatment groups.

**Anti-C5a ELISA**
Anti-C5a (4C9) levels were determined by ELISA. Briefly, mC5a (no. 2150-c5; R&D Systems, Minneapolis, MN, USA) was incubated at 4°C overnight in 96-well plates. After washing unbound protein and blocking with 2% BSA, a 1:1000 dilution of plasma was incubated overnight at 4°C. Following wash steps, the plate was sequentially incubated with biotinylated anti-mouse IgG (H and L) HRP (1:5000; BD Biosciences, San Jose, CA, USA), TMB substrate reagent (BD Biosciences) and the OD 450 nm read; 0.31 to 200 ng/ml anti-C5a (4C9) was used as a standard curve.

**Analysis of Sub-RPE Basal Laminar Deposits by Transmission Electron Microscopy**
Quantification of sub-RPE basal laminar deposits was performed as previously described. Briefly, thin sections of 60 to 80 nm were cut from sections obtained from the center of the posterior eyecup and stained with uranyl acetate and Sato’s lead. Images containing the basal RPE and BrM were taken on the parts of the section adjacent to the grid’s bar throughout the length of the retina. For each image, the thickness of the deposits was measured from the central elastin layer of BrM to the top of the deposit using ImageJ software.

**Analysis of RPE Damage Based on Flat Mount Quantification of Multinucleated Cells**
RPE flat mount preparation was performed as previously described. Briefly, RPE flat mounts were stained with a rabbit antibody against zona occludens-1 (ZO-1) (40–2200, 1:100; Invitrogen, Carlsbad, CA, USA) and Hoechst 33342, and confocal images were captured on a Nikon Eclipse C1 microscope (New York, NY, USA). Morphometric analysis was performed by a masked grader from each central quadrant. The number of enlarged, multinucleated (nuclei ≥ 5) cells per central field view was counted.

**In Vivo Visual Function Analysis by Electroretinography**
Electroretinography was performed as previously described. Briefly, mice were dark adapted overnight; pupils were dilated with 0.5% tropicamide and 1.25% phenylephrine, and mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). Scotopic ERGs were recorded using an Espion E2 system (Diagnosys LLC, Lowell, MA, USA) at increasing flash intensities. The data points from the b-wave stimulus-response curves were fitted using the least-square fitting procedure (OriginPro 9.0, OriginLab, Northampton, MA, USA).

**Inflammation and Extracellular Matrix Pathway-Focused RNA Arrays**
Total RNA from four eyecups (RPE/choroid/sclera) from each Cby+/− group was extracted using an RNeasy lipid tissue mini kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer’s instructions. RNA concentrations were determined using the Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Eyecup RNA (600 ng) was used to synthesize cDNA using the RT² First Strand cDNA kit (Qiagen, Inc.). cDNA was combined with RT² SYBR Green qPCR Mastermix (Qiagen, Inc.) and Ultrapure RNase/DNase-free water (Invitrogen). Real-time PCR data analysis of the cDNA was performed on two mouse RT² PCR pathway-focused arrays from Qiagen, Inc. (RT² Profiler Inflammatory Response and Autoimmunity [cat. no. PAMM-077ZD] and Extracellular Matrix and Adhesion Molecule [cat. no. PAMM-013ZD]) following the manufacturer’s protocol. Prior to RNA expres-
Anti-C5a Therapy in a Dry AMD Mouse Model

Ron acceptance, quality controls for genomic contamination, reverse transcription, and positive PCR were checked for each plate to confirm that each plate met quality controls. Relative mRNA expression was normalized to five endogenous reference genes, Actb, B2m, Gapdh, GusA, and Hsp90ab1, for each group using the quantitative 2^{-ΔΔC_{T}} method. All the expression data for the Inflammatory Response and Autoimmunity and the Extracellular Matrix and Adhesion Molecule arrays are presented in Supplementary Tables S1 and S2, respectively. For these analyses, a gene was considered to be upregulated or downregulated if the fold change was greater than 1.25 as previously described and shown in Supplementary Figure S2. Expression of individual genes was examined in triplicate reactions using 25 ng cDNA from a pooled RNA sample consisting of four eyecups from each group (Gfb^−/−~ND, Gfb^+/−~HC, and Gfb^+/+~HC + antiC5a), 200 nmol/L each primer, and 12.5 l IQTM SYBR green supermix (Bio-Rad, Hercules, CA, USA) in 25 l total volume. Primer sequences used in this study are shown in Supplementary Table S3. RT-PCR reactions were run in the CFX96 system (Bio-Rad) at 95°C for 3 minutes, followed by 40 cycles at 95°C for 10 seconds and 60°C for 20 seconds, then 72°C for 15 seconds. Relative mRNA expression was normalized to the endogenous reference murine gene ribosomal protein lateral subunit P0 (Rlpl0) using the quantitative 2^{-ΔΔC_{T}} method.

Tissue and RNA Isolation, and RT-PCR

Three C57BL/6j mice were euthanized with CO2 and perfused with 15 mL PBS. After perfusion, eyes were enucleated for dissection where fat, muscle, and anterior segment were removed. Neural retina was carefully separated from the dissection where fat, muscle, and anterior segment were removed. Neural retina was carefully separated from the posterior eye, placed into a separate Eppendorf tube, and flash frozen. RPE cells were isolated using a modified protocol described previously. Briefly, RPE/chorioid/sclera was placed into an Eppendorf tube with 1 mL RNAProtect Cell Reagent (Qiagen, Inc.) and left overnight at 4°C. The next day, chorioid/sclera was removed, rinsed with PBS, and placed in a separate Eppendorf tube for RNA isolation. RPE cells were spun and RNAProtect was removed from tube. Both chorioid/sclera and RPE tissues were flash frozen. Human RPE cells from a 71-year old male human donor eye with a 3.5-hour procurement time and the BrM and choroid were peeled from the sclera. This “choroid” sample was immediately frozen. Total RNA from each of these tissues, isolated from murine retina, RPE, and chorioid/sclera as well as human RPE and choroid, was extracted using an RNasy lipid tissue mini kit (Qiagen, Inc.) according to the manufacturer’s instructions. RNA concentrations were determined using the Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific). RNA from ARPE-19 cells was generously provided by Goldis Malek (Duke University) and was isolated as previously described. RNA (150 ng) from each sample was used to synthesize cDNA using ProtoScript II Reverse Transcriptase (NEB, Ipswich, MA, USA). Each RT-PCR reaction contained 7.5 ng cDNA, 200 nmol/L each primer, and 12.5 l IQTM SYBR green supermix (Bio-Rad) in 25 l total volume. All primers used in this study span an exon–exon splice junction and are described in Supplementary Table S3. RT-PCR reactions were run in triplicate in the CFX96 system at 95°C for 3 minutes, followed by 40 cycles at 95°C for 10 seconds and 60°C for 20 seconds, then 72°C for 15 seconds. Relative mRNA expression was normalized to the endogenous reference gene beta-actin using the quantitative 2^{-ΔΔC_{T}} method.

Peripheral Blood and Extravascular RPE/Choroid MNP Analysis by Flow Cytometry

Intravascular staining of circulating immune cells was performed to differentiate circulating immune cells within the blood vessel from the extravascular immune cells that had migrated into the tissue of the posterior eye as previously described. Briefly, 5 minutes prior to euthanasia, mice were injected retro-orbitally with 3 μg/50 μL APC/Cy7 anti-mouse CD45 (BioLegend, San Diego, CA, USA) (CD45-IV) to label the intravascular immune cells. Peripheral blood samples were subjected to red blood cell lysis and staining for cell viability (no. 65-0865; eBiosciences, San Diego, CA, USA) and labeled with antibodies against CD45 (no. 110735, BioLegend), CD11b (no. 562950, BD Biosciences), CD115 (no. 61-1152, eBiosciences), CD14 (no. 562866; BD Pharmingen, San Jose, CA, USA), Ly6C (no. 128022, BioLegend), and Ly6G (no. 127621, BioLegend) to determine the percent of classical and nonclassical monocytes per CD45+ cells based on established methods. Both eyes were enucleated, retina was removed, and the RPE/chorioid/sclera from each eye was isolated by microdissection. The RPE/chorioid was mechanically removed from the subadjacent sclera. Six eyes were pooled to obtain cell numbers sufficient for analysis. RPE/chorioid samples then underwent DNase I and collagenase treatment with mechanical stimulation to free immune cells. Subsequently samples were filtered and stained for cell viability, and labeled with antibodies against CD11b, CD45, Ly6C, Ly6G, CCR2 (R&D Systems, no. FAB5538A), CD11c (BD Biosciences, no. 563048), CD64 (BioLegend, no. 139308), F4/80 (BioLegend, no. 125109), and I-A/E (BioLegend, no. 107629). All samples were run on a BD Biosciences Fortessa flow cytometer using BD FACSDiva software (BD Biosciences). Gating is shown in figures and was performed as previously described in RPE/chorioid. With N = 6 eyes per group, two independent experiments were performed to confirm the data trend; the average of the peripheral blood analysis experiments is presented, and a representative experiment of the RPE/chorioid data is presented. *χ2 statistical test for P < 0.05 was used to determine the statistical significance of the cell population frequencies normalized to total in the intra- or extravascular space CD45+ cells for a pooled sample.

RESULTS

Anti-C5a Dosage and Efficacy in an Acute Model of Retinal Degeneration and of Neovascularization

The dose response of our novel mouse monoclonal anti-C5a antibody, 4C9, was determined in the NaIO3 model of retinal degeneration. Administration of NaIO3 results in the ablation of scotopic b-wave in C57BL/6j mice. ERGs were performed 3 days after mice were treated with either an intravenous injection of 3, 10, 20, 30, or 60 mg/kg anti-C5a or 60 mg/kg isotype control and were then subjected to an intravenous injection of NaIO3. We observed a statistically significant rescue in ERG responses in mice receiving the 20, 30, and 60 mg/kg doses of anti-C5a at the 0.1 (P < 0.05) and 2.5 (P < 0.001) cd/s/m2 flash intensity (Fig. 1A).
Anti-C5a Treatment in a Dry AMD Mouse Model

We validated the intermediate anti-C5a dosage (30 mg/kg) in the laser-induced CNV model for wet AMD.\(^1\)\(^4\)\(^1\)\(^2\) The antiangiogenic effects of anti-C5a therapy were measured by fluorescein angiography in the rodent CNV model by scanning laser ophthalmoscopy. Neovascular lesion size was measured following administration of either anti-C5a and/or the standard of care, anti-VEGF, in a dose–response manner. Lesion size was used to determine whether (1) the anti-C5a antibody alone reduces CNV lesion size and (2) combination therapy with anti-VEGF synergizes with complement blockade to further enhance the therapeutic effect of VEGF inhibition (Supplementary Fig. S1). Anti-C5a therapy significantly reduced CNV lesion size in mice compared to IgG control at 30 mg/kg (P < 0.05) (Fig. 1B). In addition, administration of 30 mg/kg anti-C5a, as a combinatorial therapy, significantly enhanced the efficacy of subtherapeutic doses of anti-VEGF at 2 and 5 mg/kg (P < 0.05) (Fig. 1B). Thus, the anti-C5a antibody is able to reduce CNV lesion size as a monotherapy and as a combination therapy with anti-VEGF as supported by others.\(^8\)\(^2\)\(^5\)\(^2\)\(^4\)

Anti-C5a Treatment in Aged \(Cfb^{+/−}\)\/~HFC Mice

In our previous study of aged \(Cfb^{+/−}\) and \(Cfb^{−/−}\) mice fed a HFC diet we observed a striking correlation between complement dysregulation and RPE damage and visual function impairment.\(^1\)\(^3\) These findings make the \(Cfb^{+/−}\)~HFC a suitable model to test the effects of C5a with an anti-C5a therapy on early/intermediate dry AMD-like features. \(Cfb^{+/−}\) mice aged at least 90 weeks were switched to a HFC diet and administered weekly IP injections of 30 mg/kg anti-C5a for 8 weeks. Plasma anti-C5a antibody levels ranged from 119 to 179 \(\mu\)g/mL 24 hours post injection, and after 7 days, antibody levels ranged from 58 to 99 \(\mu\)g/mL (Fig. 2A). Following 8 weeks of weekly IP injections, anti-C5a levels ranged from 153 to 80 \(\mu\)g/mL (mean of 99.4 \(\mu\)g/mL ± 26.8) 24 hours post final injection (Fig. 2B). Thus, sufficiently high plasma levels were maintained throughout the 8 weeks of HFC diet treatment in \(Cfb^{+/−}\)~HFC mice.

Anti-C5a Therapy Does Not Reduce Early/Intermediate Dry AMD-Like Features in \(Cfb^{+/−}\)~HFC Model

Extracellular lesions that form between the RPE and BrM characterize early AMD.\(^1\)\(^3\) The effect of anti-C5a therapy on the thickness of sub-RPE basal laminar deposits that accumulate in \(Cfb^{+/−}\)~HFC mice was analyzed by quantitative electron microscopy of RPE/BrM as previously described.\(^2\)\(^2\) As we have previously shown, the majority of the sub-RPE deposits seen in the \(Cfb^{+/−}\)~HFC model were 2- to 3-\(\mu\)m basal laminar deposits, and this did not change following anti-C5a therapy (Fig. 3A).\(^1\)\(^3\) Quantitative analysis of sub-RPE basal laminar deposit thickness confirmed that anti-C5a therapy did not affect the amount of sub-RPE basal laminar deposits that accumulate in \(Cfb^{+/−}\)~HFC mice (Figs. 3B, 3C), suggesting that C5a blockade is not sufficient to inhibit the accumulation of sub-RPE basal laminar deposits in the \(Cfb^{+/−}\)~HFC mouse model.

We measured the damage to the RPE cells in aged \(Cfb^{+/−}\) mice by quantifying dysmorphic, multinucleate RPE cells on RPE flat mounts stained with Hoechst 33342 to detect the nuclei and immunostained with the tight junction-associated protein, ZO-1, to reveal the cell borders (Fig. 3D). As previously reported, exposure to a HFC diet resulted in a statistically significant increase in the number of enlarged, multinucleate RPE cells in the \(Cfb^{+/−}\)~HFC mice (P < 0.05) (Fig. 3E).\(^1\)\(^3\) We found no statistical difference between \(Cfb^{+/−}\)~HFC mice treated with and without anti-C5a therapy (P > 0.05) (Fig. 3E). These
FIGURE 2. Sustained high levels of circulating antibodies are achieved with weekly injections of 30 mg/kg anti-C5a therapy. (A) Mice were injected IP with 30 mg/kg anti-C5a at day 0 and bled at days 1, 3, and 7 to establish the circulating antibody levels maintained throughout the week following weekly 30 mg/kg anti-C5a dosing. Antibody levels were determined by anti-C5a ELISA on plasma samples. (B) At the end of 8 weeks, most mice treated with 30 mg/kg anti-C5a maintained high circulating levels of anti-C5a therapy.

FIGURE 3. Anti-C5a therapy does not affect dry AMD-like features in Cfh+/− HFC mice. (A) Representative transmission electron micrograph images of basal laminar deposits along Bruch’s membrane (BrM). Large (>2 μm) deposits were often seen in the Cfh+/− HFC and Cfh+/− HFC mice treated with 30 mg/kg anti-C5a, while only minimal sub-RPE deposit could be detected in aged-matched Cfh+/− HFC mice fed a normal diet (Cfh+/− ND). Scale bars: 1 μm. (B) Quantitative analysis of sub-RPE basal laminar deposits using cumulative frequency curves shows that no change in the size of sub-RPE basal laminar deposits was detected between Cfh+/− HFC mice and Cfh+/− HFC mice treated with anti-C5a therapy. (C) Quantitative analysis of mean deposit size per mouse shows no difference could be detected between Cfh+/− HFC mice and Cfh+/− HFC mice treated with anti-C5a therapy. Asterisk (*) indicates statistical significance at $P = 0.05$ by Student’s t-test analysis. (D) Confocal fluorescence images of central RPE flat mounts from >90-week-old Cfh+/− mice fed a ND or HFC diet treated with 0 or 30 mg/kg anti-C5a that were stained with Hoechst 33342 (blue, nuclei) and anti-ZO-1 (green) and imaged with the RPE apical side up with the neural retina removed. In Cfh+/− HFC mice there are many more enlarged, multinucleate cells following HFC diet, whereas in some mice RPE cells appear to be protected from damage in Cfh+/− HFC mice treated with 30 mg/kg anti-C5a. Scale bars: 20 μm. (E) Quantification of multinucleate (nuclei ≥ 3) RPE cells per field view demonstrates that some Cfh+/− HFC mice treated with 30 mg/kg anti-C5a appear to be protected, but no statistically significant differences are detected. Asterisk (*) indicates statistical significance at $P < 0.05$ by Student’s t-test analysis. (F) Scotopic electroretinogram (ERG) flash responses in Cfh+/− fed a ND or HFC diet treated with 0 or 30 mg anti-C5a. Data are expressed as mean ± SE of the stimulus-response curve overlaid with $B = (B_{max1} \times I / I + 11) + (B_{max2} \times I / I + 112)$ comparing ND (black), HFC (green), and HFC treated with 30 mg/kg anti-C5a (pink). Cfh+/− HFC mice ≥ anti-C5a treatment were significantly worse following HFC diet. Asterisk (*) indicates statistical significance at $P < 0.05$ by Student’s t-test analysis for the indicated flash intensities comparing Cfh+/− ND and Cfh+/− HFC.
Cfh-/- mice develop a statistically significant increase in the expression of these genes in C5aR1-/- mice compared to Cfh-/- ND mice (Fig. 4). Interestingly, of these there was only a statistically significant increase in the expression of the inflammatory genes Ccl2, Ccl8, Ccl12, Cxcl11, Cxcl10, Il-1beta, Icam1, Ctnn1, Col2a1, Mmp13, and Vcan were detected in both Cfh-/- and Cfh-/- HFC mice treated with anti-C5a antibody and suggest that the HFC-associated increases of these genes is C5a independent. Interestingly, statistically significant increases in Ccl2 were observed in Cfh-/- HFC mice treated with anti-C5a antibody compared to Cfh-/- HFC mice. Data are presented as normalized averages of triplicate RT-PCR reactions relative to four pooled eyecups from normal diet age-matched Cfh-/- mice (Fig. 4). Error bars represent standard deviations. Asterisk (*) represents statistical significance at P < 0.05 using ANOVA statistical analysis with post hoc Tukey test comparing gene expression between four pooled eyecups of normal and HFC diet-fed mice or mice fed a HFC diet and those fed a HFC diet and treated with anti-C5a.

Anti-C5a Therapy Does Not Ameliorate HFC-Induced Inflammatory and ECM Gene Expression in the Eyecups of Aged Cfh-/- Mice

After obtaining a profile of inflammatory and ECM gene expression in the eyecups isolated from aged Cfh-/- mice fed the HFC diet, we validated the expression of a subset of these genes that had a fold change of 1.25 or more (Fig. 4). We also investigated the effect of blocking C5a on the expression of these same genes in Cfh-/- HFC mice (Fig. 4). There was a statistically significant increase in the expression of the inflammatory genes Ccl2, Ccl8, Ccl12, Cxcl11, Cxcl10, Il-1beta, Icam1, Ctnn1, Col2a1, Mmp13, and Vcan plus Icam1 (gene for an intracellular adhesion molecule on ECM array) in the pooled eyecups of Cfh-/- HFC mice compared to Cfh-/- ND mice (Fig. 4). Expression of these sampled genes was increased in eyecups of the Cfh-/- HFC mice treated with anti-C5a, but no decreases were observed comparing Cfh-/- HFC mice and Cfh-/- HFC mice treated with anti-C5a (Fig. 4). Interestingly, of these there was only a statistically significant increase in Ccl2 in Cfh-/- HFC mice treated with anti-C5a antibody compared to Cfh-/- HFC mice (Fig. 4).

Complement Component 5a (C5a) Receptor Is Predominantly Expressed in Mouse and Human Choroid but Not in Mouse and Human RPE

C5a has been shown to increase the expression of inflammatory and ECM remodeling genes such as Ccl2 and Icam1 in the murine posterior eye.13,26 but the anti-C5a therapy did not change the expression of these genes in the eyecups of Cfh-/- HFC mice. To localize the effect of the anti-C5a therapy in the posterior eye we investigated the relative expression levels of the C5a receptor, complement component 5a receptor 1 (C5aR1), in mouse and human retina, RPE, and choroid/sclera and human choroid tissues. The relative expression of C5aR1 was measured by RT-PCR in different regions of the mouse eye using posterior eye tissue-enriched RNA isolated from the neural retina, RPE, and choroid. We examined the expression of rhodopsin (Rbo) (Fig. 5A), retinaldehyde-binding protein 1 (Rhbp1) (Fig. 5B), RPE-specific protein 65 kDa (Rpe65) (Fig. 5B), and tyrosine kinase with immunoglobulin and epidermal growth factor homology domain-2 (Tie2) (Fig. 5C) to confirm the enrichment of the retinal, RPE, and choroidal/sclera and human choroid tissues. The majority of C5aR1 (Fig. 5D) was in choroidal/sclera-enriched samples compared to RPE-enriched and neural retina samples.

Based on the low levels of C5aR1 transcript detected in the mouse RPE-enriched sample compared to the amount detected in the choroid/sclera, it is possible that the RPE signal is due to contamination of the mouse RPE-enriched RNA with choroidal RNA, and that murine RPE cells do not express the C5aR1. To support this, we examined the relative expression of the human C5aR1 in human RPE and choroid-enriched RNA samples as well as ARPE-19 cells, an immortalized human RPE...
cell line that has been shown to express C5AR.22 The purity of the RPE- and choroid-enriched tissues we isolated was tested by RT-PCR, as described above, for human RPE65 (Supplementary Fig. S3A), RLBPI (Supplementary Fig. S3A), and TIE2 (Supplementary Fig. S3B). Expression of RLBPI, but not RPE65, was detected in ARPE-19 cells (Supplementary Fig. S3A) and confirms previously published findings.47 Examination of C5AR1 expression using these samples revealed no C5AR1 expression in the human RPE-enriched sample while C5AR1 was abundantly expressed in the human choroid-enriched sample (Supplementary Fig. S3C). Interestingly, there was no detection of C5AR1 in ARPE-19 cells (Supplementary Fig. S3C). In summary, we conclude that the majority of C5AR1 occurs in the choroid while there is little or no expression of C5AR1 in the RPE.

Anti-C5a Therapy Ameliorates Classical Monocytosis Seen in Cbfβ−/− HFC Mice

In our previous study of aged Cbfβ−/− and Cbfβ+ mice fed a HFC diet we found a correlation between complement dysregulation in the Cbfβ−/− mice with peripheral monocytosis and increased MNPs in the choroid.13 These findings made the Cbfβ−/− HFC a relevant model to test the effects of anti-C5a therapy on monocyte levels and MNP recruitment to the choroid. In the peripheral blood an established gating strategy13,38 was used to determine the percentage of classical and nonclassical monocytes (Fig. 6A).13 As expected, polymorphonuclear leukocytes (PMNs) (P < 0.05), classical monocytes (P < 0.05), and nonclassical monocyte populations were increased in old Cbfβ−/− mice following 8 weeks of HFC diet compared to age-matched controls maintained on a ND. Interestingly, the classical monocyte population in the Cbfβ−/− HFC mice was decreased following anti-C5a treatment, while PMNs were elevated and nonclassical monocytes were unchanged (Fig. 6B). Thus, systemic anti-C5a therapy ameliorates the classical monocytosis (P < 0.05) detected in the peripheral blood of Cbfβ−/− mice following HFC diet.

Anti-C5a Therapy Blocks Recruitment of Monocytes Into the Choroid

Intra- and extravascular flow cytometry was used to test the hypothesis that anti-C5a therapy blocks MNP recruitment in the RPE/choroid. We used a previously established gating strategy to distinguish the intra- and extravascular space using an intravascularly injected anti-CD45 antibody (Fig. 7A).13 Within the extravascular space there were statistically significant increases in total leukocytes (P < 0.05), in PMNs, and within all MNP populations (Ly6Ch/CD64hi classical monocytes, Ly6Ch/CD64hi, and CD64+, P < 0.05) in Cbfβ−/− mice following HFC diet (Fig. 7B). Anti-C5a therapy blocked the increases in total leukocytes (P < 0.05), PMNs, classical monocytes, and Ly6Ch/CD64hi MNPs (P < 0.05) but not CD64+ MNPs within the RPE/choroid extravascular space (Fig. 7B). Together these results demonstrate that systemic anti-C5a therapy blocks monocyte recruitment to the choroid of Cbfβ−/− HFC mice.

DISCUSSION

AMD is a multifactorial disease characterized by early features including reduced rod-mediated light sensitivity, slower dark adaptation, RPE pigmentary changes, and drusen accumulation.2–4,48 Genetic, biochemical, and clinical studies have converged to implicate the complement system in the pathogenesis of AMD development and progression. However, understanding the role of complement in the pathogenesis of AMD is critical for the development of novel therapies. We recently developed an early/intermediate dry AMD mouse model based on advanced age, alternative pathway dysregulation, and consumption of a HFC diet.13 This aged Cbfβ−/− HFC mouse model of early/intermediate dry AMD can be used to elucidate the roles of activated complement components and to test complement-based therapies in a model of AMD.

We have previously demonstrated that systemic immunotherapy targeting amyloid beta, a known component of drusen,49–51 that induces complement activation,49,50,52,53 can protect from development of an AMD phenotype in the APOE4 mouse model of AMD.34 Using a similar approach, in this study we tested the pharmacologic effects of a monoclonal anti-C5a therapy in aged male Cbfβ−/− HFC mice. Aged Cbfβ−/− fed a HFC diet have decreased scotopic ERG b-wave responses, increased RPE damage, increased sub-RPE basal laminar deposits, and increased expression of inflammatory and ECM genes. We show that anti-C5a therapy fails to ameliorate these HFC-induced pathologies in aged Cbfβ−/− mice. As a proof of drug efficacy, we used the NaIO3 model of retinal degeneration and the laser-induced CNV model of wet AMD to confirm the therapeutic potential of systemically delivered anti-C5a antibody in the posterior eye. Although no complement therapies have been tested in the NaIO3 model to date, our results from the laser-induced CNV model are supported by others.8,23,24 Our data suggest that C5a blockade is not sufficient to stop the
Figure 6. Increase in blood classical monocytes in Cfh+/−HFC mice is ameliorated by anti-C5a therapy. (A) Flow cytometry was performed on peripheral blood mononuclear phagocytes in Cfh+/−ND, Cfh+/−HFC, and Cfh+/−HFC + anti-C5a therapy. (B) An increase in the percentage of classical and nonclassical monocytes (Mo) was seen in Cfh+/− mice following HFC diet, and anti-C5a therapy ameliorated the classical monocytosis. Asterisk (*) represents statistical significance using $\chi^2$ statistical analysis for $P < 0.05$. The data presented represent two independent experiments, each with a single pooled sample with three mice per group.

Figure 7. Extravascular choroidal mononuclear phagocytes are increased in Cfh+/−HFC mice and are ameliorated by anti-C5a therapy. (A) RPE/choroid leukocytes were gated for an intravascularly injected anti-CD45 antibody to distinguish the intra- and extravascular space. Gating for polymorphonuclear leukocytes (PMNs) and three previously established populations of mononuclear phagocyte (MNP) (Ly6Chi [Class Mo], Ly6Clo, and CD64+ MNP) was performed. (B) Within the extravascular space significant increases were seen in total leukocytes, PMNs, and all the MNP subpopulations (Class Mo, CD64+ MNP, and Ly6Clo MNP). Interestingly, anti-C5a therapy significantly blocked the extravasation of total leukocytes and PMNs, as well as Class Mo and Ly6Clo MNP subpopulations. $\chi^2$ test for $P < 0.05$. Data presented represent a single experiment using a pooled sample from three mice per group. A separate independent experiment was performed to confirm trends shown above. Class Mo, classical monocyte.
development of AMD-like pathologies in Cbf<sup>b</sup>/~HFC mice and therefore may not be a viable treatment in early/intermediate dry AMD, although it shows promise as a potential mono- or combination therapy with anti-VEGF in wet AMD.

C5a is a potent anaphylatoxin critical in the recruitment and activation of immune cells to sites of tissue damage. In wet AMD a prominent pathologic hallmark is CNV where choroidal blood vessels proliferate and migrate through BrM into the sub-RPE and subretinal space causing leakage, fibrosis, and eventual tissue damage. A role of immune cells such as dendritic cells, γδ T-cells, neutrophils, and macrophages in CNV has been implicated using the laser-induced CNV mouse model of wet AMD. All of these immune cells have been shown to express the C5a receptor, C5aR1. Therefore, targeting C5a with either antibodies or aptamers should diminish the exudative lesion size by targeting immune cell populations in the laser-induced CNV mouse model of wet AMD as confirmed by our results and others.

Recruitment of immune cells in early/intermediate dry AMD mouse models has been reported, but the role of immune cells on the development of pathologies in these mouse models is not well understood. This study is the first to examine a role for immune cells specifically recruited by C5a during the development of early/intermediate dry AMD-like pathologies in vivo. Our results suggest that recruitment of total leukocytes, PMNs, classical monocytes, and Ly6Clo MNPs is sensitive to C5a in the CNV mouse model of wet AMD. All of these immune cells have been shown to express the C5aR1, C5ar1. Therefore, targeting C5a with either antibodies or aptamers should diminish the exudative lesion size by targeting immune cell populations in the laser-induced CNV mouse model of wet AMD as confirmed by our results and others.

In the current study, there was no significant protective effect from RPE damage or vision loss in Cbf<sup>b</sup>/~HFC mice treated with systemic immunotherapy blocking C5a. There was increased expression of a subset of inflammation- and ECM-related genes in the RPE/choroid/sclera isolated from Cbf<sup>b</sup>/~HFC compared to Cbf<sup>b</sup>/~ND, but this increase was not affected by anti-C5a treatment. Because anti-C5a therapy successfully blocked immune cell recruitment to the choroid we suspect that the increased inflammatory and ECM gene expression detected originates from the choroid and/or RPE, and therefore, we measured the relative expression of the C5AR1 in posterior eye tissues. Previous studies have examined the expression of the C5AR1 in human donor eye tissue and found C5AR1 expression in the choroid and retina as well as in the immortalized human RPE cell line, ARPE-19. We therefore measured the relative C5ar1 levels in RPE, choroid, and retina isolated from mouse eyes. We found much higher C5ar1 expression in isolated choroid RNA compared to the RPE and retina RNA obtained from C57BL/6j mice. Although low levels of C5ar1 expression were detected in the isolated RPE RNA, it is likely that this C5ar1 expression is due to contamination from the choroid based on the signal detected for the endothelial cell marker, Tie2, in the RPE-enriched sample. We obtained similar results using RPE and choroid RNA isolated from a human donor eye and failed to detect C5AR1 expression in ARPE-19 cells. The C5AR1 expression in ARPE-19 measured by RTPCR by Cortright et al. may well be due to genomic DNA contamination since the primers used were nested within a single exon (exon 2). Thus, under normal conditions the majority of C5ar1 expression is confined to the choroid while RPE cells do not appear to express C5AR1 in vivo. As a consequence, blocking C5a would not affect the HFC-mediated damage occurring to the RPE and may help explain why anti-C5a therapy did not ameliorate the AMD-like pathologies seen in Cbf<sup>b</sup>/~HFC mice.

Our results suggest that other components of the complement cascade such as the anaphylatoxin C3a and/or the terminal membrane attack complex (MAC) may be more important for the development of early AMD-like pathologies in Cbf<sup>b</sup>/~HFC mice. A model of Malattia Leventinese/Doyne honeycomb dystrophy, an inherited macular degeneration, using EFEMP1<sup>R146W</sup> knock-in mice and RPE cell culture, suggests that sub-RPE deposit formation is dependent on complement component 3a (C3a), but not on C5a. These results are consistent with our findings that anti-C5a therapy did not affect sub-RPE deposit formation. In addition, we have previously shown that Cfh levels regulate lipoprotein binding and remove endogenous human lipoproteins in BrM. In the absence of CFH or decreased Cfh, lipoproteins can accumulate and provide a scaffold in which complement activation can occur in the sub-RPE region of the posterior eye and lead to the development of early/intermediate dry AMD. Furthermore, there could be a role of the MAC in our animal model that would not be blocked by anti-C5a therapy.

In summary, while no mouse model can faithfully recapitulate all the insults and responses likely to play a role in the development of early/intermediate dry AMD, this model does combine the effects of aging, complement dysregulation, and changes in lipid homeostasis—three known factors involved in AMD pathogenesis. With this caveat, targeting the complement activation product C5a is not sufficient to ameliorate the pathologies seen in the aged Cbf<sup>b</sup>/~HFC mouse model of early/intermediate dry AMD; however, it is able to block the recruitment of MNPs to the RPE-choroid and decrease lesion size in the laser-induced CNV mouse model of wet AMD. Future studies will investigate the role of C5a and MAC in the development of the AMD-like phenotype observed in Cbf<sup>b</sup>/~HFC and determine if pharmacologically targeting C5a or MAC or both could be a viable therapy for early/intermediate dry AMD.

**Acknowledgments**

The authors thank Ying Hao and Marybeth Groelle for their expert technical assistance and Rose Matthews and Joan Kalnitsky for their assistance with flow cytometry. We acknowledge Wei Li, Gabriel Berstein, Huiian Gao, and Laird Bloom of Pfizer, Inc., for providing the neutralizing anti-C5a antibody 4c9 and Li Mei for helping engineer the higher-affinity variant of 4c9 used in this study. We also thank Karen Christopherson of Pfizer, Inc., for her insights and helpful suggestions during the initiation and development of this project.

Supported by National Eye Institute Grants R01 EY026161 (CBR) and P30 EY005722, T32 GM007171-Medical Scientist Training Program (CBR), an unrestricted grant from Research to Prevent Blindness (to the Duke Eye Center), a grant from the BrightFocus Foundation (MK), and a grant from the Foundation Fighting Blindness (CBR). Pfizer, Inc., owns the intellectual properties of the antibodies described in this study.

Disclosure: C.B. Toomey, None; M. Landowski, None; M. Klingeborn, None; U. Kelly, None; J. Deans, None; H. Dong, Pfizer, Inc. (E); O. Harrabi, Pfizer, Inc. (E); T. Van Blarcom, Pfizer, Inc. (E); Y.A. Yeung, Pfizer, Inc. (E); R. Grishanin, Pfizer, Inc. (E); J.C. Lin, Pfizer, Inc. (E); D.R. Saban, None; C. Bowes Rickman, Pfizer, Inc. (F)

**References**


