AMERICAN ACADEMY OF OPHTHALMOLOGY®

Cell-specific Systemic Immune Signatures Associated with Treatment Burden in Neovascular Age-related Macular Degeneration

Joseph B. Lin, BS,[1,2] *Andrea Santeford, MS,*[1] *Darksha Usmani, MS,*[1] *Aaditya V. Shah, MD,*[1] *Philip A. Ruzycki, PhD,*[1] *Rajendra S. Apte, MD, PhD*[1,3,4,5]

Purpose: Choroidal neovascularization (CNV) accounts for the majority of severe vision loss in neovascular age-related macular degeneration (AMD). Despite therapies that target VEGF, patients are often under-responsive, require frequent eye injections to control disease, and eventually lose some vision despite chronic therapy implicating a multifactorial etiology in treatment response. Genetic studies implicate systemic immunity in AMD and systemic immune cells accumulate within CNV lesions, yet a role for these cells in anti-VEGF response remains undetermined. The purpose of this study was to identify transcriptional signatures of circulating immune cells that are associated with high anti-VEGF treatment burden.

Design: Experimental pilot study.

Participants: Patients with neovascular AMD seen at Washington University School of Medicine in St. Louis and BJC Health System.

Methods: We profiled by single cell RNA sequencing the peripheral blood mononuclear cells of 27 treatment-experienced patients with wet AMD. We stratified this cohort into 2 groups with low and high treatment burden ($\leq$ 5 or $\geq$ 6 injections in the past 12 months, respectively).

Main Outcome Measures: Identification of immune cells associated with high treatment burden.

Results: Gene expression signature of CD16+ monocytes may be associated with high treatment burden.

Conclusions: These studies delineate potential signatures of circulating immune cells that may be associated with high treatment burden in neovascular AMD, potentially informing the development of diagnostic predictors of anti-VEGF response and new precision medicine-based approaches to complement anti-VEGF therapies.

Financial Disclosure(s): Proprietary or commercial disclosure may be found in the Footnotes and Disclosures at the end of this article. *Ophthalmology Science 2024;4:100410*

Supplemental material available at www.aaojournal.org.

In the industrialized world, age-related macular degeneration (AMD) is the leading cause of blindness in individuals $>$ 50 to 60 years of age.[1] Age-related macular degeneration affects the central retina, called the macula, and early/intermediate stages are characterized by focal or diffuse lipoprotein-rich deposits called drusen and retinal pigmentary alterations. Genetic factors including polymorphisms in $>$ 30 genes are associated with increased risk of developing AMD. Smoking, uncontrolled hypertension, and a body mass index $>$ 25 are also associated with increased risk of AMD progression. Advanced AMD is classified as neovascular (wet) AMD or atrophic (advanced dry) AMD. Wet AMD is characterized by development of abnormal blood vessels underneath the retina called choroidal neovascularization (CNV) while advanced dry AMD is characterized by atrophic neurodegeneration. Vision loss ultimately occurs from photoreceptor loss in either form of advanced AMD.[2–5] Although wet AMD represents only 10% to 15% of disease burden, it accounts for 80% to 90% of severe vision loss. VEGF targeted pharmacotherapies have revolutionized care in wet AMD.[6] VEGF-A antagonists delivered with intraocular injections reduce the risk of severe vision loss as seen in multiple, randomized clinical trials.[6,7] Despite these advances, several challenges remain in patients with wet AMD. These include, (a) high treatment and caregiver burden with the need for multiple injections in the eye potentially over several years of therapy; (b) rare risk of procedure-related infection; (c)

https://doi.org/10.1016/j.xops.2023.100410
ISSN 2666-9145/23

high cost of some of the medications; (d) suboptimal or transient therapeutic effect in a significant proportion of patients; and (e) partial regression of the initial therapy-associated visual acuity gains at ≥ 5 years of treatment.[6,8–10] A large proportion of patients need continued intraocular injections to maintain treatment effect over many years. At the present time, clinicians are not able to predict *a priori* the treatment response or the need for high treatment burden in a particular patient. As such, there is a significant unmet need to identify molecular biomarkers that may accurately identify factors associated with high treatment burden.

The retina is an immune privileged organ. In neovascular AMD, CNV allows systemic immune cells a conduit to the subretinal space, violating ocular immune privilege. Immune cells are a major component of CNV lesion complexes.[11] Both cellular and soluble components of the innate immune system have been implicated in AMD risk and disease progression. These include polymorphisms in genes that encode complement cascade proteins[5] and dysregulated myeloid cell immune effector function.[8,12] Therefore, immune cells may be an important component of CNV and play a critical role in the development, proliferation, and sustenance of CNV lesions, along with their response to anti-VEGF therapies.

Here, we hypothesized that specific peripheral blood immune cell signatures may be associated with high anti-VEGF treatment burden. In a cohort of anti-VEGF treatment-experienced neovascular AMD patients, we examined systemic immune signatures using single cell RNA sequencing (scRNAseq) to profile peripheral blood mononuclear cells (PBMCs). We stratified this cohort into low and high treatment burden groups using criteria based on randomized clinical trial data[7] and assessed for cell type-specific gene expression trends and whether these were predictive of anti-VEGF treatment burden. In doing so, we potentially identified peripheral immune signatures associated with high anti-VEGF treatment burden. This information may hold prognostic value by potentially enabling early treatment stratification and may generate insights into specific immune mechanisms that contribute to under/nonresponsiveness to anti-VEGF therapy and can be specifically targeted.

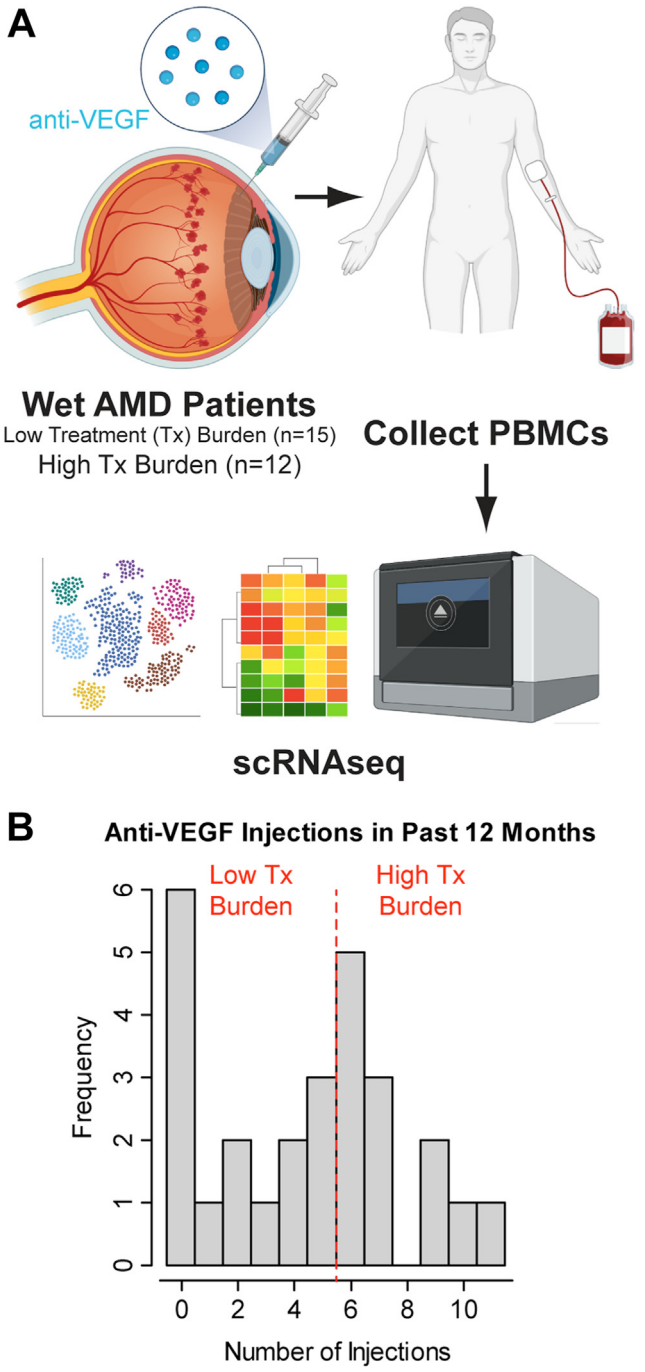

Figure 1. Study scheme. **A,** Schematic of experimental workflow. Created in BioRender. **B,** Histogram showing distribution of anti-VEGF treatment burden in the patients under study. AMD = age-related macular degeneration; PBMCs = peripheral blood mononuclear cells; scRNAseq = single cell RNA sequencing; Tx = treatment.

Methods

Collection of PBMCs From Patients

We obtained informed consent from patients with neovascular AMD (i.e., CNV in ≥ 1 eye) that were undergoing anti-VEGF therapy for management for their disease. We identified any patients undergoing immunotherapy, chemotherapy, or radiation for autoimmune and immune-mediated diseases or cancer. We excluded these patients from further study because these treatments and conditions could affect circulating immune cells. We drew blood by venous blood draw into K_2EDTA-coated BD Vacutainer Venous Blood Collection Tubes. To isolate PBMCs, we centrifuged the blood to isolate the buffy coat and lysed red blood cells to leave nucleated PBMCs behind. We kept PBMCs in 90% heat inactivated fetal bovine serum (FBS) + 10% dimethyl sulfoxide (DMSO) in liquid nitrogen for long-term storage.

scRNAseq of PBMCs

We thawed frozen PBMCs in a 37°C water bath, washed cells twice in Roswell Park Memorial Institute (RPMI) 1640

media containing 10% FBS and 200 KU/mL DNase I. We counted live and dead cells using trypan blue dye exclusion assay with a Tecan automated cell counter (median cell viability 69%). We adjusted the total cell concentration to approximately 700 to 1200 cells/μl. We then performed scRNAseq on the microfluidic-based 10× Genomics platform according to the manufacturer's instructions. We used the Chromium Single Cell 3'v3.1 Reagent Kit. In brief, we used the 10× Genomics Chromium Controller to separate cells into individual droplets containing a barcoded gel bead. Then, we lysed cells and reverse transcribed RNA to cDNA, broke the emulsion, amplified and fragmented cDNA, and added Illumina sequencing adapters. We sequenced the libraries on the Illumina NovaSeq 6000 platform at the McDonnell Genome Institute at Washington University School of Medicine in St. Louis.

Analysis of scRNAseq Data

Import, Quality Control, and Cell Type Annotation of PBMC Dataset. We processed the raw FASTQ sequencing files using CellRanger 4.0.0 with alignment to the 10× Genomics human reference genome (refdata-gex-GRCh38-2020-A). We imported the filtered count matrices into Seurat v4,[13] and assigned each cell a unique identifier to prevent overlap of barcodes between different samples. Using default parameters, we normalized, log-transformed, and scaled the count matrices to remove unwanted sources of variation such as discrepancies in sequencing depth. We identified the top 2000 highly variable genes for principal component analysis. We integrated across samples to account for any sample- or experiment-specific batch effects using the Harmony package.[14] We utilized these harmony embeddings to run uniform manifold approximation and projection dimensional reduction to 2 dimensions.[15] We clustered cells according to the Louvain algorithm and identified marker genes for each cluster using Wilcoxon rank sum tests comparing each cluster to all other clusters. We validated that our manual annotation of cell types was consistent with automated annotation assigned using Azimuth, using the "pbmcref" reference.[13]

Differential Gene Expression Analysis. To identify differentially expressed genes for each cell type comparing control to AMD patients, we used a pseudobulked approach where gene expression of all cells of a particular cell type are averaged by patient. Seurat's differential gene expression analysis with the FindMarkers function was designed for sample sizes of 100 to 1000s of cells and uses the conservative Bonferroni correction. For our pseudobulked comparisons of 10s of patients, we elected to use a less stringent approach by use of the unadjusted P values to determine statistical significance. The usage of unadjusted P values for pseudobulked scRNAseq datasets has been previously described.[16] We used the top 50 gene expression trends with unadjusted P values < 0.05.

Gene Expression Signature Scores. We calculated gene expression signature scores for all PBMCs as well as individual scores for each cell population. We summed the |z-score| of the top 50 gene expression trends.

Pathway Analysis. We performed pathway analysis for the top 50 gene expression trends in CD16+ monocytes using Enrichr.[17–19]

Statistics

Statistical methods for analysis of scRNAseq data are described in the previous section. We assessed for differences between the low and high treatment burden groups: for sex we used a chi-square test; for age and genetic risk scores we used a multiple logistic regression; for injections prior to July 2020, disease chronicity, and for PBMC collection latency we used Mann–Whitney tests. To assess whether the aggregate PBMC gene expression score was predictive of treatment burden, we used a simple logistic regression. To assess whether cell type-specific expression scores were predictive of treatment burden, we used a simple logistic regression for each cell type and corrected for multiple comparisons using a Sidak single-step P value correction.

Table 1. Patient Demographic Data

	Low Tx Burden	High Tx Burden	P Value
Number of injections (July 2020–July 2021)	2 (0–5)	7 (6–11)	
Total, n	15	12	
Male	6	6	0.60*
Female	9	6	
Age	81 (73–93)	76 (64–93)	0.07†
BMI	26.5 (19.1–43.8)	26.9 (17.3–40.2)	
Geographic atrophy	7/15	5/12	
Genetic Risk (Arctic, 0–100)	82 (15–99)	89.5 (65–99)	0.42†
Injections (prior to July 2020)	9 (2–10+)	10 (4–10+)	0.03‡
Disease duration (years prior to July 2020)	2 (1–5+)	4.5 (2–5+)	0.15‡
PBMC collection latency after anti-VEGF Tx (days)	63 (18–365+)	41 (4–365+)	0.15‡

Median (min - max). BMI = body mass index; PBMC = peripheral blood mononuclear cell; Tx = treatment.
*χ^2 test.
†Multiple logistic regression.
‡Mann Whitney test.

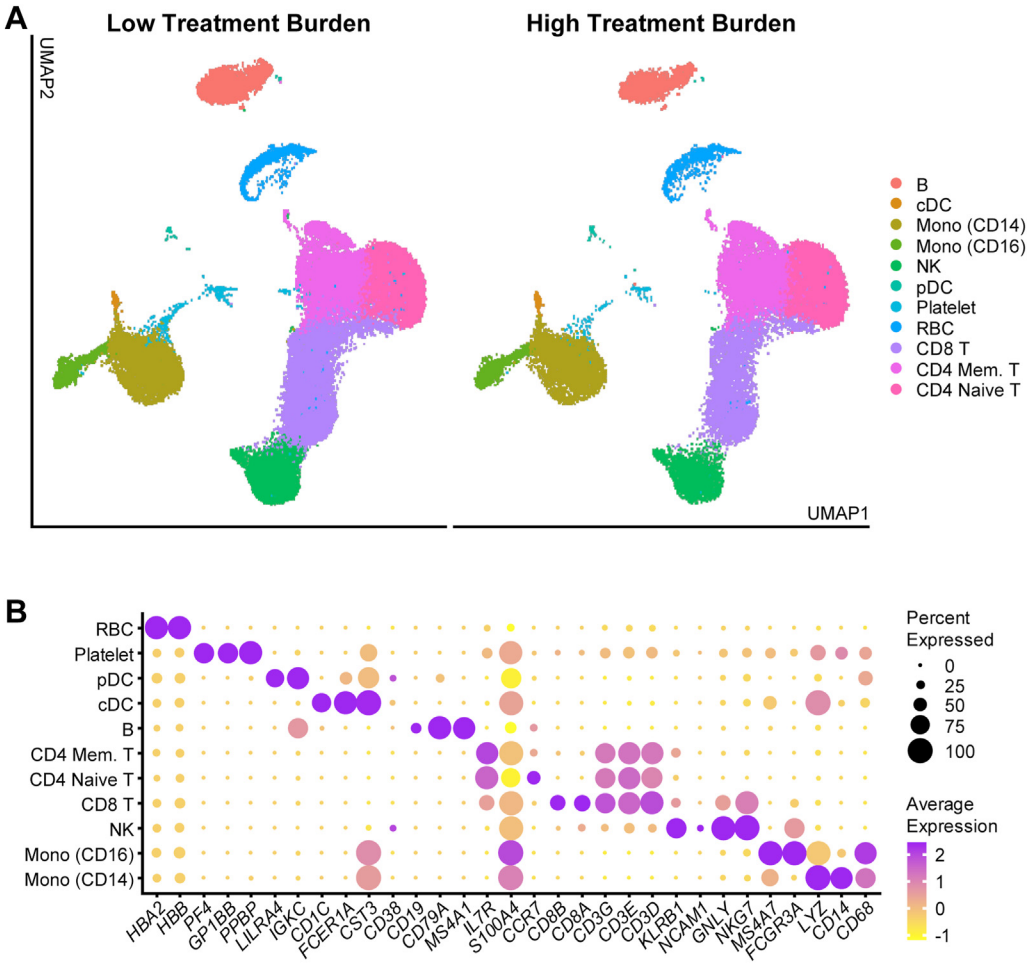

Figure 2. Cell heterogeneity of the peripheral blood immune cells in neovascular age-related macular degeneration patients. **A,** Uniform manifold approximation and projection (UMAP) plot showing the cell heterogeneity of peripheral blood mononuclear cells. **B,** Dot plot showing marker gene expression for each cell type. B = B cell; CD8 T = CD8+ T cells; cDC = classical dendritic cell; CD4 Mem. T = CD4+ memory T cell; CD4 Naive T = CD4+ naive T cell; Mono (CD14) = CD14+ monocyte; Mono (CD16) = CD16+ monocyte; NK = natural killer; pDC = plasmacytoid dendritic cells; RBC = red blood cells.

Study Approval

This study adheres to the Declaration of Helsinki and was approved by the Human Research Protection Office of Washington University School of Medicine in St. Louis. We obtained written informed consent from all subjects prior to enrollment in the study.

Resource Availability

Lead Contact. Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Rajendra Apte (apte@wustl.edu).

Materials Availability. This study did not generate new unique reagents.

Data and Code Availability. Genomic data have been deposited in the Gene Expression Omnibus under the following accession number: GSE222647 (scRNAseq). Patients with neovascular AMD in the low anti-VEGF treatment burden group were: R001, R020, R022, R023, R025, R028, R031, R035, R039, R045, R054, R056, R064, R066, and R073. Patients with neovascular AMD in the high treatment burden group were: R007, R008, R014, R018, R026, R043, R055, R061, R063, R069, R076, and R077.

Results

Patient Characteristics

We recruited patients with neovascular AMD undergoing anti-VEGF therapy (Fig 1A). Blood samples were collected from a period of August 2019 to March 2020. These are patients with chronic, neovascular AMD with a median disease history of 3 years prior to July 2020 (range: 1−5+ years). Additionally, these are treatment-experienced patients with a median number of injections received ≥ 10 prior to July 2020 (range: 2−10+ injections).

In this "prior treatment-experienced group," we assessed their current treatment burden by electronic chart review to

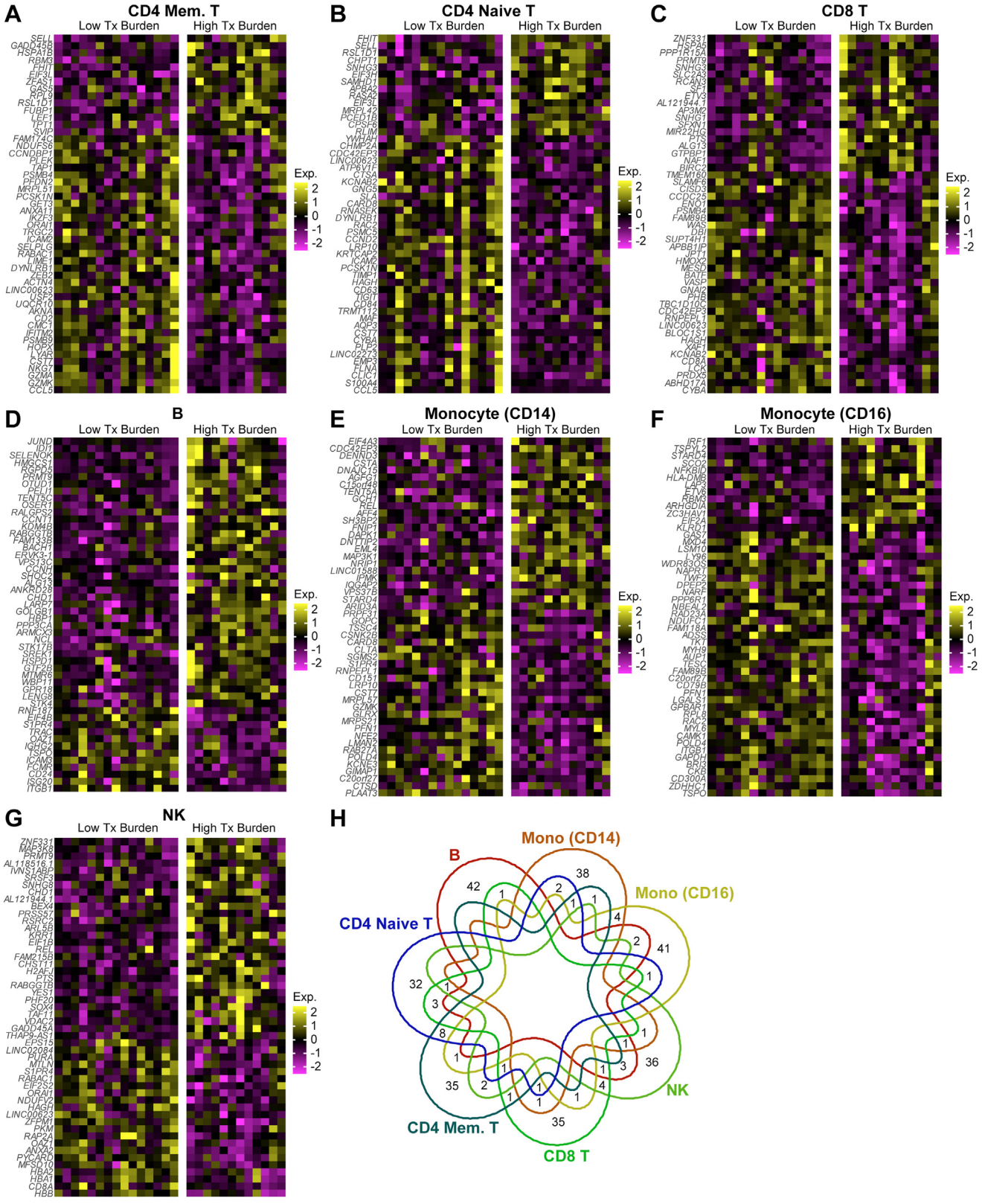

Figure 3. Cell type-specific gene expression signatures associated with anti-VEGF treatment burden in age-related macular degeneration. Heatmaps for top 50 gene expression trends in CD4 memory T cells (**A**); CD4 naive T cells (**B**); CD8 T cells (**C**); B cells (**D**); CD14+ monocytes (**E**); CD16+ monocytes (**F**); and natural killer (NK) cells (**G**). **H,** Venn diagram showing number of gene expression trends in 1 or > 1 cell types. Tx = treatment.

Table 2. Cell Type-specific Gene Signatures Predictive of High Anti-VEGF Treatment Burden

	Odds Ratio	*P* Value (Adjusted*)
CD4 Mem. T	1.08	0.07
CD4 Naive T	1.11	0.09
CD8 T	1.05	0.06
B	1.18	0.20
Mono (CD14)	1.20	0.13
Mono (CD16)	1.09	0.04
NK	1.25	0.26

CD = cluster of differentiation; NK = natural killer.
*Adjusted using Sidak single-step method.

determine the total number of anti-VEGF injections received in a 12 month period between July 2020 and July 2021 (Fig 1B). Based on the number of injections received from July 2020 to July 2021, we stratified patients into 2 groups as guided by prior clinical trial data. We based our criteria on a prior study regarding the average number of anti-VEGF injections in 1 year when using a *pro re nata* approach for making treatment decisions.[7] In this multicenter randomized controlled trial, the "as needed" treatment arms received an average of 6 to 7 injections per year.[7] Given that all the patients in our cohort were treated with a similar approach (i.e., as needed based on the presence or absence of fluid), we stratified treatment burden groups based on an average of 6 injections per year. The low treatment group received 0 to 5 injections during that period (n = 15), whereas the high treatment group received 6 to 11 injections (n = 12).

We first assessed if treatment burden was associated with disease duration, past number of injections received, age, or genetic risk (Table 1). There was no difference in disease chronicity between the low and high treatment groups (Mann Whitney test, *P* value = 0.15). The low treatment burden group had received significantly fewer anti-VEGF injections than the high treatment burden group prior to July 2020 (Mann Whitney test, *P* value < 0.05). We found that neither age nor AMD Vita Risk genetic score (Arctic Medical Laboratories) was predictive of high treatment burden (multiple logistic regression, *P* value for age = 0.07, *P* value for genetic risk score = 0.42).

Anti-VEGF therapy is delivered locally to the eye via intravitreal injections. The volume delivered is small compared to total body blood volume (ie, 0.05 mL vs. 5 L, respectively) and it is unlikely that anti-VEGF agents delivered locally to the eye would have substantial effects on the bulk population of peripheral blood immune cells. Nonetheless, we compared the latency from anti-VEGF injection to PBMC collection, which ranged from 4 to > 365 days, and found that there was no significant difference between the low and high treatment burden groups (Mann−Whitney test, *P* value = 0.15).

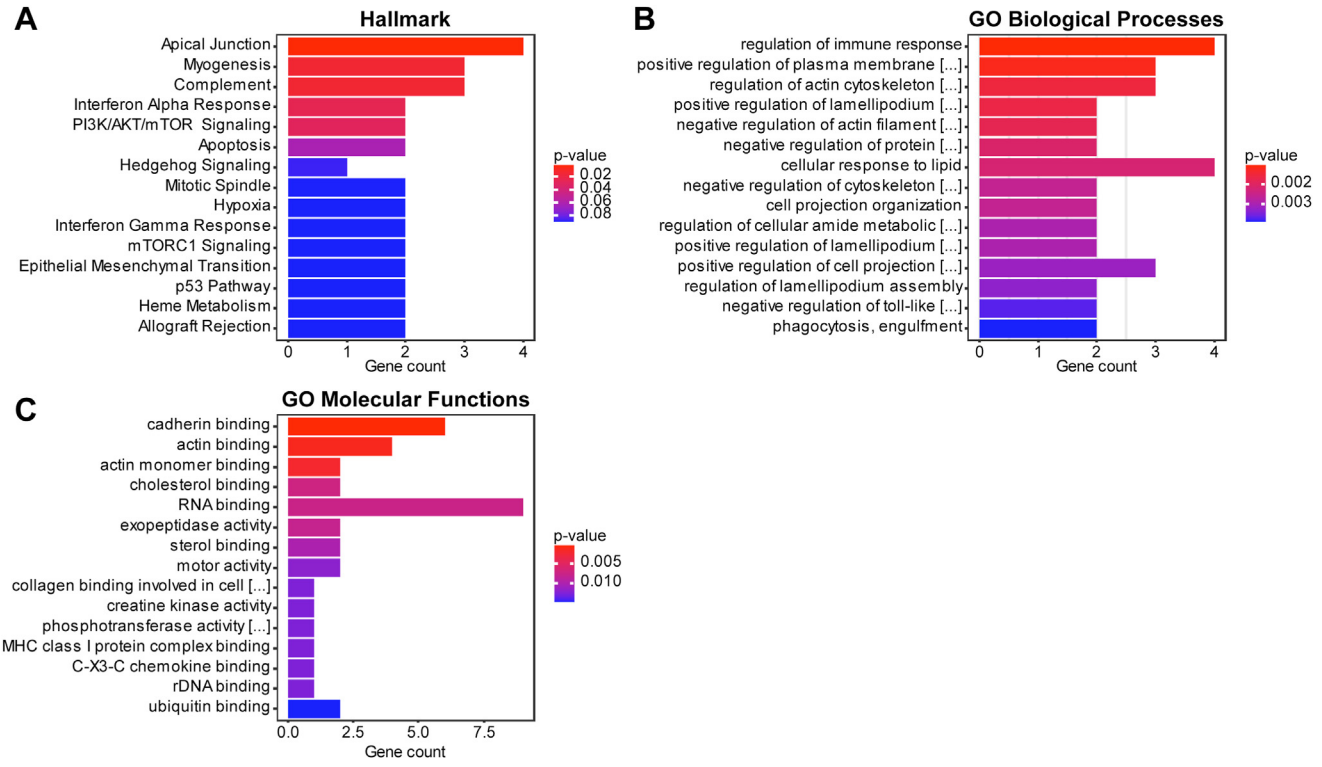

Figure 4. Pathways dysregulated in CD16+ monocytes in age-related macular degeneration (AMD) patients with high anti-VEGF treatment burden. Pathway analysis for the top 50 gene expression trends in CD16+ monocytes in AMD patients with high anti-VEGF treatment burden including hallmark pathways (**A**); Gene Ontology (GO) biological processes (**B**); and GO molecular functions (**C**). AKT = protein kinase B; CD = cluster of differentiation; mTOR = mammalian target of rapamycin; mTORC1 = mammalian target of rapamycin complex 1; PI3K = phosphoinositide 3-kinase; rDNA = ribosomal DNA.

Systemic Immune Cell Heterogeneity in Treatment-experienced Wet AMD Patients

To profile circulating immune cells, we isolated PBMCs from blood samples. We performed scRNAseq on the 10× Genomics Chromium platform. After filtering out low quality cells, we obtained gene expression data for 65 863 cells across 36 601 genes. On average, we detected 5358 transcripts and 1616 unique genes per cell. After integration using Harmony to account for batch effects,[14] we identified 11 distinct immune populations based on known cell type markers (Figs 2A, B and S1A–C). These were: 3 populations of T cells including CD8+ T cells, CD4+ memory T cells, and CD4+ naïve T cells; natural killer cells; B cells; 2 populations of monocytes including CD14+ monocytes and CD16+ monocytes; classical dendritic cells and plasmacytoid dendritic cells; red blood cells; and platelets (Fig 2A, B). All cell types were present in all patient samples, except 1 patient had 0 classical dendritic cells. In general, the cell type distribution was similar between low and high treatment burden groups (Fig S1A). Because we were specifically interested in immune cell signatures, we excluded platelets and red blood cells from all further analyses. We also excluded plasmacytoid dendritic cells and classical dendritic cells due to sparse detection.

Cell Type-specific Immune Cell Transcriptional Signatures Associated With Anti-VEGF Treatment Burden

We performed differential gene expression analysis for each cell type comparing low versus high treatment burden. The most common approach to scRNAseq analysis treats each individual cell as an individual replicate, but in this analysis of highly variable patient samples, this may erroneously identify gene signatures associated with only 1 to 2 patients.[16] Therefore, we adopted a pseudobulk approach.[16,20] This approach involves averaging each cell type's gene expression for each patient, enabling the identification of gene signatures broadly associated with an experimental group. We then identified differentially expressed genes for each cell type, comparing the low treatment group and high treatment group (n = 15 vs. n = 12, respectively). By treating each patient as a replicate rather than each individual cell, this pseudobulk approach sacrifices statistical power. Indeed, there were no genes that passed adjusted P value < 0.05. In order to cast a wide net to identify any gene expression trends associated with anti-VEGF treatment burden in wet AMD, we identified for each cell type the top 50 genes by fold-change with unadjusted P-values < 0.05 (Fig 3A–G). Out of the 350 cell type-specific genes total, there were 301 unique genes (Fig 3H and Table S1). Of these, 259 genes (86%) had changes that were specific to 1 cell type, highlighting the utility of scRNAseq to resolve cell-specific gene expression as compared to bulk methods for studying PBMCs.

To determine whether these gene expression trends are broadly predictive of treatment burden in wet AMD, we calculated an aggregate PBMC gene score for each patient. The PBMC gene score represents the summation of relative expression (|z-score|) for each of the top 50 cell type-specific genes (50 z-scores for each of 7 cell types = 350 z-scores were summed). This total PBMC gene score was a statistically significant predictor of anti-VEGF treatment burden (simple logistic regression: odds ratio = 1.02, 95% confidence interval = [1.01, 1.04], P value < 0.05). The odds ratio of 1.02 indicates that for every 1 increase in PBMC gene score, which ranged from −346 to +356, there is a 2% additional risk for requiring ≥ 6 anti-VEGF injections per year.

To determine if gene signatures of individual cell types were predictive of anti-VEGF treatment burden, we calculated cell type-specific gene scores in a similar manner to the aggregate score (Table 2). After Sidak correction for multiple comparisons, only the gene score for CD16+ monocytes was predictive of treatment burden (simple logistic regression: odds ratio 1.09, adjusted P value = 0.04).

Pathway Analysis of CD16+ Monocytes

To determine whether the gene expression trends for CD16+ monocytes suggested abnormalities in specific pathways, which may provide insight into the mechanism by which these immune cells may regulate treatment response to anti-VEGF agents in neovascular AMD, we performed pathway analysis using Enrichr for enrichment of hallmark gene sets, Gene Ontology (GO) biological processes, and GO molecular function.[17–19] Numerous pathways were enriched, including many potentially relevant to AMD (Fig 4A–C). Of interest, the third highest enriched hallmark gene set was "complement," consistent with the known association between complement factor H genetic variants and AMD.[21–23] Additionally, "cellular response to lipid" was the seventh highest enriched GO biological process and "cholesterol binding" and "sterol binding" were the fourth and seventh highest enriched GO molecular function, consistent with (1) the fact that drusen are lipid-rich[24]; (2) association of polymorphisms in lipid-related genes with AMD[25–31]; and (3) a known role for lipid metabolism in regulating monocyte/macrophage function.[12,32,33]

Discussion

VEGF antagonists have revolutionized our ability to treat neovascular AMD.[6] Despite these advances, the need for repeated intraocular injections creates a high treatment burden that is necessary to maintain the therapeutic effect. In addition, there is a paucity of molecular biomarkers that can stratify *a priori* patients into groups based on low or high treatment burden. Immune cells are a dominant component of CNV lesions[11] and multiple genome-wide association studies have implicated polymorphisms in genes within the immune system with increased AMD risk.[5] Here, we use scRNAseq to examine systemic immune cells in anti-VEGF treatment-experienced patients with chronic, neovascular AMD that are stratified based on injection burden into low or high treatment burden groups. In order to identify gene expression signatures associated with high

treatment burden, we used a pseudobulk approach as previously described.[16] This type of approach sacrifices statistical power as compared to treating each cell as an individual replicate. Indeed, using this approach we found that there were no genes with adjusted P values < 0.05. However, it is critical to adopt a pseudobulk approach because it more accurately identifies gene expression signatures associated across an entire patient group. In this small proof-of-concept pilot study limited by sample size, we used unadjusted P values to identify gene expression trends associated with high treatment burden. We demonstrate that the gene expression trends of a specific immune cell, the CD16+ monocyte, was predictive of treatment burden. This is especially interesting because monocyte-derived macrophages are the predominant immune cell type in CNV lesions isolated from patients in the submacular surgery trials and examined by histopathology.[11] To identify CD16+ monocyte pathways that may be relevant to response to anti-VEGF treatment, we performed pathway analysis. Pathway analysis linked the gene expression trends of CD16+ monocytes with complement components and lipid & cholesterol metabolism. While complement activation is linked to AMD risk and progression, dysregulated lipid and cholesterol homeostasis is associated with drusen biogenesis.[33] Our results suggest a potential link to how the immune system in AMD may interface with lipid homeostasis and drusen biogenesis in the context of anti-VEGF therapy.

One limitation of our study is that there is not a consensus definition of high treatment burden in AMD. For our small pilot study, we stratified our patient cohort into either low or high treatment burden groups based on the number of injections they received in a 1-year time frame when clinical management used a *pro re nata* approach. We adopted a cutoff of 6 injections per year based on a previous multicenter randomized controlled trial in which the treatment arm receiving anti-VEGF therapy "as needed" received on average 6 to 7 injections per year.[7] However, the threshold for high versus low treatment burden warrants further discussion in studies incorporating a larger number of patients that may be able to define treatment burden as a continuous variable while also directly accounting for the burden of these therapies on patients and their caretakers.

In the current study, we study peripheral blood immune cells as a model to evaluate a role for immune cells in AMD treatment burden, a disease whose manifestations are highly localized to the eye. This is supported by previous studies that have demonstrated gene expression signatures of circulating monocytes that are related to both neovascular and atrophic AMD.[34] Functional assays of peripheral blood monocytes have also demonstrated deficits in phagocytosis in AMD.[35] Furthermore, a more recent study demonstrated that there were bulk PBMC gene expression signatures associated with poor versus good responders to anti-VEGF therapy in neovascular AMD.[36] The results of the current work build on these past findings by providing insight into the cell type-specific gene expression signatures that may be associated with treatment response or burden. Nonetheless, it is likely that hematopoietic immune cells adopt tissue-specific activation states upon ingress into the eye. For instance, a recent study demonstrated that in the subretinal space, monocytes from genetically-at-risk individuals were more likely to express osteopontin contributing to monocyte persistence in ocular tissues.[37] Furthermore, another study demonstrated that retinal pigment epithelium and myeloid cells were the most disease-relevant cell types with accessible chromatin coinciding with genome-wide association studies risk loci,[38] further supporting that in situ activation of hematopoietic immune cells potentially mediated by local ocular cells may be critically relevant in AMD. Taken together with these previous studies, our current findings suggest that CD16+ monocytes warrant further investigation in the context of AMD, especially their role when localized to ocular tissue. Furthermore, our findings do not necessarily exclude a role for other immune cells in AMD or in disease response to anti-VEGF therapy since peripheral blood gene expression may not reflect expression or functions when cells are located in ocular tissue.

Nonetheless, this precision medicine-based molecular approach to examining the role of cellular components of the immune system provides potential evidence to suggest a nexus between the gene expression pattern of peripheral blood CD16+ monocytes and neovascular AMD treatment burden. Our findings warrant further validation of this type of analysis using a larger cohort of patients. Future studies that investigate the specific genes whose expression is altered in CD16+ monocytes may identify novel therapeutic targets for patients with neovascular AMD that complement current approaches and potentially reduce the treatment burden while improving visual outcomes.

Acknowledgments

The authors thank patients and their families for participating in this study. This work was supported by the BrightFocus Foundation (P.A.R.); a pilot project grant from the Washington University Genome Technology Access Center (P.A.R.); Carl Marshall and Mildred Almen Reeves Foundation (P.A.R. and R.S.A.); National Institutes of Health grants R01 EY019287 (R.S.A.) and P30 EY02687 (Vision Core Grant); Jeffery T. Fort Innovation Fund (R.S.A.); Starr Foundation AMD Research Fund (R.S.A.); Siteman Retina Research Fund (R.S.A.); Retina Associates Research Fund (R.S.A.); Research to Prevent Blindness Career Development Award (P.A.R.); and an unrestricted grant from Research to Prevent Blindness to the John F. Hardesty, MD Department of Ophthalmology and Visual Sciences at Washington University School of Medicine in St. Louis. J.B.L. was supported by National Institutes of Health grant F30 DK130282 and the Washington University in St. Louis Medical Scientist Training Program (National Institutes of Health grant T32 GM07200). Research reported in this publication was supported by the Washington University Institute of Clinical and Translational Sciences (National Institutes of Health grant UL1 TR002345).

Footnotes and Disclosures

Originally received: May 31, 2023.
Final revision: August 28, 2023.
Accepted: October 6, 2023.
Available online: October 11, 2023. Manuscript no. XOPS-D-23-00118.

[1] John F. Hardesty, MD, Department of Ophthalmology & Visual Sciences, Washington University School of Medicine, St. Louis, Missouri.

[2] Neurosciences Graduate Program, Roy & Diana Vagelos Division of Biology and Biomedical Sciences, Washington University School of Medicine, St. Louis, Missouri.

[3] Department of Developmental Biology, Washington University School of Medicine, St. Louis, Missouri.

[4] Center of Regenerative Medicine, Washington University School of Medicine, St. Louis, Missouri.

[5] Department of Medicine, Washington University School of Medicine, St. Louis, Missouri.

Rajendra S. Apte, MD, PhD, an Associate Editor of this journal, was recused from the peer-review process of this article and had no access to information regarding its peer-review.

Disclosure(s):

All authors have completed and submitted the ICMJE disclosures form.

The author(s) have made the following disclosure(s): J.B.L.: All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.)—National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of General Medical Sciences, Washington University.

P.A.R.: All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.)—BrightFocus Foundation, Genome Technology Access Center, Carl Marshall and Mildred Almen Reeves Foundation, Research to Prevent Blindness, Washington University.

R.S.A.: All support for the present manuscript (e.g., funding, provision of study materials, medical writing, article processing charges, etc.)—BrightFocus Foundation, Genome Technology Access Center, Carl Marshall and Mildred Almen Reeves Foundation, National Institutes of Health, Jeffrey T. Fort Innovation Fund, Starr Foundation, Siteman Retina Research Fund, Retina Associates Research Fund, Washington University.

The other authors have no conflicts of interest.

HUMAN SUBJECTS: Human subjects were included in this study. This study adheres to the Declaration of Helsinki and was approved by the Human Research Protection Office of Washington University School of Medicine in St. Louis. The authors obtained written informed consent from all subjects prior to enrollment in the study.

No animal subjects were used in this study.

Author Contributions:

Conception and design: Ruzycki, Apte

Data collection: Lin, Santeford, Usmani, Shah, Ruzycki, Apte

Analysis and interpretation: Lin, Ruzycki, Apte

Obtained funding: Other than the grant funding listed in the acknowledgments section in support of the research, the authors performed the research as part of their regular duties at Washington University.

Overall responsibility: Lin, Santeford, Usmani, Shah, Ruzycki, Apte

Abbreviations and Acronyms:
AMD = age-related macular degeneration; **CNV** = choroidal neovascularization; **GO** = Gene Ontology; **PBMCs** = peripheral blood mononuclear cells; **scRNAseq** = single cell RNA sequencing.

Keywords:
AMD, CNV, Macrophage, Monocyte, VEGF.

Correspondence:
Rajendra S. Apte, 660 S. Euclid Ave (MSC-8096-06-07), St. Louis, MO 63110. E-mail: apte@wustl.edu; and Philip A. Ruzycki, 4523 Clayton Ave (MSC 8096-06-1104), St. Louis, MO 63110. E-mail: p.ruzycki@wustl.edu.

References

1. Apte RS. Age-related macular degeneration. *N Engl J Med*. 2021;385:539−547.
2. Seddon JM, Willett WC, Speizer FE, Hankinson SE. A prospective study of cigarette smoking and age-related macular degeneration in women. *JAMA*. 1996;276:1141−1146.
3. Seddon JM, Cote J, Davis N, Rosner B. Progression of age-related macular degeneration: association with body mass index, waist circumference, and waist-hip ratio. *Arch Ophthalmol*. 2003;121:785−792.
4. Seddon JM, George S, Rosner B. Cigarette smoking, fish consumption, omega-3 fatty acid intake, and associations with age-related macular degeneration: the US Twin Study of Age-Related Macular Degeneration. *Arch Ophthalmol*. 2006;124:995−1001.
5. Strunz T, Kiel C, Sauerbeck BL, Weber BHF. Learning from fifteen years of genome-wide association studies in age-related macular degeneration. *Cells*. 2020;9:E2267.
6. Apte RS, Chen DS, Ferrara N. VEGF in signaling and disease: beyond discovery and development. *Cell*. 2019;176:1248−1264.
7. Comparison of Age-related Macular Degeneration Treatments Trials (CATT) Research Group; Writing Committee, Martin DF, et al. Ranibizumab and bevacizumab for treatment of neovascular age-related macular degeneration: two-year results. *Ophthalmology*. 2020;127:S135−S145.
8. Sene A, Chin-Yee D, Apte RS. Seeing through VEGF: innate and adaptive immunity in pathological angiogenesis in the eye. *Trends Mol Med*. 2015;21:43−51.
9. Bhisitkul RB, Mendes TS, Rofagha S, et al. Macular atrophy progression and 7-year vision outcomes in subjects from the ANCHOR, MARINA, and HORIZON studies: the SEVEN-UP study. *Am J Ophthalmol*. 2015;159:915−924.e2.
10. Comparison of Age-related Macular Degeneration Treatments Trials (CATT) Research Group, Maguire MG, Martin DF, et al. Five-year outcomes with anti-vascular endothelial growth factor treatment of neovascular age-related macular degeneration: the comparison of age-related macular degeneration treatments trials. *Ophthalmology*. 2016;123:1751−1761.
11. Grossniklaus HE, Miskala PH, Green WR, et al. Histopathologic and ultrastructural features of surgically excised subfoveal choroidal neovascular lesions: submacular surgery trials report no. 7. *Arch Ophthalmol*. 2005;123:914−921.
12. Sene A, Apte RS. Eyeballing cholesterol efflux and macrophage function in disease pathogenesis. *Trends Endocrinol Metab*. 2014;25:107−114.

13. Hao Y, Hao S, Andersen-Nissen E, et al. Integrated analysis of multimodal single-cell data. *Cell*. 2021;184:3573−3587.e29.
14. Korsunsky I, Millard N, Fan J, et al. Fast, sensitive and accurate integration of single-cell data with Harmony. *Nat Methods*. 2019;16:1289−1296.
15. McInnes L, Healy J, Melville J. UMAP: uniform manifold approximation and projection for dimension reduction. arXiv: 180203426 [cs, stat]. http://arxiv.org/abs/1802.03426; 2020. Accessed August 5, 2021.
16. Voigt AP, Mullin NK, Mulfaul K, et al. Choroidal endothelial and macrophage gene expression in atrophic and neovascular macular degeneration. *Hum Mol Genet*. 2022;31:2406−2423.
17. Chen EY, Tan CM, Kou Y, et al. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC Bioinformatics*. 2013;14:128.
18. Kuleshov MV, Jones MR, Rouillard AD, et al. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids Res*. 2016;44:W90−W97.
19. Xie Z, Bailey A, Kuleshov MV, et al. Gene set knowledge discovery with Enrichr. *Curr Protoc*. 2021;1:e90.
20. Lun ATL, Marioni JC. Overcoming confounding plate effects in differential expression analyses of single-cell RNA-seq data. *Biostatistics*. 2017;18:451−464.
21. Edwards AO, Ritter R, Abel KJ, et al. Complement factor H polymorphism and age-related macular degeneration. *Science*. 2005;308:421−424.
22. Haines JL, Hauser MA, Schmidt S, et al. Complement factor H variant increases the risk of age-related macular degeneration. *Science*. 2005;308:419−421.
23. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. *Science*. 2005;308:385−389.
24. Wang L, Clark ME, Crossman DK, et al. Abundant lipid and protein components of drusen. *PLoS One*. 2010;5:e10329.
25. Chen W, Stambolian D, Edwards AO, et al. Genetic variants near TIMP3 and high-density lipoprotein-associated loci influence susceptibility to age-related macular degeneration. *Proc Natl Acad Sci U S A*. 2010;107:7401−7406.
26. Neale BM, Fagerness J, Reynolds R, et al. Genome-wide association study of advanced age-related macular degeneration identifies a role of the hepatic lipase gene (LIPC). *Proc Natl Acad Sci U S A*. 2010;107:7395−7400.
27. Klaver CC, Kliffen M, van Duijn CM, et al. Genetic association of apolipoprotein E with age-related macular degeneration. *Am J Hum Genet*. 1998;63:200−206.
28. McKay GJ, Patterson CC, Chakravarthy U, et al. Evidence of association of APOE with age-related macular degeneration: a pooled analysis of 15 studies. *Hum Mutat*. 2011;32:1407−1416.
29. Souied EH, Benlian P, Amouyel P, et al. The epsilon4 allele of the apolipoprotein E gene as a potential protective factor for exudative age-related macular degeneration. *Am J Ophthalmol*. 1998;125:353−359.
30. Fritsche LG, Chen W, Schu M, et al. Seven new loci associated with age-related macular degeneration. *Nat Genet*. 2013;45:433−439, 439e1-2.
31. Yu Y, Reynolds R, Rosner B, et al. Prospective assessment of genetic effects on progression to different stages of age-related macular degeneration using multistate Markov models. *Invest Ophthalmol Vis Sci*. 2012;53:1548−1556.
32. Sene A, Khan AA, Cox D, et al. Impaired cholesterol efflux in senescent macrophages promotes age-related macular degeneration. *Cell Metab*. 2013;17:549−561.
33. Ban N, Lee TJ, Sene A, et al. Impaired monocyte cholesterol clearance initiates age-related retinal degeneration and vision loss. *JCI Insight*. 2018;3:e120824.
34. Grunin M, Hagbi-Levi S, Rinsky B, et al. Transcriptome analysis on monocytes from patients with neovascular age-related macular degeneration. *Sci Rep*. 2016;6:29046.
35. Gu BJ, Huang X, Avula PK, et al. Deficits in monocyte function in age related macular degeneration: a novel systemic change associated with the disease. *Front Med (Lausanne)*. 2021;8:634177.
36. Oca AI, Pérez-Sala Á, Pariente A, et al. Predictive biomarkers of age-related macular degeneration response to anti-VEGF treatment. *J Pers Med*. 2021;11:1329.
37. Beguier F, Housset M, Roubeix C, et al. The 10q26 risk haplotype of age-related macular degeneration aggravates subretinal inflammation by impairing monocyte elimination. *Immunity*. 2020;53:429−441.e8.
38. Orozco LD, Owen LA, Hofmann J, et al. A systems biology approach uncovers novel disease mechanisms in age-related macular degeneration. *Cell Genomics*. 2023;3:100302.