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Liquid Biopsy for Proliferative Diabetic Retinopathy: Single-Cell Transcriptomics of Human Vitreous Reveals Inflammatory T-Cell Signature

Rachana Haliyur, MD, PhD,[1] *David H. Parkinson, MS,*[2] *Feiyang Ma, PhD,*[3] *Jing Xu, PhD,*[1] *Qiang Li, MD, PhD,*[1] *Yuanhao Huang,*[4] *Lam C. Tsoi, PhD,*[3] *Rachael Bogle, MS,*[3] *Jie Liu, PhD,*[4] *Johann E. Gudjonsson, MD,*[3] *Rajesh C. Rao, MD*[1,4,5,6,7,8,9,10]

Purpose: Current therapies for proliferative diabetic retinopathy (PDR) do not specifically target VEGF-independent, cell-type–specific processes that lead to vision loss, such as inflammatory pathways. This study aimed to identify targetable cell types and corresponding signaling pathways by elucidating the single-cell landscape of the vitreous of patients with PDR.

Design: Case series.

Subjects: Vitreous and peripheral blood obtained from 5 adult patients (6 eyes) undergoing pars plana vitrectomy for vision-threatening PDR.

Methods: Single-cell RNA sequencing (scRNA-seq) was performed on vitreous cells obtained from diluted cassette washings during vitrectomy from 6 eyes and peripheral blood mononuclear cells (PBMCs, n = 5). Droplet-based scRNA-seq was performed using the Chromium 10x platform to obtain single-cell transcriptomes. Differences in tissue compartments were analyzed with gene ontology enrichment of differentially expressed genes and an unbiased ligand–receptor interaction analysis.

Main Outcome Measures: Single-cell transcriptomic profiles of vitreous and peripheral blood.

Results: Transcriptomes from 13 675 surgically harvested vitreous cells and 22 636 PBMCs were included. Clustering revealed 4 cell states consistently across all eyes with representative transcripts for T cells (*CD2*, *CD3D*, *CD3E*, and *GZMA*), B cells (*CD79A*, *IGHM*, *MS4A1* (CD20), and *HLA-DRA*), myeloid cells (*LYZ*, *CST3*, *AIF1*, and *IFI30*), and neutrophils (*BASP1*, *CXCR2*, *S100A8*, and *S100A9*). Most vitreous cells were T cells (91.6%), unlike the peripheral blood (46.2%), whereas neutrophils in the vitreous were essentially absent. The full repertoire of adaptive T cells including CD4+, CD8+ and T regulatory cells (Treg) and innate immune system effectors (i.e., natural killer T cells) was present in the vitreous. Pathway analysis also demonstrated activation of CD4+ and CD8+ memory T cells and ligand–receptor interactions unique to the vitreous.

Conclusions: In the first single-cell transcriptomic characterization of human vitreous in a disease state, we show PDR vitreous is primarily composed of T cells, a critical component of adaptive immunity, with activity and proportions distinct from T cells within the peripheral blood, and neutrophils are essentially absent. These results demonstrate the feasibility of liquid vitreous biopsies via collection of otherwise discarded, diluted cassette washings during vitrectomy to gain mechanistic and therapeutic insights into human vitreoretinal disease.

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Proliferative diabetic retinopathy (PDR), a complication of diabetes mellitus, is a major cause of vision loss in working-age adults worldwide characterized by retinal ischemia and neovascularization.[1] Acute vision loss in PDR occurs from either vitreous or preretinal hemorrhage secondary to retinal neovascularization that obstructs the visual axis and/or macular traction or detachment due to contracture of fibrovascular membranes.[1,2] Current therapies for vision loss due to PDR involve pars plana vitrectomy (PPV) to surgically remove hemorrhage, dissection with removal of fibrovascular membranes, anti-vascular endothelial growth factor (VEGF) intravitreal treatment, and panretinal photocoagulation to regress pathologic retinal neovascularization. Panretinal photocoagulation, the most durable treatment to

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prevent retinal neovascularization and reduce ischemia, does so by destruction of retinal cells.[3] Thus, given the risks and limitations of surgery, destructive photocoagulation, and nonresponse or incomplete response to anti-VEGF therapies, there is a need for additional pathophysiologic insights and therapeutic modalities that target the diverse underlying disease process in PDR.

Although a break in the blood−retina barrier is a hallmark of neovascularization, the mechanisms that contribute to an abnormal vitreoretinal interface in PDR and subsequent vision loss are poorly understood.[4] Structural and molecular alterations to the vitreous can result secondary to hypoxia, inflammation, and oxidative stress occurring within the retina in diabetic retinopathy (DR), whereas concurrently the vitreous may exert pathologic effects on the diabetic retina that contribute to the progression of the disease. Existing data suggest that the innate immune system predominates in DR, specifically through activation of local microglia and complement.[5−8] It has been hypothesized that the proinflammatory retinal microenvironment in DR results in T-cell lymphocyte activation leading to retinal vascular dysfunction and fibrovascular membrane formation seen in later stages; however, the role of the adaptive immune system in DR is not well understood.[9−11] Vitreous fluid obtained from patients with PDR undergoing vitreoretinal surgery has revealed growth factors and cytokines that have a proangiogenic and inflammatory profile.[1,12,13] These and similar reports suggest the biological activity of vitreous samples is critical to our understanding of the pathogenesis of key events in PDR, as well as insights into novel therapies.

Despite the hypothesized importance of the vitreous and vitreoretinal interface, to our knowledge, no reports have comprehensively characterized the cell types in the human vitreous in PDR. In contrast to the high abundance of protein content allowing vitreous proteomics,[12,14] the lack of vitreous cellular data is partly due to the difficulty of obtaining viable samples, which traditionally have been limited to postmortem collection, enucleation, or with therapeutic vitrectomy.[15] Traditional histopathologic methods and flow cytometry have provided insights into the identities of cells that infiltrate the vitreous in PDR, but lineage-specific markers employed for elucidating cell-type composition in human PDR vitreous must be individually selected a priori, which introduces bias. Furthermore, sample collection has been limited by the concern of blood-borne molecules, such as from vascular leakage or hemorrhage, which may "contaminate" vitreous specimens and lack of concurrent peripheral blood specimens for proper comparison of cell-type proportions in the hemorrhagic PDR vitreous versus the peripheral blood.[14]

To address these gaps in knowledge, we report the first transcriptomic characterization at the single-cell level of the human vitreous in a disease state. Specifically, we conducted single-cell RNA sequencing (scRNA-seq) from surgically harvested vitreous (n = 6 eyes) and peripheral blood (n = 5) from 5 patients undergoing PPV for vision-threatening PDR. We show that T cells, a critical component of adaptive immunity, constitute the highest proportion of cell types in the human PDR vitreous with activity and proportions distinct from T cells within the peripheral blood and that certain innate immune cell populations, such as innate-like mucosal-associated invariant T (MAIT) cells, and CD16+ and CD56+ populations of natural killer (NK) cells can be found in the PDR vitreous. We also demonstrate that collection of diluted cassette washings (i.e., vitreous diluted by infusion fluid [i.e., balanced salt solution] required to maintain intraocular pressure during vitrectomy) provides foundational insights into the cell types present in PDR, an approach that may be feasible from otherwise discarded vitreous washings from a variety of ocular disease states.

Methods

Peripheral Blood Mononuclear Cell and Vitreous Sample Collection and Single-Cell Preparation

The study was approved by the Institutional Review Board and Ethics Committee at the University of Michigan Kellogg Eye Center, and informed consent was obtained from each patient in accordance with requirements of the University of Michigan Institutional Review Board. All described research adhered to the tenets of the Declaration of Helsinki. Patients were included if they had a diagnosis of PDR and underwent necessary vitrectomy for vision-threatening disease (i.e., nonclearing vitreous hemorrhage and/or tractional retinal detachment). All relevant demographic variables and histories regarding their DR are included in Table 1. Time from vitreous hemorrhage (VH) onset and vitrectomy were estimated based on clinical documentation. The clinical stage of DR for each participant was classified according to the International Clinical Diabetic Retinopathy Severity Scale.[16] Individuals with autoimmune disease, cancer, and other causes of VH such as trauma, uveitis, and other forms of retinopathy were excluded. Patients who had undergone prior PPV were excluded from the study. Visual acuity was converted to the logarithm of the minimum angle of resolution (logMAR). Snellen vision count fingers, hand motion (HM), light perception (LP), and no-light perception (NLP) were converted to logarithm of the minimum angle of resolution as follows: count fingers 1.85, HM 2.3, LP 2.75, NLP 3.2.[17] Approximately 150 ml of vitreous fluid samples were collected at the conclusion of PPV surgery from the diluted cassette washings. The average vitreous cut rate for all included vitrectomies was 20 000 cuts per minute. All surgeries were performed with a standard 3-port 25-gauge transscleral trocar cannular system PPV (Alcon Laboratories Inc). Peripheral blood was obtained intravenously from patients ahead of surgery (5 ml). Samples were collected into sterile tubes, stored on dry ice, and then immediately processed into single cells. Peripheral blood mononuclear cell (PBMC) samples were diluted at a ratio of 1:4 using phosphate-buffered saline (PBS), and a 100-μM strainer was used to remove debris before red blood cell lysing. Ficoll PAQUE Plus (1.077 mg/ml, GE Healthcare Bio-Sciences AB) was added to the diluted blood sample and then centrifuged. The cell pellet was resuspended in 1XRBC lysis buffer and incubated on a rocker for 10 minutes at room temperature. The sample was again centrifuged, and the cell pellet was resuspended in PBS with 0.04% bovine serum albumin. Vitreous samples were centrifuged initially, resuspended in PBS, and passed through the cell strainer (100 μM), and then centrifuged again and resuspended in PBS with 0.04% bovine serum albumin. Once resuspended, both samples were then counted with the hemocytometer ahead of 10x genomics scRNA-seq.

Table 1. Summary of demographic variables and relevant ocular history for included eyes from human participants with proliferative diabetic retinopathy included in single cell sequencing analysis

Eye	Age (YR)	Sex (M/F)	Race (W/A/UK)	DM Type (1/2)	Insulin Use (Y/N)	DM Duration (YR)	HbA1C near PPV (%)	# of prior IVI anti-VEGF	Prior PRP (Y/N)	Time from VH onset to PPV (days)	PPV Findings	PVD Status (Y/P/N)	VA prior to PPV (logMAR)	Final VA (logMAR)
ID #1	35	F	W	1	Y	20	9.8	1	N	289	VH, TRD, ERM	Partial	0.4	0.2
ID #2*	62	M	UK	2	Y	28	8.0	0	Y	346	VH, ERM	Partial	2.3	0.6
ID #3	53	F	W	2	Y	UK	7.5	1	Y	147	VH, LMH, VMT, TRD, DME, ERM	Partial	0.8	2.3
ID #4	50	F	W	2	N	3	8.3	1	N	78	VH, DME, ERM, TRD, VMT	Partial	1.4	0.3
ID #5*	62	M	UK	2	Y	28	8.0	1	Y	388	VH, TRD, ERM	Partial	2.3	0.54
ID #6**	66	M	A	2	N	UK	7.3	4	Y	105	VH, ERM	Y	1.9	0.1
Summary	**53.2 (10.8)**	**40%**	**60%**	**20%**	**60%**	**17.0 (10.4)**	**8.2 (0.8)**	**1.3 (1.2)**	**67%**	**225.5 (120.7)**		**16.7%**	**1.5 (0.8)**	**0.7 (0.8)**

Summary values are represented as mean (SD) or % of first group. VA is reported in logMAR scale.
Abbreviations: A, Asian; DM, Diabetes Mellitus; DME, Diabetic macular edema; DR, Diabetic Retinopathy; ERM, Epiretinal membrane; IVI, Intravitreal injection; LMH, Lamellar Macular Hole; N, sample size; N/A, not available; F, female; HbA1c, Hemoglobin A1c; logMAR, logarithm of the minimum angle of resolution; M, male; PBMC, Peripheral Blood Mononuclear Cells; PPV, Pars Plana Vitrectomy; PRP, Panretinal Photocoagulation; PVD, Posterior Vitreous Detachment; TRD, Tractional Retinal Detachment; UK, unknown; VA, Visual Acuity; VEGF, Vascular Endothelial Growth Factor; VH, Vitreous Hemorrhage; VMT, Vitreomacular traction; W, White.
*IDs #2 and #5 represent each eye from the same donor thus summary demographic data is identical across these IDs.
**Only vitreous and not peripheral blood was collected from ID #6

scRNA-Seq and Cluster Identification

Droplet-based scRNA-seq with data processing, including quality control, read alignment, and gene quantification, was conducted using the Cell Ranger software (10x Genomics) to obtain single-cell transcriptomes. Seurat was used for normalization, data integration, and clustering analysis.[18] All subtype cell and cell−cell interaction analyses utilized data filtered for quality of gene profile. The R package DESeq2 was then used to identify differentially expressed genes within clusters. The cutoff criteria for determining differentially expressed genes were log2 fold change (FC) > 0 and false discovery rate (FDR) < 0.05. Clustered cells were mapped to corresponding cell types by matching cell cluster gene signatures with cell-type−specific markers identified with FC > 2 and $P < 0.05$. Differentially expressed genes were further analyzed using gene ontology enrichment analysis (https://geneontology.org/docs/go-enrichment-analysis/) applying filters for FC $> \pm 1$ (i.e., up- and down-regulated) and FDR < 0.05 (Table S2, available at www.ophthalmologyscience.org).

Analysis of Cell-Type Differences between Tissue Types

Counts for cell-type for each sample were normalized to the total number of cells obtained for that sample. Cell-type proportions were then calculated, and the average proportions were compared between PBMCs and vitreous tissue types. Statistical significance was evaluated using a 2-sided Wilcoxon rank sum test. *P* values were adjusted for multiple tests using the Bonferroni correction. Chi-square tests were used to evaluate for differences in cell make-up, including T cells, B cells, and myeloid subtype cell groups.

Cell−Cell Communication Analysis

Ligand−receptor interactions were determined using Dimer Signal Receptor Analysis.[19] Expression of specific ligands and receptors in each cell-type informed what cell−cell interactions were most likely. These estimations were then compared within the vitreous and PBMC samples. Considering the differences in cell populations, to better compare between the vitreous and peripheral blood samples, the cell−cell interaction analysis of PBMCs were limited to the interactions possible in the vitreous. Differences were shown by subtracting the PBMC interaction scores from the vitreous interaction scores

Results

A total of 5 patients with PDR seen at a single academic tertiary eye care center were included (Table 1). Patients were on average 53 ± 11 years of age, majority female sex (n = 3), and White (n = 3). Most patients were diagnosed with type 2 diabetes (n = 4) that was long-standing (average duration of diabetes 17 ± 10 years with an average hemoglobin A1c of 8.2 ± 0.8) and insulin-dependent (n = 4). A total of 5 of the 6 eyes had received ≥1 injection of anti-VEGF, and two thirds were treated with panretinal photocoagulation at some point before surgery. Intraoperative findings were notable for evidence of VH and epiretinal membrane (ERM) in all included eyes. A tractional ERM and/or vitreomacular traction were noted in 4 of the 6 eyes, whereas 3 eyes had a tractional retinal detachment on examination. Only 1 eye had a complete posterior vitreous detachment at the time of surgery.

scRNA-seq Resolution of 4 Cell Types in the PDR Vitreous and Peripheral Blood

After quality control filters, we collected transcriptomes from 13 675 cells in the vitreous and 22 636 PBMCs from the peripheral blood, with a normally distributed average of 1926 ± 677 and 1291 ± 779 genes read per cell, respectively (mean reads and median genes per cell in Table S3, available at www.ophthalmologyscience.org). Single cells from vitreous and peripheral blood were projected onto an Uniform Manifold Approximation and Projection space (Fig 1B, D). Clustering revealed 4 cell states: T cells, B cells, myeloid, and neutrophils (Fig 1B). These clusters appeared generally consistently across batches (Fig S2A, available at www.ophthalmologyscience.org), and distinct differences in cell-type composition across sample type (Fig 1D). Representative transcripts for T cells (*CD2*, *CD3D*, *CD3E*, and *GZMA*), B cells (*CD79A*, *IGHM*, *MS4A1* [CD20], and *HLA-DRA*), myeloid (*LYZ*, *CST3*, *AIF1*, and *IFI30*), and neutrophils (*BASP1*, *CXCR2*, *S100A8*, and *S100A9*) are described in Figure 1F. Interestingly, T cells comprise a large majority of the cells obtained from the PDR vitreous tissue, whereas neutrophils are nearly absent (Figs 1C, S2B), and, again, this was consistent among the 6 eyes (Fig S2A)

Because vitreous cells (13 675 cells) make up 38% of the total cells (36 311 cells) studied from both compartments, we would expect a similar distribution within each cell-type cluster for both the vitreous and PBMCs if hemorrhaging into the vitreous was random spillover. However, the distribution of each cell type from the 2 compartments was notably different (Table S4, available at www.ophthalmologyscience.org), with 54.5% of T cells from the vitreous compared with 45.5% from PBMCs, 17% of myeloid cells from the vitreous compared with 83% from PBMCs, 11.8% of B cells from the vitreous compared with 88.2% from PBMCs, and 1.4% of neutrophils from the vitreous compared with 98.6% from PBMCs. We next assessed the total number and proportion of each cell-type in vitreous versus PBMC samples (Figs 1C, E). Among the vitreous cells, 91.6% were T cells with 5.9% myeloid cells, 1.9% B cells, and 0.5% neutrophils compared with the peripheral blood, where 46.2% were T cells, 26.6% myeloid cells, 5.7% B cells, and 21.5% were neutrophils (Table S4).

PDR Vitreous and Peripheral Blood T-Cell Subtypes

We next refined the single-cell data clusters to map subclusters within each T-cell state: CD4+ naïve T, CD4+ memory T, CD8+ naïve T, CD8+ memory T, Tregs, CD16+ natural killer (NK) cells, CD56+ NK cells, and MAIT cells in the vitreous and peripheral blood (Fig 3A, B) and correspond to cell-type−specific transcripts (Fig 3C). Each sample source was mapped onto these subclusters,

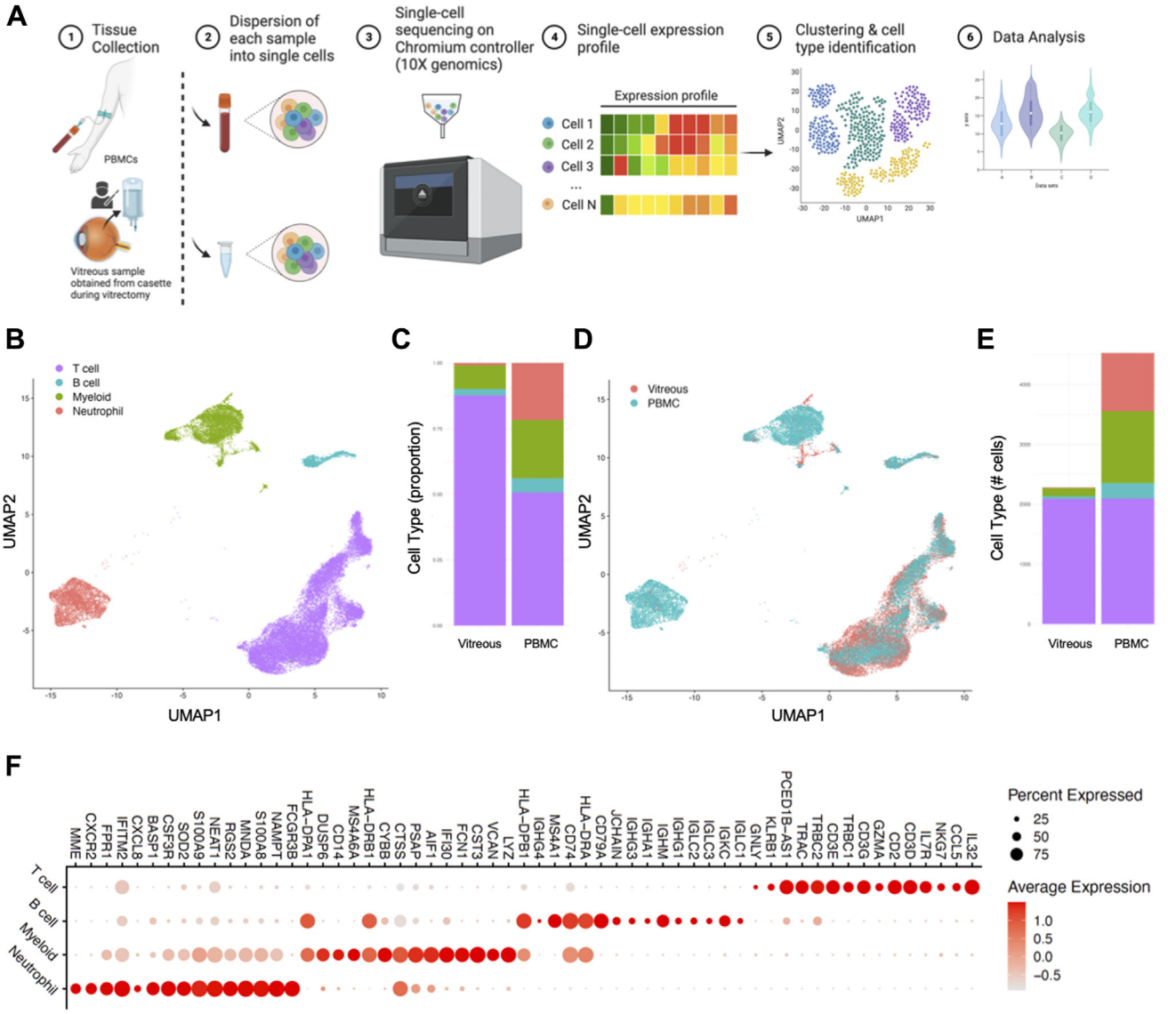

Figure 1. Single-cell atlas of the human vitreous and peripheral blood mononuclear cells (PBMCs) in individuals with proliferative diabetic retinopathy (PDR) **A,** Schematic diagram of study design with sample collection from vitreous and PBMCs from each human donor undergoing vitrectomy for proliferative diabetic retinopathy (PDR). Uniform Manifold Approximation and Projection (UMAP) of integrated scRNA-seq analysis of the vitreous (13 675 cells) and PBMCs (22 636 cells) from all 6 eyes resulting in 36 311 cells in total. Major immune cell populations are shown in (**B**) with comparison of these populations between the vitreous and peripheral samples in (**D**). The retrospective distribution of major immune cell populations by proportion (**C**) and cell numbers (**E**) per donor eye. **F,** Dot plot of differentially expressed genes in each identified cluster and associated immune cell population. This figure was created with BioRender.com.

and we assessed the average numbers and proportion of each T-cell subtype for each sample category (Fig S2C, D). We found that CD4+ naïve and memory T cells, CD8+ memory T cells, and CD56+ NK cells in the PDR vitreous comprised $\geq$ 50% of the total cells (PDR vitreous plus PBMCs) in each corresponding subcluster (Table S5, available at www.ophthalmologyscience.org). A chi-square test revealed significant differences in T-cell composition between the peripheral blood and PDR vitreous tissue when comparing the T-cell subtypes between the 2 compartments ($P < 0.001$; Fig S2). Finally, we assessed gene ontology (GO) terms enriched in the vitreous in CD4+ and CD8+ memory T-cell populations in the PDR vitreous when compared with PBMCs. We found that cytokine-mediated signaling, oxidative phosphorylation, neutrophil activation, cell−cell adhesion signaling, leukocyte migration, cell growth, and response to hypoxia were enriched the vitreous CD4+ memory cells and CD8+ memory cells supporting a unique inflammatory milieu in the PDR vitreous compared with the peripheral blood. When looking at NK subtypes, pathways associated with cell killing and neutrophil chemotaxis were negatively differentially expressed in CD16+ NK cells, whereas CD56+ NK cells had upregulation of pathways associated with cell proliferation and NK

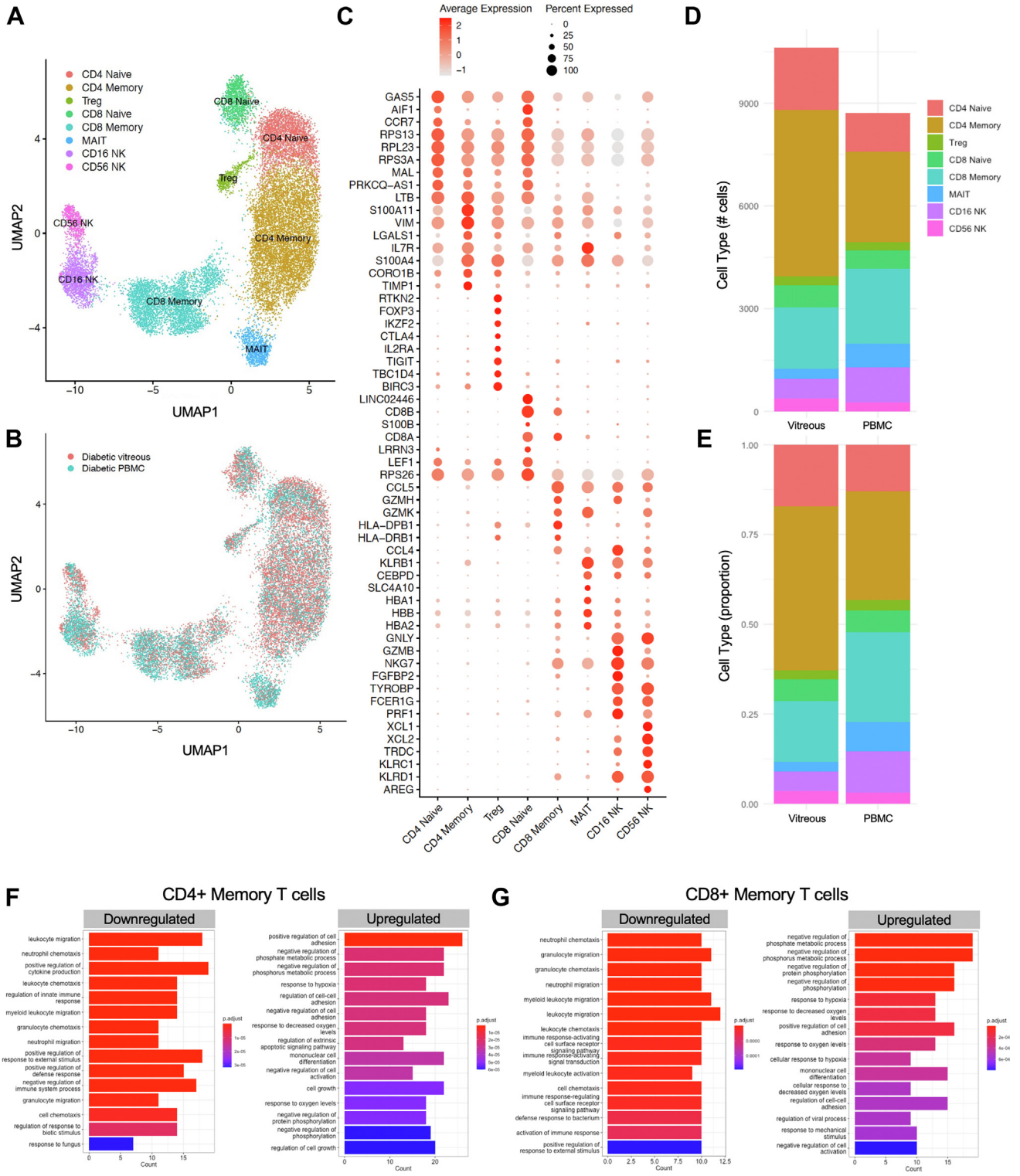

Figure 3. Distinct effector profiles of T-cell populations within vitreous and PBMC populations from individuals with PDR. **A,** UMAP plot of the cluster identifications for effector T-cell populations and their cytotoxic profiles and (**B**) respective UMAP plot of cell location from the vitreous and peripheral blood. (**C**) Dot plot of differentially expressed genes in each identified cluster and effector T-cell population. Comparison of (**D**) cell counts and (**E**) proportion of T cells per cluster in vitreous and PBMC tissues. Enriched gene ontology biological processes found in (**F**) CD4 memory T cells and (**G**) CD8 memory T cells when comparing vitreous to PBMCs. NK = natural killer; MAIT = mucosal-associated invariant T; Treg = regulatory T cell; FC = fold change; FDR = false discovery rate; PBMC = peripheral blood mononuclear cell; PDR = proliferative diabetic retinopathy; UMAP = Uniform Manifold Approximation and Projection.

cell chemotaxis (Fig S4A, B). These data indicate the composition of T and NK subtypes is distinct in the PDR vitreous when compared with PBMCs and suggest differences in T-cell activation between these two compartments.

PDR Vitreous and Peripheral Blood Myeloid and B-Cell Subtypes

Similarly, although fewer in absolute numbers, we next investigated the cell-type subclusters of myeloid cells and B cells in the vitreous and peripheral blood of individuals with PDR (Fig 3; Fig S4). There was not enough genetic detail to further investigate subpopulations of the vitreous neutrophils in any eye or the subtypes of B cells or myeloid cells for 2 eyes (Fig S2E, G). Chi-square tests revealed significant differences in both myeloid and B-cell compositions between the peripheral blood ($P < 0.001$) and PDR vitreous tissue ($P < 0.01$). Within the myeloid single-cell population, subclusters were identified for the following cell types: monocytes (mono), macrophages (mac), classical dendritic cells (cDCs), plasmodial dendritic cells (pDCs; Fig 5A, B) (Fig S2). Macrophages and monocytes expressing interferon (IFN) and nonclassical monocytes were also subclassified and identified by cell-type–specific transcripts (Fig 5C). Macrophages made up the primary myeloid subtype (54.9%) in the vitreous compared with the peripheral blood, whereas monocytes make up the primary myeloid cell-type in the peripheral blood (48.5%; Fig 5D, E). Interferon-expressing monocytes and macrophages were uncommon within the vitreous when compared with the peripheral compartment (Fig 5D, E). No myeloid subtypes were enriched in the combination of known hyalocyte or microglial markers (such as *TREM2*, *TMEM119*, *CX3CR1*, *P2RY12*, *CD68*, *FTL*, *CD11b*, *IBA-1*, *OLFML3*, and *SALL1*) and thus are likely not captured in the vitreous.[20,21] Gene ontology-term analysis showed activation of dendritic cell and macrophage populations within the vitreous compared with the peripheral blood (Fig S4C, D). Subcluster analysis revealed 5 B-cell subpopulations: naïve B cells, intermediate B cells, memory B cells, and plasma cells, as well as B cells expressing IFN (IFN B cells) (Fig 5 F-H) (Fig S2). Proliferative diabetic retinopathy vitreous B cells had a similar cell-type distribution to the peripheral blood with naïve B cells being the predominant type in both (Fig 5I, J).

Immune Cell–Cell Interactions within the PDR Vitreous and Peripheral Blood

To assess immune cell–cell interactions, we used Dimer Signal Receptor Analysis to evaluate the crosstalk mediated by ligand–receptor interactions occurring within the vitreous compared with the peripheral blood. The intercellular communication in the PDR vitreous was distinct from the peripheral blood despite the overlap in patients. Specifically, interactions between myeloid cells and T cells or dendritic cells (DCs) seem to predominate in the PDR vitreous (Fig 6A), whereas myeloid cell subtype interactions such as macrophage–cDC, monocyte–macrophage, and macrophage–macrophage interactions are enriched in both compartments (Fig 6A, B). Interactions between the myeloid and B cells are more prominent in the PBMC compartment, which also sees more interactions between myeloid cells and B cells, T regulatory cells, and NK cells. Specific interactions between the ligand on myeloid cells with receptors on CD4+ memory and dendritic cells were enriched in the vitreous compared with the PBMC, ultimately identifying targetable pathways between chemokines and receptors unique to the vitreous compartment (Table S6). To better visualize the differences between the two compartments, the PBMC interaction score was subtracted from the vitreous interaction score, which further emphasized the intermyeloid cell interactions and CD4+ memory T-cell-macrophage predominate in the vitreous PDR with minimal B-cell–specific interactions with macrophages and monocytes (Fig 6C). Even if we considered all possible interactions within each compartment (Fig S7), the same processes between myeloid and T cells were enriched in the vitreous compared with the peripheral tissue.

Discussion

Interactions at the vitreoretinal interface contribute to vision loss in PDR, making understanding of the vitreous space vital to uncovering disease mechanisms. Using scRNA-seq of the vitreous from patients with PDR undergoing vitrectomy, we report the single-cell landscape within the vitreous in PDR and achieve a cellular resolution that uncovers immune cell subtypes previously underappreciated or undescribed. Our findings highlight that T cells, a critical component of adaptive immunity, constitute the highest proportion of cell types in the human PDR vitreous, and that neutrophils, important in innate immune response, are notably lacking in the PDR vitreous cavity. Although fewer in number, we also identify the presence of B cells and myeloid-lineage cells within the vitreous and that intermyeloid cell and macrophage-CD4+ memory T-cell interactions are enriched in the vitreous space when compared with PBMCs.

Consistent with previous studies utilizing flow cytometry analysis of PDR vitreous, we confirmed the presence of T-cell infiltration, including CD4+ and CD8+ T cells[22,23] and further identified CD4+ naïve and memory T cells, as well as CD8+ naïve and memory T cells. Moreover, we demonstrate the presence of Tregs, and innate-like MAIT cells, and CD16+ and CD56+ populations of NK T cells from the innate immunity, all of which have not yet been described before in the PDR vitreous. The average proportion of T cells in vitreous samples is 45.4 percentage points higher than that of PBMC samples, and the average proportion of neutrophils in vitreous samples is 21 percentage points lower than what was seen in PBMC samples. Despite the limitations associated with the lower absolute number of B cells, myeloid cells, and neutrophils identified by scRNA-seq in PDR vitreous, these data highlight T cells as the predominant cell-type present in the PDR vitreous, especially because VH was present in all PDR vitreous samples.

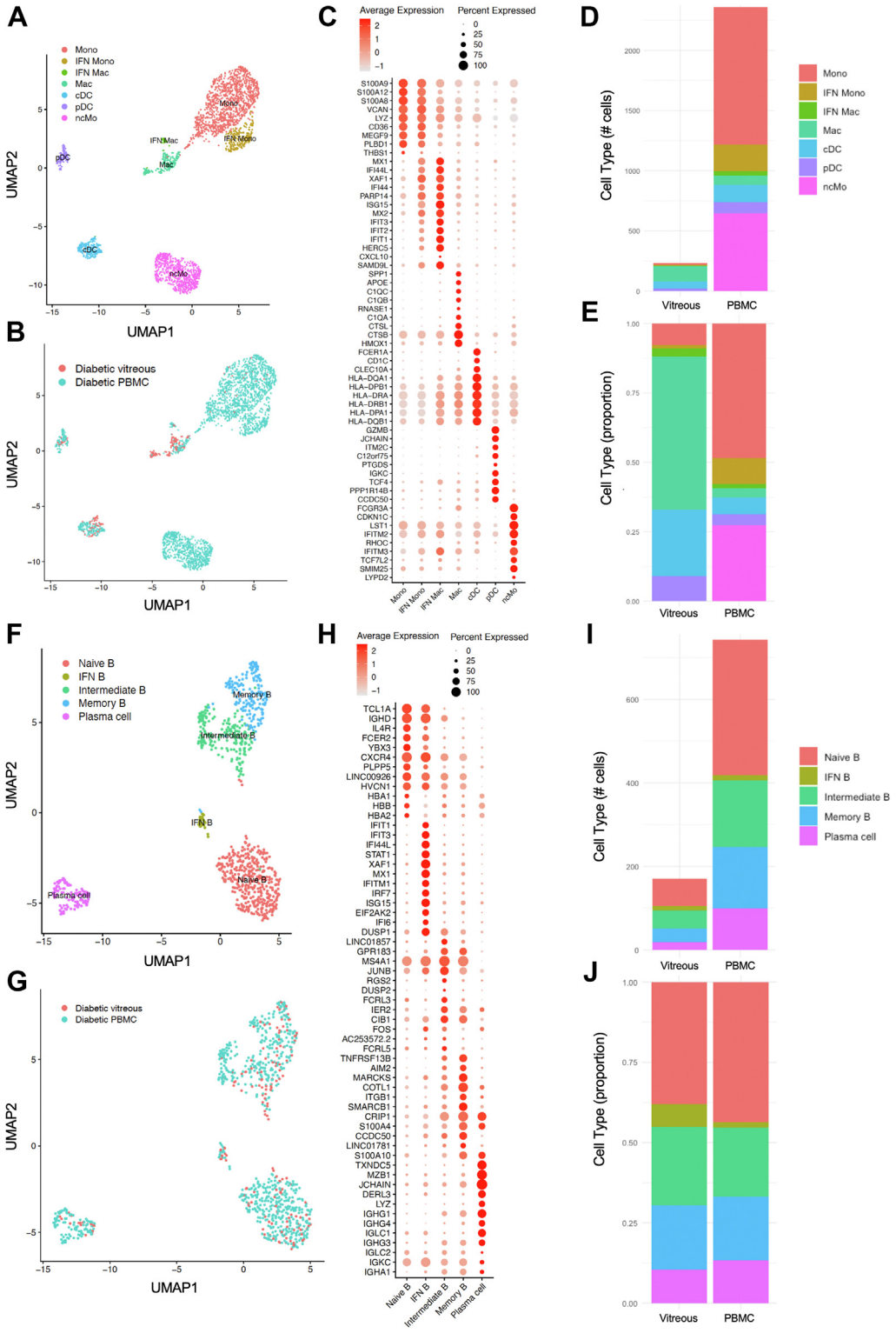

Figure 5. Myeloid-lineage cell and B-cell populations within vitreous and PBMCs from individuals with PDR. **A,** UMAP plot of the cluster identifications for macrophage and dendritic cell populations and **(B)** respective UMAP plot of cell location from the vitreous and peripheral blood. **(C)** Dot plot of differentially expressed genes in each identified cluster and monocyte population. **D, E,** Comparison of myeloid cell subtypes per cluster based on cell location by cell numbers and proportion. **F,** UMAP plot of the cluster identifications for B-cell populations and **(G)** respective UMAP plot of cell location from the vitreous and peripheral blood. **(H)** Dot plot of differentially expressed genes in each identified B-cell cluster. **I,** Comparison of cell numbers and **(J)** cell proportion of B-cell type per cluster based on cell location. IFN = interferon; Mono = monocyte; Mac = macrophage; DC = dendritic cell; FC = fold change; FDR = false discovery rate; PDR = proliferative diabetic retinopathy; UMAP = Uniform Manifold Approximation and Projection.

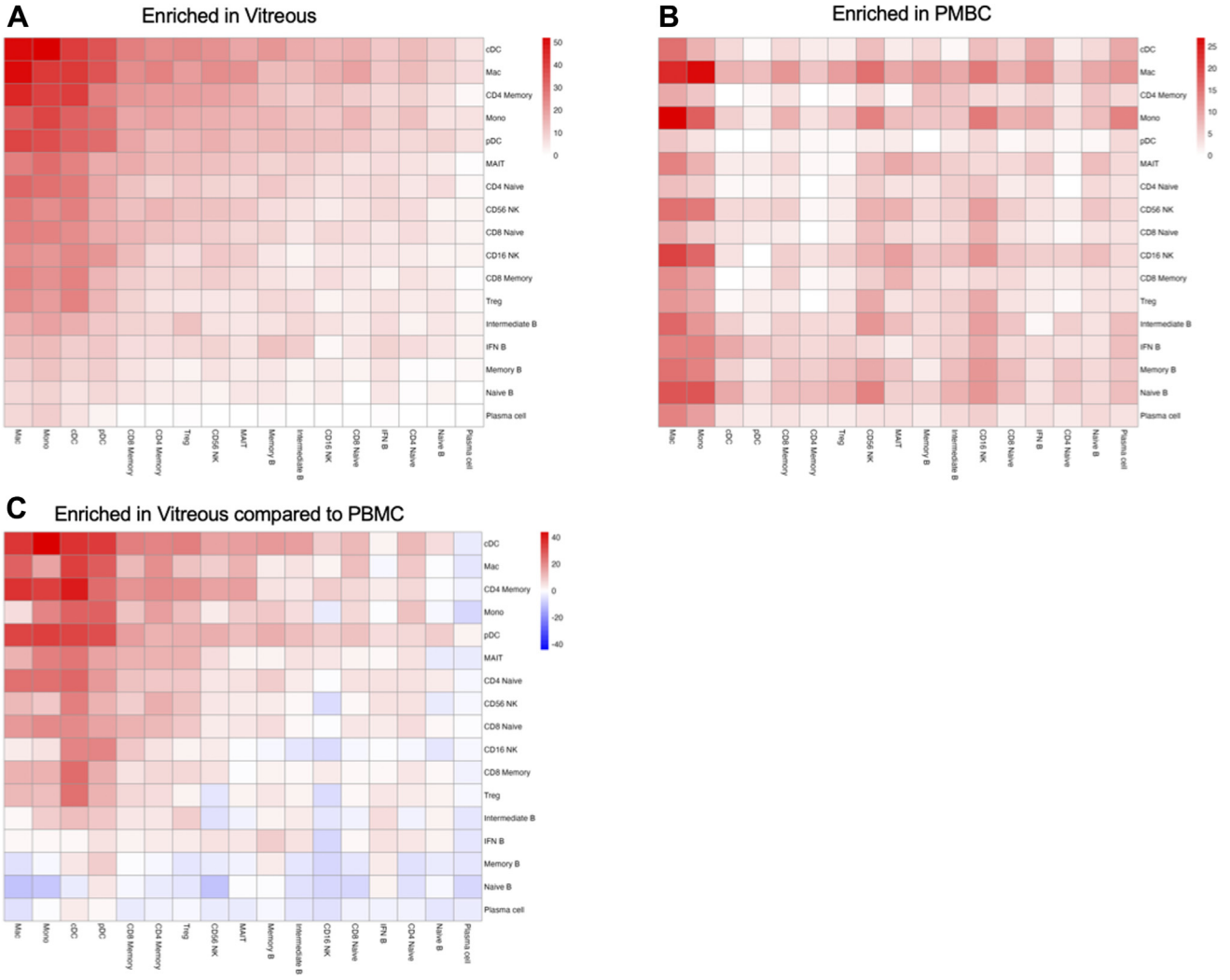

Figure 6. Cell–cell interactions within the vitreous and PBMC compartments. **A,** Heatmap representing cell–cell interactions identified within the vitreous compartment using Dimer Signal Receptor Analysis (DiSiR) which predicts ligand–receptor interactions at the cell-type level using the scRNA-seq data. Similarly, cell–cell interactions within the PBMC compartment were identified (**B**) with the data presented limited to interactions also possible in the vitreous compartment. **C,** Cell–cell interactions more prominent in the vitreous compartment are highlighted in this heatmap. PBMC = peripheral blood mononuclear cell.

Although innate components of the immune system in DR have been appreciated, the role of adaptive immunity is much less understood. This gap in knowledge may result from parts of the posterior segment, such as the retina being considered "immune-privileged" and the fact that animal models of DR do not develop PDR. What might be the role of T cells in the PDR vitreous? Studies using nondiabetic, murine oxygen-induced retinopathy (OIR) to model neovascularization, increased vascular permeability, and vision loss seen in PDR ascribe important functions to Treg cells (i.e., a subset of CD4+ T-cells enriched in FOXP3), CD4+, and CD8+ T cells that invade the retina.[23–25] Specifically, Treg cells initially increase in the OIR retina and then subside as neovascularization progresses. If Treg cell loss is avoided by interleukin-2 antagonism or adoptive transfer of Treg cells, there is a subsequent reduction of vaso-obliteration, neovascularization, and retinal ischemia.[25] Furthermore, CD4+ and CD8+ T cells were found to invade into the retina during neovascularization in OIR with CD8+ T cells at neovascular tufts and migrate there through a CXCR3-dependent process. Depletion of CD8+ T cells, but not CD4+ T cells, was found to reduce retinal neovascularization and leakage, suggesting that unlike Treg cell or other CD4+ T-cell populations, CD8+ T cells play a role in these key pathologic features present in PDR. Moreover, transplantation of CD8+ T cells with deficient expression of granzymes A/B, tumor necrosis factor (TNF), and interferon gamma, blocked retinal pathology, highlighting the central role CD8+ T cells play to worsen OIR.[24] Indeed ligand–receptor analysis showed enrichment in the vitreous over the PBMC compartment of CXCL12 ligand, expressed by macrophages, with the CXCR3 receptor, known to be expressed by T cells and pDCs.[26,27] In addition, granzyme transcripts were identified in our

scRNA-seq, and CD8+ T-cell interactions with myeloid-lineage cells were more prominent in the PDR vitreous (Fig 6C). These data suggest the animal OIR does model some aspect of T-cell infiltration into the human vitreous in PDR. The identified vitreous T-cell repertoire raises the possibility of new therapeutic targets in PDR: increasing the pool of invading Tregs and/or reducing CD8+ T-cell and pDC activity through CXCR3 blockade.

We also show for the first time that several effectors of the innate immune system, such as NK and MAIT cells, are present in the vitreous of humans with diabetes. These cell types have not been described in the vitreous of animal models of DR. Natural killer cell dysfunction has been theorized as a contributor to pathology in diabetes mellitus, but previous studies have largely been focused on their changes in the peripheral blood of humans and animal models, rather than their infiltration into the vitreous.[28,29] We also highlight the absence of neutrophils within the PDR vitreous compartment identified by our data set (Fig 1C). In the OIR animal model of PDR, early rise of neutrophils within the retina leads to vascular damage by priming a deleterious inflammatory and angiogenic response, and retinal neovascularization can be reduced with early depletion of neutrophils.[30] This interestingly contrasts with neutrophils within the peripheral blood, which have been previously described as a systemic inflammatory biomarker and predictor for worsening diabetic eye disease in patients.[31–33] The reason for this depletion of neutrophils in late-stage PDR may indicate inflammatory processes such as neutrophil NETosis or extracellular traps, which cannot be captured by single-cell analysis, may be contributing to retinal vasculopathy and that timing for neutrophil targeting may be particularly important in this disease process.[30,34–36] These data reveal the knowledge gap related to the roles of the activity of these components of the innate immune system in the PDR vitreous and highlight opportunities for future studies.

Our investigation revealed other components of the adaptive and innate immune system in the PDR vitreous, although these effector cells were less in number than T cells. These include various types of macrophages, dendritic cells, and B cells. The presence of macrophages and interferon macrophages we observed is consistent with the elevated levels of monocyte chemoattractant protein frequently upregulated in the PDR vitreous,[37–39] which promotes monocytes migration from the peripheral blood across the vascular endothelium, presumably into the vitreous. Indeed, higher numbers of vitreous resident macrophages have been reported by immunohistochemistry at the vitreoretinal interface in a VEGF-dependent manner, as occurs in the PDR vitreous.[40] We also identified 2 populations of dendritic cells, specifically cDCs and pDCs, antigen presenting cells that, after activation, migrate to lymphoid tissues to promote a T-cell–dependent adaptive immune response. Ligand–receptor pair analyses revealed the nature of intercellular communication in the PDR vitreous was distinct from the peripheral blood of the corresponding patients, specifically between myeloid-lineage cells. Although dendritic cell–epithelium interplay has been described in wound healing of the cornea, very little is known about the role of these cells in the diabetic vitreous.[41,42]

There are several limitations to this study. First, this study includes only 5 patients and 6 eyes with PDR, and cell counts of certain cell types are appreciably small, requiring further investigation with a larger study population to confirm the reported findings in comparison to patients without diabetes. Despite the small sample size, we demonstrate the consistency of our findings, with all 6 eyes showing a similar distribution of immune cell types (Fig S2A), which contrasts with the heterogeneity seen in the peripheral blood. However, we do note that even in the same donor, measurable differences in each eye were captured in our analysis, for example in myeloid cell populations (Fig S2E, IDs 2 and 5), that may reflect underlying pathology associated with tractional retinal detachment or prior anti-VEGF. This study also uses a convenient but previously undescribed approach to sample collection: collection of single cells from the diluted cassette washings of the vitreous during surgery. These washings are typically discarded as surgical waste; however, recent reports have demonstrated these otherwise discarded washings, via polymerase chain reaction and next-generation sequencing, can enhance and improve diagnosis for vitreoretinal lymphoma, a deadly CNS lymphoma that is a notorious masquerader that can take a year or more to properly diagnose.[43–45] Our findings demonstrate the utility of our methodological approach to better understand vitreous cell biology and function in a human disease state. Because vitreous is a biofluid that may, in the future, be amenable to serial sampling, this approach also opens the potential for precision medicine approaches by tracking disease progression and treatment response. Flow cytometry-based approaches of the PDR vitreous have not revealed the presence of B-cell lymphocytes[22,23,46]; however, scRNA-seq revealed, albeit in low numbers, naïve, IFN, memory, and intermediate B cells, as well as plasma B cells.

We intentionally included patients with VH in our study design. This contrasts with previous studies that used flow cytometry to interrogate inflammatory cell types, which have excluded or separated cellular analyses of human vitreous samples containing hemorrhage. Presumably, the assumption to exclude these samples is that cell types in VH simply reflect "spillover" (i.e., cell types that comprise a similar proportion to those in the peripheral circulation, such as PBMCs [lymphocytes and monocytes], polymorphonuclear cells [eosinophils and neutrophils], and red blood cells). However, the disrupted blood–retina barrier may favor invasion of specific cell types, and/or the vitreous compartment may promote selective survival or proliferation of cell types that is distinct from the milieu of the peripheral blood. Finally, cells present in the PDR VH may have interactions in the posterior segment among themselves or with resident retinal cells that contribute to the progression of PDR and vision loss. Thus, it is important to know the full repertoire of cells resident in the hemorrhagic PDR vitreous. We find that T cells are enriched to a higher degree than would be suspected by simple "spilling" of T cells within the peripheral blood into the vitreous. This may represent preexisting T-cell infiltration into the PDR

vitreous, before VH, and/or that T cells from hemorrhagic origin selectively persist in the PDR. Moreover, our results suggest a selection against neutrophils in the hemorrhagic PDR vitreous, in contrast to their abundance in the peripheral blood of the PDR-affected patients in our study.

In summary, we report our findings for the first single-cell vitreous atlas in the human PDR vitreous and identify an immune cell-type landscape that provides new insights of pathophysiology and potential pharmacologic targets in PDR. These data suggest that immune shifts are not just in numbers of immune cells but also in terms of immune subtypes, activity, and function. It is likely that the inflammatory changes occurring within the PDR vitreous involve pathways of immune activation and antigen presentation rather than a single or specific cytokine pathway. Furthermore, these results demonstrate the feasibility of liquid vitreous biopsies via collection of functional vitreous cell types from diluted cassette washings during vitrectomy, to make mechanistic and therapeutic insights, at single-cell transcriptomic resolution, of human retinal and vitreous diseases that contribute to vision loss.

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[1] Department of Ophthalmology and Visual Sciences, Kellogg Eye Center, University of Michigan, Ann Arbor, Michigan.

[2] Medical Scientist Training Program, University of Michigan Medical School, University of Michigan, Ann Arbor, Michigan.

[3] Department of Dermatology, University of Michigan Medical School, Ann Arbor, Michigan.

[4] Department of Computational Medicine and Bioinformatics, University of Michigan, Ann Arbor, Michigan.

[5] Rogel Cancer Center, University of Michigan, Ann Arbor, Michigan.

[6] Department of Human Genetics, University of Michigan, Ann Arbor, Michigan.

[7] Department of Pathology, University of Michigan, Ann Arbor, Michigan.

[8] Center for RNA Biomedicine, University of Michigan, Ann Arbor, Michigan.

[9] A. Alfred Taubman Medical Research Institute, University of Michigan, Ann Arbor, Michigan.

[10] Section of Ophthalmology, Surgery Service, Veterans Administration Ann Arbor Healthcare System, Ann Arbor, Michigan.

R.H. and D.H.P. contributed equally to this work.

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Author Contributions:

Conception and design: Haliyur, Parkinson, Liu, Gudjonsson, Rao

Data collection: Parkinson, Ma, Xu, Li, Huang, Tsoi, Bogle, Liu, Gudjonsson, Rao

Analysis and interpretation: Haliyur, Parkinson, Gudjonsson, Rao

Obtained funding: Rao

Overall responsibility: Haliyur, Parkinson, Rao

Abbreviations and Acronyms:
cDC = classical dendritic cell; **DR** = diabetic retinopathy; **ERM** = epiretinal membrane; **FC** = fold change; **FDR** = false discovery rate; **GO** = gene ontogeny; **HM** = hand motion; **IFN** = interferon; **LP** = light perception; **MAIT** = mucosal-associated invariant T; **NK** = natural killer; **OIR** = oxygen-induced retinopathy; **PBMC** = peripheral blood mononuclear cell; **PBS** = phosphate-buffered saline; **PDR** = proliferative diabetic retinopathy; **PPV** = pars plana vitrectomy; **scRNA-seq** = single-cell RNA sequence; **Treg** = T regulatory; **VEGF** = vascular endothelial growth factor; **VH** = vitreous hemorrhage.

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Correspondence:
Rajesh C. Rao, MD, 1000 Wall St., Ann Arbor, MI 48105. E-mail: rajeshr@umich.edu.

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Further Reading

1. Single cell RNA sequencing data associated with this study was deposited at Spectacle: a single cell atlas of gene expression in the eye at URL: singlecell-eye.org/app/spectacle (The University of Iowa Institute for Vision Research).