Elevation of Granulocyte Colony Stimulating Factor in Human AMD Donor RPE-Choroid

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Purpose. Choroidal inflammation, complement deposition, and accumulation of C-reactive protein (CRP) are involved in age-related macular degeneration (AMD) pathology. The pro-inflammatory signals that regulate immune cell recruitment in the choroid of patients with AMD remain to be determined. We performed cytokine profiling of human AMD and age-matched control donor tissue to identify inflammatory molecules upregulated in AMD tissue.

Methods. Protein was isolated from 25 AMD and 21 control donor RPE/choroid macular punches. Total protein was quantified, and 50 µg assayed for expression of 40 cytokines using an inflammation array. We validated the elevated expression of granulocyte colony stimulating factor (G-CSF) protein by ELISA in a second cohort of 22 control and 26 AMD donors. To identify an AMD associated stressor responsible for upregulating G-CSF we assayed for changes in G-CSF protein secretion in RPE/choroid organ cultures treated with the monomeric (m)CRP, an inflammatory protein elevated in AMD.

Results. Using a multiplex array, we identified elevated G-CSF protein in the choroid of AMD donors compared to age-matched non-AMD controls. Differential expression of G-CSF was confirmed via ELISA in an independent cohort of samples ($P = 0.01$). The mCRP, which is deposited in AMD choroids, increased G-CSF protein secretion in RPE/choroid organ cultures. Single nuclei RNA sequencing identified choroidal endothelial cells and fibroblasts as the primary cell types responsible for increased G-CSF secretion in response to mCRP. The G-CSF receptor is expressed primarily by choroidal macrophages and dendritic cells and anti-G-CSFR colocalizes with anti-CD45 and anti-CD68 in human donor choroid tissue.

Conclusions. Elevated G-CSF expression in AMD donor tissue as a result of increased levels of mCRP may be involved in immune cell recruitment in AMD contributing to inflammatory stress in the choroid.

Keywords: age-related macular degeneration (AMD), cytokine, inflammation, granulocyte colony stimulating factor (G-CSF), C-reactive protein (CRP)

Age-related macular degeneration (AMD) is a leading cause of central vision loss worldwide. Loss of cells in the choroid and RPE leads to death of the light sensing photoreceptor cells in the macular region, causing progressive vision loss. Clinical and histopathologic evidence indicate degeneration of the choriocapillaris early in AMD progression.[1–3] In addition to early choroidal endothelial loss, hallmarks of increased choroidal inflammation have been associated with early/intermediate AMD.

Histological studies have shown that increased numbers of choroidal macrophages[4] and degranulated mast cells[5] are present in the submacular region, the primary site of AMD pathology, at all stages of AMD. Interestingly macrophages appear more rounded in the submacular region suggesting an altered activation status locally at the site of AMD pathology. In addition to early AMD, in cases in which AMD progresses to a neovascular end stage, inflammation contributes to the pathogenesis of choroidal neovascularization. The role of myeloid cells has been studied extensively in the wet form of the disease, in which monocytes/macrophages are present in neovascular membranes and both monocytes and their adhesion molecules are essential for the neovascular response in animal models.[4,6–17]

A number of systemic studies have been performed to characterize pro-inflammatory cytokine and chemokine molecules in association with AMD. Cytokine levels have

been studied in the serum and plasma of AMD patients and elevation of systemic cytokines, such as IL-6, have been linked to AMD progression.[18] Furthermore, increased levels of pro-inflammatory cytokines IL-6, IL-8, and MCP-1[19,20] have been reported in the aqueous fluid of patients with AMD. In addition to systemic changes, cytokine secretion has been observed in vitro from RPE cells under oxidative stress conditions mimicking AMD pathogenesis.[21] Cytokines signal to immune cells promoting inflammation and draw mononuclear cells to sites of tissue injury. A recent study has identified that local alterations in the extracellular matrix protein CYR61 in choroidal veins may be involved in the recruitment of circulating monocytes,[22] supporting the hypothesis that local alterations in inflammatory molecules may contribute to increased mononuclear cell infiltration and activation in AMD and thus could be responsible for the increased numbers of activated macrophages observed in the submacular region of AMD donor eyes.[4] Although there is significant evidence for a local inflammatory response in early/intermediate AMD, less is known about local alterations in inflammatory cytokines in the human RPE and choroid. As there are increased numbers of inflammatory cells in the submacular choroid, we sought to investigate cytokine changes in macular human donor RPE/choroid tissue obtained from the macula of AMD and non-AMD eye donors. To this end, we quantified a series of 40 cytokine and chemokine proteins and found that granulocyte-colony stimulating factor (G-CSF) was elevated in AMD samples as compared to non-AMD controls.

Methods

Tissue Collection

Human donor eyes were obtained by the Iowa Lions Eye Bank (Iowa City, IA, USA). All experiments were conducted in accordance with the Declaration of Helsinki and with full consent of the donors' next of kin for the evaluation of medical records and the use of these tissues for biomedical research.

Donor eyes were prepared as described previously.[23] Briefly, anterior segments were removed and 4 full thickness incisions at 90 degrees were made from anterior to posterior such that the posterior poles were flattened for photomicrography. Trephine punches of 8 mm diameter were collected from the macula, centered on the fovea centralis, and were hemisected with half of the sample preserved for biochemical experiments (retina and RPE/choroid flash frozen separately) and the other half was preserved in freshly generated 4% paraformaldehyde. RPE-choroid samples used in this experiment were stored at –70 C.

Phenotyping

In most cases, donor affection status was obtained by ophthalmic chart review with verification from histology. In cases where no medical records were available, phenotyping was performed using a combination of evaluating the gross photographs and histology of the fixed portion of the macula.

Protein Isolation

For the samples used in the Quantibody array, RPE/choroid punches obtained from the macula of human donors were homogenized in PBS with 1% Triton X-100 containing protease inhibitor cocktail (Roche). Protein was quantified using Bio-Rad DC protein assay.

Single Nuclei Protein Fraction

Samples used for the G-CSF ELISA replication study were obtained as part of a nucleus extraction experiment. Tissue was homogenized by pestle and nuclei were isolated using a commercial nuclear isolation kit (10x Genomics Chromium Nuclei Isolation Kit), according to the manufacturer's instructions. The first supernatant fraction was collected from the pelleted nuclei and stored at –70°C until used for quantification and ELISA.

Quantibody Human Inflammation Array 3

RPE/choroid protein samples were thawed on ice and diluted to 50 µg in assay buffer. Then, 50 µg of total protein was incubated on the Raybiotech Quantibody human inflammation array 3 (QAH-INF-3). Each glass array detects 40 cytokines; BLC (CXCL13), Eotaxin-1 (CCL11), Eotaxin-2 (MPIF- 2/CCL24), GCSF, GM-CSF, I-309 (TCA-3/CCL1), ICAM-1 (CD54), IFN-gamma, IL-1 alpha (IL-1 F1), IL-1 beta (IL-1 F2), IL-1 ra (IL-1 F3), IL-2, IL-4, IL-5, IL-6, IL-6 R, IL-7, IL-8 (CXCL8), IL-10, IL-11, IL-12 p40, IL-12 p70, IL-13, IL-15, IL-16, IL-17A, MCP-1 (CCL2), M-CSF, MIG (CXCL9), MIP-1 alpha (CCL3), MIP-1 beta (CCL4), MIP-1 delta (CCL15), PDGF-BB, RANTES (CCL5), TIMP-1, TIMP-2, TNF alpha, TNF beta (TNFSF1B), TNF RI (TNFRSF1A), and TNF RII (TNFRSF1B). Cytokine values were interpolated from Log-Log regression Standard Curves. All detectable values for G-CSF were included in the analysis of healthy controls versus patients with AMD.

Granulocyte-Colony Stimulating Factor ELISA

There were 22 healthy control (no evidence of AMD in clinical history or via phenotyping) and 26 AMD donor protein lysates containing cytoplasmic and membrane proteins that were collected. The nuclear fraction from each donor was utilized in separate experiments. The amount of protein in each sample was quantified and 20 µg of total protein was resuspended in ELISA dilutant buffer for a final volume of 100 µl. The concentration of G-CSF was measured in each protein sample using ELISA (RayBiotech).

Immunohistochemistry

Immunohistochemistry was performed on frozen cryosections of paraformaldehyde-fixed human donor tissue sections. Immunofluorescence with antibodies directed against CD45 and G-CSFR. Each tissue section was blocked with 0.1% bovine serum albumin for 15 minutes, followed by a 1-hour incubation with a 1:200 dilution of anti-CD45 antibody conjugated to Alexa Fluor 488 (BD Biosciences Catalog number 567401) and a 1:50 dilution of anti-G-CSFR antibody (F-11) conjugated to Alexa Fluor 546 (Santa Cruz Biotechnology SC-393689). Sections were subsequently washed, and nuclei were counter stained with 4′-6-diamidino-2-phenylindole (DAPI). Colabeling of CD68 and G-CSFR was performed using a Fab Fragment secondary antibody. Each tissue section was blocked with 0.1% bovine serum albumin for 15 minutes, followed by a 1-hour incubation with a 1:50 dilution of anti-G-CSFR (F-11; Santa Cruz Biotechnology

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SC-393689) antibody. Sections were subsequently washed and incubated with Alexa Fluor 488 Fab fragment donkey anti-mouse antibody (Jackson ImmunoResearch Laboratories) for 30 minutes and were then washed and incubated with a 1:100 dilution of anti-CD68 antibody (Thermo Fisher Scientific) for 1 hour. Sections were subsequently washed and incubated with Alexa Fluor 546 donkey anti-mouse antibody (Invitrogen) for 30 minutes. Nuclei were counter stained with DAPI. After an additional wash, the sections were mounted and coverslipped with Aquamount. Sections were imaged on an Olympus BX41 microscope.

Organ Culture

A series of paired, 4 mm biopsy punches of human donor RPE/choroid tissue collected from 6 donors were plated into 24-well plates in DMEM with Primocin containing either 20 µg/mL of recombinant monomeric CRP or media alone containing vehicle (PBS) and were maintained at 37°C, 5% CO2. After 22 hours of incubation, the media was collected for G-CSF cytokine ELISA.

Single Nuclei RNA Sequencing of Human Organ Culture

As described above, paired 4 mm biopsy punches of human donor RPE/choroid tissue were collected into 24-well plates in DMEM with Primocin containing either 20 µg/mL of recombinant monomeric CRP or media alone containing PBS and were maintained at 37°C, 5% CO2. After 22 hours of incubation, the tissue was snap frozen and stored at –80°C. Frozen tissue samples were prepared for single nuclei RNA sequencing using the Chromium Nuclei Isolation Kit (PN-1000493 10x Genomics). In brief, frozen tissue samples were dissociated in lysis buffer with a sterile plastic pestle. During the dissociation the samples were stored on ice. Dissociated tissue was pipette mixed and incubated for 10 minutes on ice. Dissociated tissue was passed through a 100 µm filter prior to transferring to a nuclei isolation column. Samples were centrifuged and the flowthrough which contained nuclei was collected. Nuclei were washed and resuspended in 25 to 50 µL. Nuclei were counted using a hemocytometer. Nuclei were barcoded with the Chromium GEM-X single cell 3′ Kit (10X Genomics). The libraries were sequenced on the Novaseq Sequencer platform with an S2 flowcell and a run length of 100 cycles.

Computational Analysis of scRNA-Seq Data

Sequenced reads were mapped to the CellRanger human genome build GRCh38 with CellRanger (version 8.0.1) using the Gencode human transcript GTF (version 32) and the following parameter: –expect-cells = 8000. Background removal was performed using CellBender (version 0.3), and the resulting samples imported into Seurat (version 4.3.0) objects. Each object was filtered per cell requiring at least 2000 genes identified (nFeature_RNA > 2000), fewer than 10,000 genes identified (nFeature_RNA < 10000), and less that 1% of the reads from a mitochondrial origin (mito.genes < 1). Mitochondrial genes were identified with the following command: PercentageFeatureSet(my_initial_object, pattern = "^MT-"). Each Seurat object was normalized (NormalizeData) using the LogNormalize method and a scale factor of 10,000, and the set of variable genes identified (FindVariableFeatures) using the VST method and 2000 genes. The resulting objects were then integrated using FindIntegrationAnchors and IntegrateData, scaled and centered (ScaleData), had dimensionality reduction performed (RunPCA and RunUMAP), and clusters identified (FindNeighbors and FindClusters with a granularity resolution of 0.9). Clusters judged to represent the same cell type (e.g. RPE and fibroblast) were merged together.

Statistics

Experiment one consisted of screening 21 control and 25 AMD donors for 40 cytokines. The AMD group contained 20 early-intermediate AMD donors, 2 geographic atrophy (GA) donors, and 3 choroidal neovascularization (CNV) donors. Given the low number of donor tissue samples with either GA or CNV, all AMD donors were treated as a single group and compared with the control group.

Experiment two evaluated the levels of G-CSF in a second cohort of 22 control and 26 AMD donors. The AMD group was comprised of 14 early-intermediate AMD donors, 3 GA donors, and 9 CNV donors. All AMD donors were again treated as a single group and compared to the age-matched control group. Statistical significance was determined using the nonparametric Wilcoxon rank sum test.

Results

Cytokine Profiling Identifies an Increase in G-CSF Protein in AMD Donor RPE-Choroid Tissue

Inflammation has been shown to play a role in the pathogenesis and progression of AMD. Altered cytokine secretion has been reported both systemically in serum and plasma and locally in aqueous humor samples of patients with AMD. Local changes in the affected tissues are less well appreciated. We aimed to identify cytokines that were altered in donor RPE/choroid tissue from human donor eyes with AMD, as this compartment is the site of the cardinal events in AMD pathogenesis (e.g. drusen formation, choriocapillaris loss, and RPE pigmentary changes). To identify alterations in cytokine secretion in human donor RPE/choroid tissue we isolated protein from 21 control and 25 AMD macular RPE/choroid biopsy punches (Figs. 1A, 1B, Table 1). A total of 50 micrograms of protein from each donor was analyzed for abundance of 40 cytokine and chemokine proteins using a Quantibody human inflammation array. Differences between control and AMD donor tissue was detected for several of the cytokines evaluated (Supplementary Files S1–S5). A representative array depicting control and AMD donor cytokine expression is shown in Figure 1C. Following quantification of each cytokine, the greatest change observed was a 6.5-fold increase in the chemokine G-CSF (Fig. 1D), although this did not reach statistical significance when corrected for multiple measurements (P value = 0.06).

AMD Donor RPE-Choroid Tissue has Significantly Increased Level of G-CSF Protein

Having identified an average 6.5-fold increase in G-CSF using cytokine array profiling we sought to further validate this observation in independent samples of AMD RPE-choroid tissue. For this follow-up study, we utilized protein depleted of nuclei obtained during the processing of nuclei

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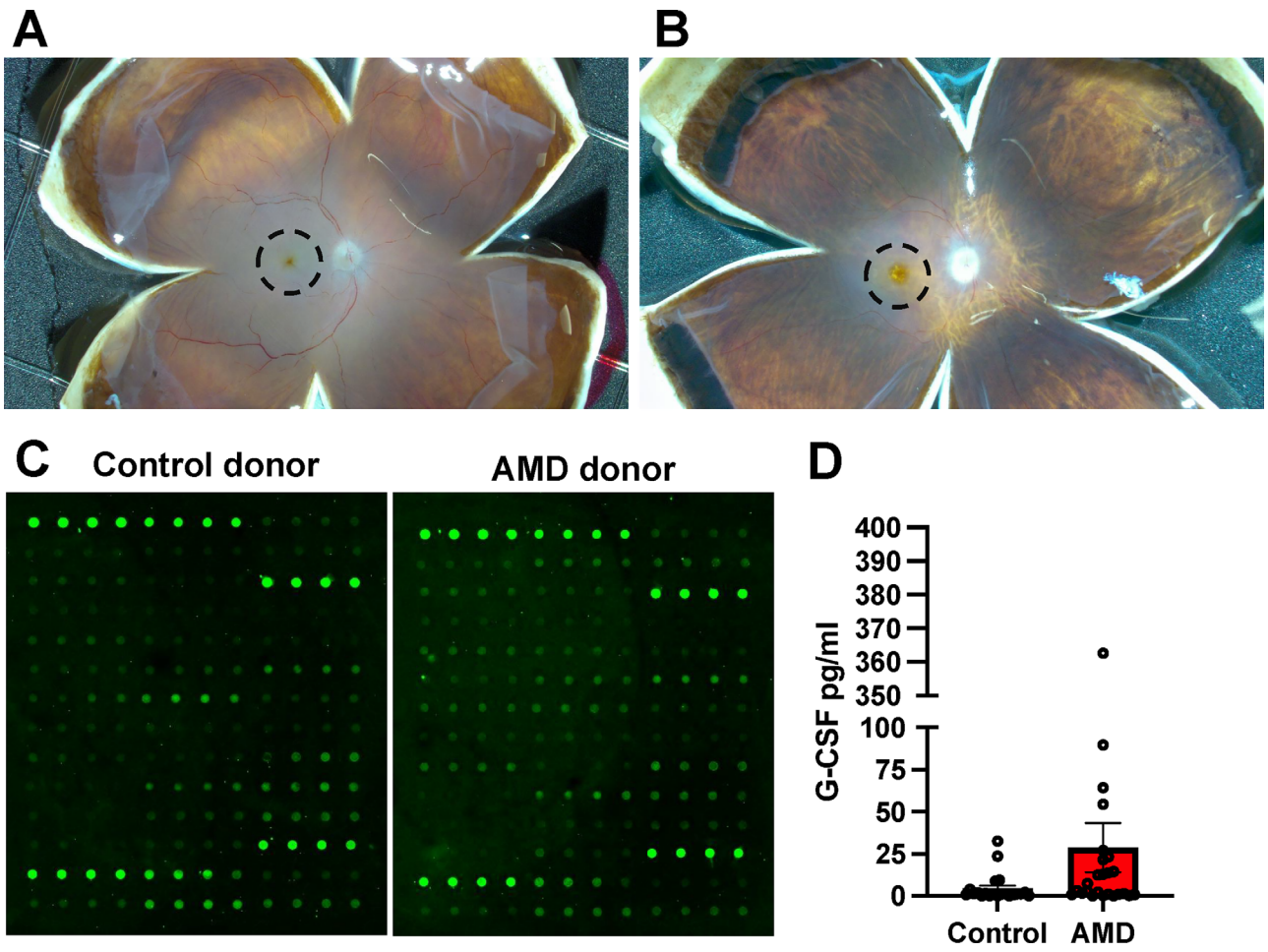

Figure 1. Protein was isolated from macular RPE/choroid punches collected from 21 age-matched control (**A**) and 25 AMD donor (**B**) eyes. Cytokine array analysis of bulk protein detected altered cytokine levels in control and AMD donor RPE/choroid tissue (**C**). The chemokine G-CSF showed a trend toward elevation (6.5-fold) in AMD donor tissue P value = 0.06.

for separate experiments. This allowed us to screen a second cohort of 22 control and 26 AMD donors for the presence or absence of G-CSF chemokine expression. Due to processing the protein collected from this second group of donors was more dilute and therefore we tested 20 µg of total protein for G-CSF expression using a G-CSF ELISA, which has sensitivity to detect 500 to 0.69 pg/mL of G-CSF protein. We detected G-CSF protein in 3 control donors and 12 AMD donors, and G-CSF was significantly elevated in the AMD donors RPE/choroid tissue compared to the control tissue using the nonparametric Wilcoxon rank sum test $P = 0.01$ (Fig. 2A, Table 2).

The Receptor for G-CSF is Expressed by Inflammatory Macrophages in Human Donor Choroid

G-CSF is a glycoprotein initially characterized to be involved in the recruitment of granulocytes and neutrophils in response to tissue injury.[24] Since its identification, a broad range of additional roles have been discovered for G-CSF, including neuroprotective, anti-inflammatory, and anti-apoptotic roles.[25] Interestingly, G-CSF has been reported to regulate macrophage polarization toward a tumor promoting phenotype.[26] To identify the cell types within human donor choroid tissue that expresses both the chemokine G-CSF (*CSF3*) and its receptor (*CSF3R*), we utilized our online single cell expression atlas[27] to query the expression of *CSF3* and *CSF3R* in the human choroid of 11 AMD and 10 control donors.[28] We observed that expression of *CSF3*, the gene encoding for the G-CSF chemokine protein, was enriched in the choriocapillaris, artery, and veins of the human choroid, and the chemokine receptor *CSF3R* was expressed predominantly by inflammatory macrophages and dendritic cells (Fig. 2B). Furthermore, we observed a subset of cells modestly positive for CD45 that labeled positively for G-CSFR in the choroid of control human donor (Fig. 2C). In follow-up studies, we observed a subset of CD68 positive choroidal macrophages in AMD donor tissue that co-labeled with G-CSFR (Supplementary Fig. S1). These observations suggest that vascular derived G-CSF may signal to recruit inflammatory macrophages or activate dendritic cells during AMD progression.

mCRP Treatment Induces CSF3 Expression and G-CSF Secretion From Human RPE/Choroid Organ Culture

Having identified elevated G-CSF chemokine levels in AMD donor RPE/choroid tissue we next investigated whether

Table 1. Donors for Cytokine Array and Detected G-CSF Values

Donor	Age	Sex	Disease Status	Cause of Death	Time to Preservation	G-CSF pg/mL
1	78	F	Control	Cancer	5:15	1.98335562
2	83	F	Control	Respiratory failure	5:42	1.091050592
3	96	F	Control	Respiratory failure	6:09	1.276894479
4	82	F	Control	Unknown	7:08	0.1328041
16	88	F	Control	Cancer	5:33	1.241305911
18	78	M	Control	Renal failure	8:41	0.669392404
19	84	F	Control	Cancer	8:58	0
20	87	M	Control	Cancer	7:04	1.053363913
21	97	F	Control	Pulmonary edema	6:34	1.133053702
22	74	M	Control	Cardiac arrest	6:10	1.36687924
23	91	F	Control	Pulmonary embolism	6:50	3.984082946
24	68	F	Control	Cancer	6:30	0
25	90	F	Control	Cholecystitis	6:40	0.864173693
26	82	F	Control	GI bleed	6:18	1.530924494
28	78	F	Control	Cancer	5:12	0.114653296
29	94	M	Control	Vagal episode	6:12	2.142524098
30	68	M	Control	Heart failure	6:10	0
31	77	M	Control	Cardiac arrest	5:14	9.444057432
39	77	M	Control	Pneumonia	5:16	23.59424446
41	75	M	Control	Arrhythmia	6:03	32.52168219
42	86	M	Control	Subdural hematoma	7:34	8.761358462
6	88	F	AMD	Pneumonia	7:34	1.044369983
7	90	F	AMD	Respiratory failure	5:49	362.6
8	99	M	AMD	Heart failure	7:14	27.02421766
9	77	M	AMD	Cardiac arrest	10:34	1.7399911
10	83	F	AMD	Cancer	7:13	2.90193461
11	71	M	AMD	Lymphoma	6:22	0.732961182
13	90	M	AMD	Traumatic brain injury	5:41	0
14	76	M	AMD	Lung disease	5:48	0.976842693
17	81	F	AMD	Cancer	6:53	3.253524288
27	88	M	AMD	Cardiac arrest	7:02	23.32882149
33	66	F	AMD	Subdural hematoma	5:03	7.480336186
34	92	F	AMD	Aspiration	4:44	64.39588684
35	86	M	AMD	Stroke	5:41	54.5198243
36	93	F	AMD	Small bowel obstruction	6:25	12.41846903
37	82	F	AMD	Renal failure	5:48	89.60744304
38	83	F	AMD	Cardiac arrest	5:10	13.70817868
40	76	F	AMD	Subarachnoid hemorrhage	6:18	12.77536969
43	83	F	AMD	Melanoma	5:13	0.095295752
44	97	F	AMD	Respiratory failure	5:55	0.892110548
45	86	M	AMD	Cerebral vascular accident	6:40	0
5	87	F	Neovascular	Cardiac arrest	4:35	0.20716515
12	83	M	Neovascular	Cancer	6:41	2.32891587
15	84	M	Neovascular	Lung disease	6:19	1.423074791
32	85	F	GA	Cardiogenic shock	4:50	14.51633765
46	97	F	GA	Cerebral vascular accident	6:33	21.7008142

GA, geographic atrophy; GI, gastrointestinal.

inflammatory monomeric CRP, which is elevated and accumulates around the choriocapillaris of AMD donors,[29] contributes to stimulating G-CSF cytokine secretion. To this end, we obtained 4 mm biopsy punches of human donor RPE/choroid at the time of arrival to the laboratory and placed the fresh tissue directly into media containing mCRP or vehicle control. Each of the donors had no indication of AMD pathology at the time of tissue collection (Table 3). After 22 hours of culture, the media was collected from the donor organ cultures to assay for the secretion of G-CSF in response to mCRP stimulation. We collected three paired punches per donor starting directly adjacent to the macula and working out toward the periphery (Fig. 3A). We collected organ cultures for 6 total donors and observed significantly increased G-CSF protein secretion into the media following treatment with mCRP in each set of paired 4 mm punches independent of anatomic location (Figs. 3B–D). We observed the same trend of increased G-CSF secretion in all three anatomic punches in response to mCRP treatment. Therefore, we took an average value of PBS treated tissue and mCRP treated tissue per donor and found a significant increase in G-CSF protein secretion across the 6 donors in response to mCRP (Fig. 3E). To determine which cell type in the heterogenous RPE/choroid organ culture was responsible for increased secretion of G-CSF in response to mCRP treatment, we dissociated frozen organ culture punches treated with either PBS or CRP from a single donor and conducted single nuclei RNA sequencing. We identified that both fibroblasts and endothelial cells upregulate the expression of *CSF3* in response to mCRP (Fig. 3F). Processed

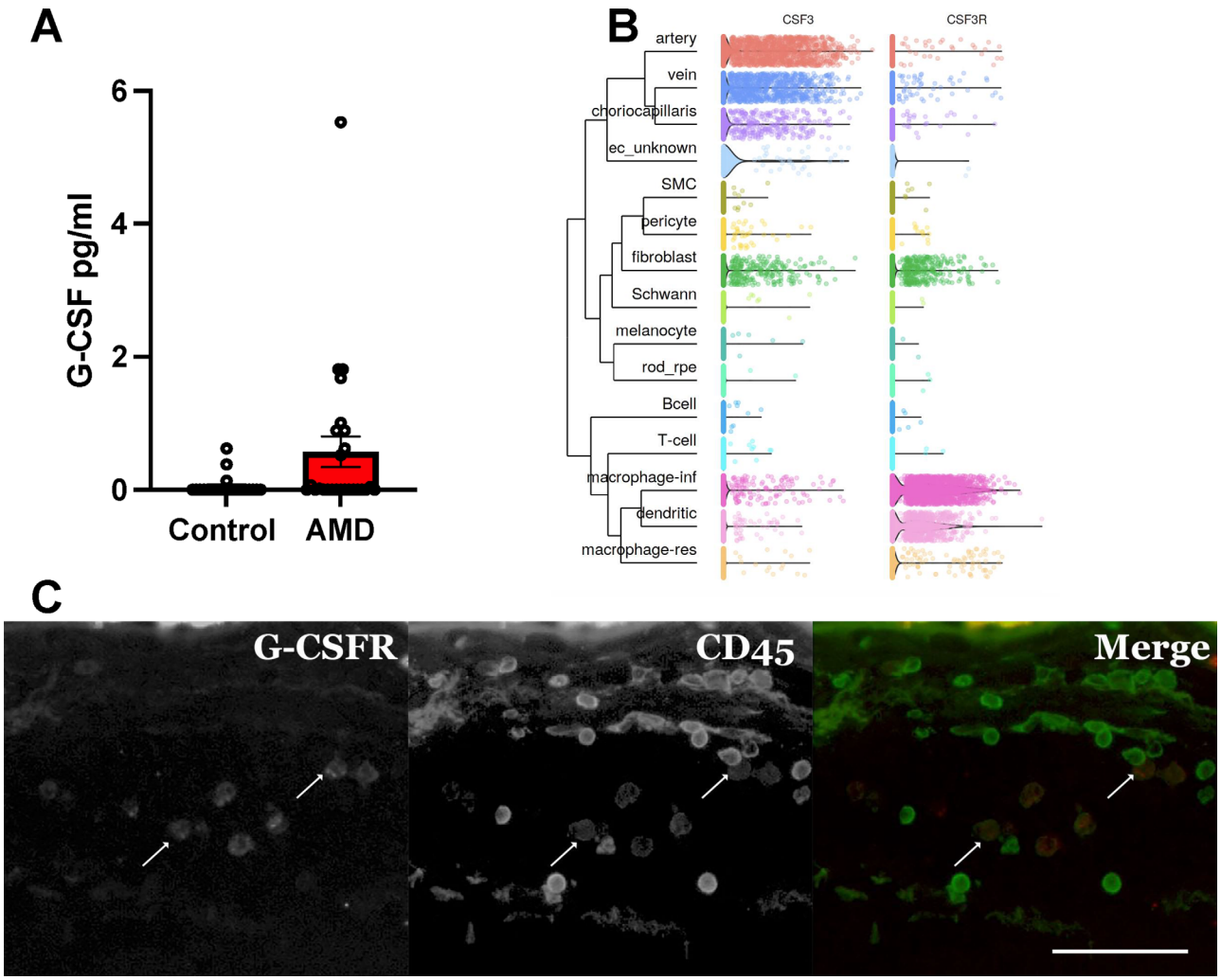

Figure 2. Elevation of G-CSF cytokine was validated by ELISA in an additional cohort of 22 control and 26 AMD donor protein samples *P* value = 0.01 (**A**). The gene encoding for G-CSF, *CSF3*, is expressed by artery, vein, choriocapillaris, and fibroblasts and the G-CSF receptor, *CSF3R* is expressed on fibroblasts, macrophage-inflammatory and dendritic cells in human single cell RNA sequencing datasets[27] (**B**). G-CSFR was visualized on a subset of CD45 positive cells in human donor choroid (**C**). Scale bar = 50 µm.

data from the single nuclei RNA sequencing experiment is available on singlecell-eye.org.[27]

Discussion

There is increasing evidence indicating inflammation is involved in the pathogenesis of AMD.[9,10,13–15,30] Early/intermediate AMD is associated with increased numbers of activated macrophages,[4] yet the signaling molecules elevated locally which attract these immune cells remain to be fully elucidated. Cytokines and chemokine molecules play an essential role in the recruitment of immune cells during infection or in response to tissue injury.

Whereas altered cytokine profiles have been identified in the blood of patients with AMD and in aqueous humor samples, less is known about local alterations in inflammatory cytokines in the RPE and choroid. In this study, we investigated whether inflammatory cytokine or chemokine protein expression is altered in AMD human donor RPE/choroid tissue. From a panel of 40 inflammatory molecules, we identified an association between AMD and elevated levels of G-CSF in an experiment with 25 AMD donors and 21 age-matched control donors. We further validated this finding in an additional, non-overlapping cohort of 22 control and 26 AMD donors and report a significant increase in G-CSF levels in AMD donor RPE/choroid tissue by ELISA.

G-CSF is a chemokine initially characterized to be involved in neutrophil proliferation, differentiation, and survival.[24] However, since its identification, a broad range of additional roles have been discovered for G-CSF, including neuroprotective, anti-inflammatory, and anti-apoptotic roles.[25] For example, G-CSF is produced by endothelial cells in response to stress.[31] Interestingly, G-CSF protects against vascular loss in other tissues by recruiting bone marrow-derived endothelial progenitor cells[32] and has been reported to be involved in "re-endothelialization." For example, in a rat model of carotid artery injury, administration of recombinant G-CSF supported vascular endothelial cell regeneration via the recruitment of C-kit-positive/FLK-1-positive bone marrow-derived cells.[33] Because G-CSF

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Table 2. Donors for G-CSF ELISA and Detected G-CSF Values

Donor	Age	Sex	Disease Status	Cause of Death	Time to Preservation	G-CSF pg/mL
1	87	F	Control	Stroke	5:30	0
2	86	M	Control	Aortic stenosis	4:55	0
3	83	M	Control	Cardiac arrest	5:45	0
4	92	F	Control	Stroke	5:02	0
5	77	M	Control	Renal failure	5:31	0
6	81	F	Control	Sepsis	5:00	0
7	89	F	Control	Pneumonia	3:33	0
8	87	F	Control	Sepsis	6:56	0
9	92	M	Control	Natural causes	5:45	0
10	89	F	Control	Acute subdural hematoma	5:14	0
11	72	F	Control	Sepsis	6:33	0
12	91	F	Control	Malnutrition	7:45	0
13	83	F	Control	Gastric bowel perforation	6:42	0.624690118
14	84	F	Control	Cardiac arrest	5:35	0
15	78	M	Control	Cardiac arrest	7:37	0
16	88	M	Control	Unknown	3:05	0
17	77	F	Control	Sepsis	6:43	0
18	85	M	Control	Cardiogenic shock	6:43	0.381708754
19	71	F	Control	Sepsis	6:30	0.13538161
20	97	F	Control	Sepsis	7:08	0
21	96	F	Control	Lung disease	5:26	0
22	89	M	Control	Renal disease	6:10	0
23	83	M	AMD	Motor vehicle collision	5:25	0
24	92	F	AMD	Cancer	5:25	0
25	89	F	AMD	Unknown	3:54	0
26	86	M	AMD	Lung disease	7:27	0.893153548
27	77	M	AMD	Pneumonia	6:04	0
28	89	F	AMD	Cerebrovascular accident	4:24	0
29	71	M	AMD	Cardiac arrest	4:43	1.682802869
30	92	M	AMD	Multi-organ failure	4:33	0.069167491
31	89	F	AMD	Pneumonia	6:20	0
32	89	M	AMD	Congestive heart failure	6:20	1.006622835
33	79	M	AMD	Pancreatic cancer	6:47	0
34	85	M	AMD	Acute coronary syndrome	6:06	0
35	79	F	AMD	Congestive heart failure	6:43	0
36	96	F	AMD	Sepsis	5:22	0.524112391
37	83	F	Neovascular	Subdural hematoma	4:42	0
38	93	F	Neovascular	Cardiogenic shock	7:31	0
39	97	M	Neovascular	Cardiac arrest	3:59	0
40	92	F	Neovascular	Sepsis	6:43	0.624690118
41	81	M	Neovascular	Cardiac arrest	7:23	1.814128462
42	72	F	Neovascular	Surgical complications	6:01	0.893153548
43	85	F	Neovascular	Aortic aneurysm	4:55	0
44	70	F	Neovascular	Cardiac arrest	7:50	0.016317197
45	80	M	Neovascular	Respiratory arrest	4:35	5.526315431
46	93	F	GA	Cardiogenic shock	7:59	0
47	92	F	GA	Respiratory distress	5:21	0.04058825
48	76	F	GA	Unknown	4:04	1.814128462

GA, geographic atrophy.

Table 3. Donors for Organ Culture

Donor	Age	Sex	Cause of Death	Ophthalmology Notes
1	65	F	Esophageal varices/shock	No retina disease in eye records numerous hard drusen on contralateral macula H&Es
2	78	M	Pancreatic cancer	No eye records, dot blot hemorrhages on gross examination
3	49	F	Liver disease	No retinal disease
4	62	M	Cardiac arrest/acute hypoxemic respiratory failure	BRVO contralateral eye, diabetic retinopathy
5	66	F	Urothelial cancer	No retinal disease
6	77	F	Metastatic adrenal cancer	High myopia, Hx of BRVO

BRVO, branch retinal vein occlusion; H&E, hematoxylin and eosin stain; Hx, history.

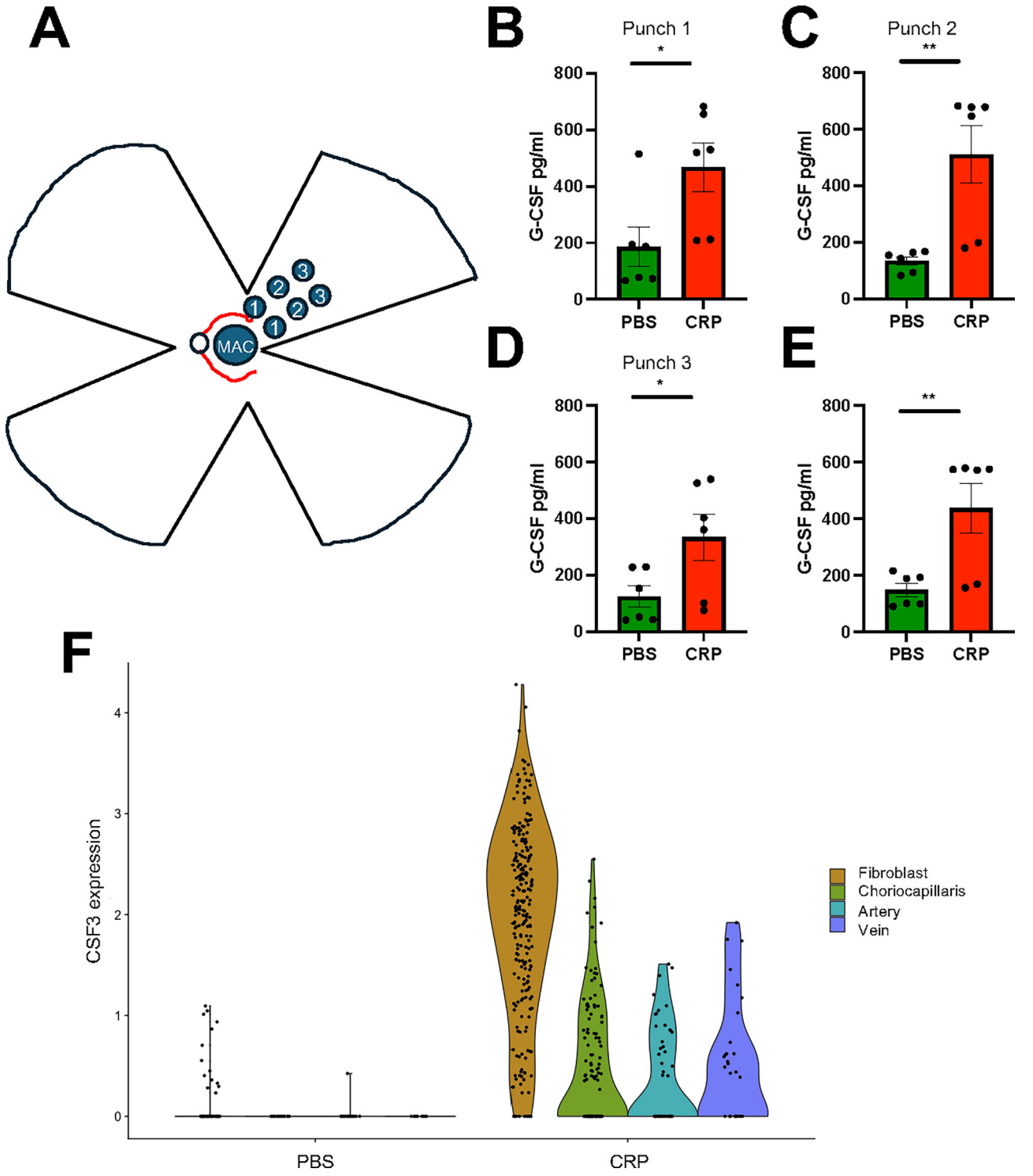

Figure 3. Organ cultures of human RPE-choroid were collected as pairwise punches during donor eye dissection illustrated in schematic (**A**) the optic nerve head is indicated by an open circle and the macula by a *circle* labeled MAC. Tissue was cultured in the presence of either PBS as vehicle or with 20 µg/mL of monomeric CRP. After 22 hours of incubation, media samples were collected, and levels of G-CSF were measured in paired punches in 6 human donors using ELISA punch 1 = P value = 0.03, punch 2 = P value = 0.004, and punch 3 = P value = 0.04 (**B, C, D**). An average value was taken per donor per treatment condition and showed a 2-fold increase in G-CSF in response to monomeric CRP P value = 0.009 (**E**). Single nuclei RNA seq on a human RPE-Choroid punch identified fibroblasts and choriocapillaris endothelial cells as the primary cell types responsible for increased G-CSF expression (**F**).

protects against vascular loss, the mechanistic relationship between choroidal vascular loss (which occurs early in AMD), and local G-CSF levels may be important in choroidal health.

Single cell RNA sequencing data identifies vascular endothelium as the main source of G-CSF in human donor choroid (see Fig. 2B). Therefore, it is possible that G-CSF may be released from damaged endothelial cells during

the progression of AMD. Interestingly G-CSF has been reported to dampen the pro-inflammatory effects of C-reactive protein,[34] an inflammatory molecule that is itself more abundant in AMD blood and choroid.[18,29,35,36] as well as in the choroid of donors with high risk CFH genotypes.[36] From re-evaluation of previous bulk RNA-sequencing data of human donor RPE/choroid organ cultures incubated with mCRP, we found that CRP treatment elevated the expression of G-CSF RNA 13-fold compared to control treated tissue (adjusted P value = 0.001).[37] Furthermore, we identified that CRP treatment of six donor organ cultures resulted in increased secretion of G-CSF. This may suggest that cells within the RPE/choroid upregulate G-CSF to counteract the inflammatory response associated with accumulating mCRP. Using single nuclei RNA-Seq, we identified endothelial cells and fibroblasts as the main cell types responsible for increased G-CSF secretion in response to mCRP.

Chemokines recruit immune cells to sites of injury. To identify which cell type may be responding to elevated G-CSF secretion in AMD donor tissue, we analyzed the expression level of the G-CSF receptor (*CSF3R*) in human donor choroid single cell data and observed expression of the chemokine receptor on a subpopulation of inflammatory macrophages and on dendritic cells. Furthermore, we observed subsets of CD45 positive cells which labeled positively for G-CSFR in human donor choroid tissue from a control donor and, in follow-up studies, identified CD68 positive macrophages which labeled positively for G-CSFR in AMD donor tissue. Therefore, G-CSFR positive mononuclear cells are observed in both control and AMD human donor choroid, which can be expected given the choroidal vasculature contains circulating mononuclear cells in addition to resident macrophages. However, the chemokine G-CSF which activates the G-CSFR is elevated in AMD donors.

There are a number of limitations to this study. Although both early and advanced AMD samples were utilized, as indicated in Table 1, the number of advanced AMD cases (2 GA and 3 macular neovascularization for arrays and 3 GA and 9 neovascularization [NV] for ELISA) was statistically underpowered for subgroup analysis and we therefore combined all AMD donors. Larger sample cohorts may help parse the distribution of G-CSF (and other cytokines) across different clinical conditions and severities. Whereas G-CSF was upregulated on average across AMD samples, most samples in both categories showed undetectable levels, suggesting a contribution of genetic background or other variable in addition to AMD affection status. We identified endothelial cells and fibroblasts as the cell types responsible for elevated G-CSF secretion in response to monomeric CRP in organ cultures generated from a single human donor, which is informative but not conclusive evidence of the cell types responsible for increased G-CSF levels in all donors. In addition to activating receptors on immune cells (specifically FcγRIIa),[38] CRP can modulate complement activation,[39,40] which is likely to further contribute to altered cytokine signaling in AMD.

In summary, we observed increased levels of G-CSF protein in AMD donor tissue compared to age-matched control tissues and identified monomeric CRP as a local insult which may be responsible for elevated secretion of G-CSF from endothelial cells and fibroblasts. The receptor for G-CSFR protein is present on CD45 and CD68 positive myeloid cells in human donor choroid and macrophages and dendritic cells express the RNA for G-CSFR. We conclude that further studies are necessary to explore the G-CSF/G-CSFR axis as it may be involved in immune cell recruitment and activation in AMD contributing to the inflammatory stress of the choroid early in AMD progression.

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Data Availability: The snRNA-seq data is publicly available from GEO as GSE278749. Processed data from the single nuclei RNA sequencing experiment is available on singlecell-eye.org.[27]

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