Induction of Chemokine Secretion and Monocyte Migration by Human Choroidal Melanocytes in Response to Proinflammatory Cytokines

Tina Jehs,1 Carsten Faber,1 Maja S. Udsen,1 Martine J. Jager,2 Simon J. Clark,3 and Mogens H. Nissen1

1University of Copenhagen, Department of Immunology and Microbiology, Eye Research Unit, Copenhagen, Denmark
2Department of Ophthalmology, Leiden University Medical Center, Leiden, The Netherlands
3Centre for Ophthalmology and Vision Sciences, Institute of Human Development, AV Hill Building, University of Manchester, Manchester, United Kingdom

Correspondence: Tina Jehs, Blegdamsvej 3, 2200 Copenhagen N, Denmark; tjehs@sund.ku.dk.
Submitted: October 28, 2015
Accepted: October 26, 2016
Citation: Jehs T, Faber C, Udsen MS, Jager MJ, Clark SJ, Nissen MH. Induction of chemokine secretion and monocyte migration by human choroidal melanocytes in response to proinflammatory cytokines. Invest Ophthalmol Vis Sci. 2016;57:6568–6579. DOI:10.1167/iovs.15-18524

PURPOSE. To determine to which extent inflammatory cytokines affect chemokine secretion by primary human choroidal melanocytes (HCMs), their capacity to attract monocytes, and whether HCMs are able to influence the proliferation of activated T cells.

METHODS. Primary cultures of HCMs were established from eyes of 13 donors. Human choroidal melanocytes were stimulated with IFN-γ and TNF-α or with supernatant from activated T cells (T-cell-conditioned media [TCM]). Gene expression analysis was performed by using microarrays. Protein levels were quantified with ELISA or cytometric bead array.

RESULTS. Stimulation of HCMs with TCM or IFN-γ and TNF-α resulted in increased expression and secretion of CXCL8, CXCL9, CXCL10, CXCL11, CCL2, CCL5 and intercellular adhesion molecule 1. Vascular endothelial growth factor and monocyte migration inhibitory factor were constitutively expressed without changes in response to proinflammatory cytokines. Supernatants derived from unstimulated cultures of 10 HCM donors induced a high initial level of monocyte migration, which decreased upon stimulation with either TCM or IFN-γ and TNF-α. The supernatants from three HCM donors initially showed a low level of monocyte attraction, which increased after exposure to proinflammatory cytokines. Direct coculture of HCMs with T cells resulted in inhibition of T-cell proliferation.

CONCLUSIONS. These results showed that normal and activated HCMs are immunologically active by secreting chemokines, and that HCMs are able to attract monocytes in addition to inhibiting T-cell proliferation.

Keywords: choroidal melanocytes, inflammation, chemokines, monocyte migration

Uveal melanocytes are the most abundant cells in the choroid.1 They contain two different types of melanin (eumelanin and pheomelanin), where particularly eumelanin has an antioxidative effect.2 Furthermore, previous studies have shown that cultured human choroidal melanocytes (HCMs) are also able to produce matrix metalloproteinases3 and tissue plasminogen activator.4

A link between HCMs and inflammatory eye diseases such as uveitis and age-related macular degeneration (AMD) has been suggested previously (see Hu et al.5), as in vitro stimulation of HCMs leads to the secretion of IL-6,1 CXCL8, and CCL2.5 In both diseases an increased level of those cytokines occurs in the aqueous humor.6,7 Additionally, in ocular tissue from AMD patients, increased levels of the chemokine CXCL10 have been found.8 However, the functions of HCMs have only been partially characterized and are currently poorly understood.

Macrophages and chemokines are central to inflammatory events in the uvea. Chemokines are a group of chemotactic cytokines, which are highly redundant and can drive the migration of monocytes from the bloodstream into the tissue where these cells then differentiate into macrophages.9 Macrophages are phagocytic cells that can modulate an inflammatory response and it has been shown that homeostatic macrophages reside in the choroid of normal human eyes.10,11 In mice, an age-dependent accumulation of macrophages in the choroid occurs.12,13 Moreover, it has been shown in mice as well as in humans that with age, the ratio of the anti-inflammatory M2 macrophages to proinflammatory M1 macrophages increases in the eye,14,15 suggesting age-dependent functional changes of macrophages in the choroid. Also, increased levels of circulating inflammatory mediators have been measured in the serum or plasma of aging individuals.16–18 Patients with AMD,19 uveitis,20,21 and uveal melanoma22 While macrophages are present in the healthy choroid, they have also been implicated in the pathogenesis of these eye diseases.14,14,23–30 In AMD, macrophages accumulate around drusen and in choroidal neovascular membranes,10,14,24 Chimeric mice in which circulating monocytes express green
fluorescent protein suggest that most of the macrophages found in choroidal neovascular lesions are derived from the peripheral circulation.\textsuperscript{51}

Previously, we have shown that stimulation of uveal melanoma cell lines with activated T cells induces the production of high levels of the chemokines CCL2, CCL3, CCL8, CXCL9, and CXCL10; the supernatant of these cells is able to attract monocytes.\textsuperscript{52} It is not known whether immune stimulation of primary HCM cultures will have a similar effect on the innate immune system or whether this is specific for malignant cells.

For human epidermal melanocytes, studies show an increased secretion of CXCL8, IL6, IL-1β, and TNF-α, and an increased expression of CCL2, CCL3, and CCL5 when stimulated with various Toll-like receptor ligands.\textsuperscript{33,34} Therefore, these cells might be of importance in the skin innate immunity and might be active players in inflammatory events. Although epidermal melanocytes and uveal melanocytes originate from neural crest cells during embryonic development,\textsuperscript{35,36} they display a number of different features: In contrast to epidermal melanocytes, uveal melanocytes do not transfer melanin to neighboring cells\textsuperscript{36} and do not express the \( \alpha \)-melanocyte-stimulating hormone. Therefore, uveal melanocytes do not respond to this hormone, which has an effect on proliferation and melanogenesis.\textsuperscript{37}

We hypothesized that HCMs, like uveal melanoma and epidermal melanocytes, can secrete chemokines and cause monocyte migration. We found that stimulation of HCM cultures led to the secretion of various chemokines, which stimulated monocyte migration. However, in some donors, the effect was the opposite, suggesting that the development of local inflammation is donor specific. Further, HCMs were able to inhibit the proliferation of stimulated T cells, potentially playing a role in the immune privilege of the eye.

\section*{Materials and Methods}

\subsection*{Ethics Statement}

Ethical approval was waived by the local ethics committee “Den Videnskabsetiske Komité Københavns og Frederiksberg Kommuner.” Samples were identified by date of blood sampling only and analyzed anonymously. Verbal informed consent of blood sampling was obtained. For the isolation of HCMs the study was performed on donor eyes: human eyes were obtained from the Manchester Royal Eye Hospital Eye Bank after removal of the corneas for transplantation and with guidelines in accordance with the manufacturer’s instructions (Complete Protease Inhibitor Cocktail; Roche Diagnostics, Mannheim, Germany). The choroid was washed once with PBS + 1% Pen/Strep. The cells were collected and spun at 100g for 5 minutes. The cells were plated in a T25 flask (Techno Plastic Products AG, Trasadingen, Switzerland) in FIC medium, which is F12 medium (Gibco) supplemented with 10% fetal calf serum (FCS; Seralab, West Sussex, UK), 2 mM glutamine (Gibco), 20 mg/mL basic fibroblast growth factor (Gibco), 0.1 mM isobutylmethylxanthine (Sigma-Aldrich Corp.) 10 ng/mL cholera toxin (Sigma-Aldrich Corp.), and 50 µg/mL gentamicin (Gibco). To release all HCMs, the choroid was further digested with 0.6 U/mL dispase II (Gibco) for 18 hours at 37°C at 5% CO\textsubscript{2} without any agitation. No morphologic difference was observed by the isolations using different enzymes. The exact number of cells was not determined but visually there was a similar degree of cell density within each flask. Addition of the protease inhibitor cocktail stopped the proteolysis. The choroid was thoroughly shaken and passed through a 70-µm cell strainer (BD Biosciences). Cells were collected, spun down as described above, and plated in FIC medium in a new T25 flask. The culture medium was supplemented for 5 to 7 days with 0.1 mg/mL genetin (Sigma-Aldrich Corp.) to remove any contaminating fibroblasts and RPE cells, which are more sensitive to the effects of genetin than HCMs.\textsuperscript{38} This treatment led to pure HCM cultures. Cell culture medium was changed two to three times a week. Human choroidal melanocytes were subcultured with 0.05% trypsin/EDTA.

\subsection*{Flow Cytometry}

The purity of HCMs from each donor was checked with flow cytometry. Human choroidal melanocytes express S-100 but no cytokeratins, whereas RPE cells express both. Fibroblasts express none of these.\textsuperscript{38} In brief, cells were harvested, fixed, and permeabilized with the Cytofix/Cytoperm Kit (BD Biosciences). The cells were incubated for 30 minutes at 4°C with an Alexa Fluor 488–conjugated antibody to the cytokerin peptides 4, 5, 6, 8, 10, 13, and 18 (pan cytokeratin) (clone C11; Exbio, Vestec u Prahy, Czech Republic), the corresponding isotype control (Invitrogen, Carlsbad, CA, USA), and the primary antibody S-100 (clone B32.1; Santa Cruz, Dallas, TX, USA). After washing, the cells labeled with the primary antibody S-100 were incubated for 30 minutes at 4°C with a FITC-conjugated F(ab')\textsubscript{2} specific secondary antibody (Sigma-Aldrich Corp.) that was diluted 1:100. Cells were analyzed on an LSR II flow cytometer (BD Biosciences). Data were analyzed with FlowJo version 7.6.5 for Windows (Tree Star, Ashland, OR, USA).

\subsection*{Preparation of T-Cell–Conditioned Media (TCM)}

T cells were purified from fresh whole blood from five healthy young volunteers, as previously described.\textsuperscript{40} The healthy status of the volunteers was self-reported well-being, thereby excluding any known acute or chronic infections. The purity of T cells was checked with an FITC-labeled CD3 antibody
HCM donors were pooled into those two groups. ICH, intracranial hemorrhage; PMP, pseudomyxoma peritonei; SAH, subarachnoid hemorrhage.

were pooled into two groups: group 1 with a fold-change of

10 ng/mL TNF-α was neutralized; fold-change of migrated monocytes from IFN-γ-stimulated HCMs/unstimulated HCMs. Based on the fold-changes the HCMs were pooled into two groups: group 1 with a fold-change of >1 (bold) and group 2 with a fold-change of ≤1 (italic). In the following graphs the HCM donors were pooled into those two groups. ICH, intracranial hemorrhage; PMP, pseudomyxoma peritonei; SAH, subarachnoid hemorrhage.

(F, Female; M, Male).

<table>
<thead>
<tr>
<th>No. CM</th>
<th>Sex</th>
<th>Age, y</th>
<th>Postmortem Time, h</th>
<th>Cause of Death</th>
<th>% Migration Unstimulated</th>
<th>% Migration IFN-γ + TNF-α</th>
<th>% Migration IFN-γ + TNF-α + Anti-IFN-γ</th>
<th>Fold-Change Value IFN-γ + TNF-α/Unstimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>001</td>
<td>M</td>
<td>56</td>
<td>33</td>
<td>SAH</td>
<td>4.0</td>
<td>19.6</td>
<td>61.7</td>
<td>6.0</td>
</tr>
<tr>
<td>003</td>
<td>F</td>
<td>59</td>
<td>30</td>
<td>ICH</td>
<td>32.2</td>
<td>6.3</td>
<td>18.5</td>
<td>0.2</td>
</tr>
<tr>
<td>006</td>
<td>F</td>
<td>56</td>
<td>28.5</td>
<td>ICH</td>
<td>29.4</td>
<td>3.8</td>
<td>11.9</td>
<td>0.4</td>
</tr>
<tr>
<td>007</td>
<td>M</td>
<td>51</td>
<td>33.5</td>
<td>PMP</td>
<td>13.6</td>
<td>1.6</td>
<td>10.0</td>
<td>0.7</td>
</tr>
<tr>
<td>009</td>
<td>F</td>
<td>76</td>
<td>49</td>
<td>Cancer</td>
<td>25.3</td>
<td>6.7</td>
<td>14.4</td>
<td>0.3</td>
</tr>
<tr>
<td>011</td>
<td>F</td>
<td>53</td>
<td>37</td>
<td>Lung cancer</td>
<td>11.1</td>
<td>1.6</td>
<td>5.8</td>
<td>0.1</td>
</tr>
<tr>
<td>013</td>
<td>M</td>
<td>47</td>
<td>25.5</td>
<td>Cancer</td>
<td>20.6</td>
<td>12.5</td>
<td>31.0</td>
<td>0.6</td>
</tr>
<tr>
<td>014</td>
<td>F</td>
<td>53</td>
<td>44.5</td>
<td>Pneumonia</td>
<td>8.7</td>
<td>16.2</td>
<td>31.0</td>
<td>1.9</td>
</tr>
<tr>
<td>015</td>
<td>M</td>
<td>64</td>
<td>29</td>
<td></td>
<td>18.3</td>
<td>3.3</td>
<td>6.0</td>
<td>0.2</td>
</tr>
<tr>
<td>016</td>
<td>F</td>
<td>59</td>
<td>46.5</td>
<td>PMP</td>
<td>8.0</td>
<td>12.9</td>
<td>30.0</td>
<td>1.6</td>
</tr>
<tr>
<td>017</td>
<td>F</td>
<td>15</td>
<td>34.5</td>
<td>Cancer</td>
<td>14.5</td>
<td>3.4</td>
<td>13.1</td>
<td>0.2</td>
</tr>
<tr>
<td>019</td>
<td>M</td>
<td>37</td>
<td>37</td>
<td>Cardiac arrest</td>
<td>10.8</td>
<td>9.4</td>
<td>24.6</td>
<td>0.9</td>
</tr>
<tr>
<td>020</td>
<td>F</td>
<td>75</td>
<td>58</td>
<td>Unknown</td>
<td>25.9</td>
<td>5.9</td>
<td>15.0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Stimulation of HCMs

For the experiments, HCMs from 13 donors (Table) were used at an early passage, which did not exceed p14. A total of 100,000 cells were plated into six-well plates (Becton Dickinson, Le Pont De Claix, France) in 3 mL X-VIVO 15 serum-free medium (Lonza BioWhittaker, Verviers, Belgium) supplemented with 2 mM glutamine and 50 μg/mL gentamicin (both from Gibco). T cells were activated by using Dynabeads CD3/CD28 T-Cell Expander (Invitrogen, Oslo, Norway) for 64 hours at 37°C and 5% CO2. The TCM from each individual donor was spun down, aliquoted separately, and immediately frozen at ~80°C.

Microarrays

RNA was labeled and hybridized to a genome-wide microarray Human Gene 2.0 ST (Affymetrix, Santa Clara, CA, USA) at the Copenhagen University Hospital Microarray Center according to Affymetrix protocols. The microarray data were used as screening tool and performed for eight HCM donors. Upregulated genes were functionally annotated with Gene Ontology terms using DAVID Bioinformatics Resources 6.7 (david.abcc.ncifcrf.gov; provided in the public domain by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA); all microarray data are Minimum Information About a Microarray Experiment (MIAME) compliant, and raw data have been submitted to the Gene Expression Omnibus (GEO, accession number GSE70762).

Multiplex Protein Determination

Protein concentration of CXCL8, CXCL9, CXCL10, CXCL11, CCL2, CCL5, vascular endothelial growth factor (VEGF), and intercellular adhesion molecule (ICAM)-1 was quantified by using the BD Cytometric Bead Array (CBA; BD Biosciences) according to the manufacturer’s instructions. Briefly, we first performed a preliminary experiment to determine the optimal dilutions for each sample type. Supernatants from untreated HCMs were assayed at 1X, 4X, and 20X dilutions, stimulated HCM supernatants at 5X, 30X, 75X, 150X, and 300X dilutions, and TCM at 1X and 4X dilutions. For each protein, the dilution that yielded a concentration within the standard curve was used. The data were analyzed by using CBA software supplied by BD Biosciences. The assay was performed in singles for all 13 donors.

Monocyte Purification and Migration Assay

Monocytes were purified from peripheral blood mononuclear cells from healthy young volunteers by CD14+ selection by using magnetic activated cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocyte purity was checked with an FITC-labeled CD14 antibody (BioLegend, San Diego, CA, USA) and found to be above 98% (data not shown). For the transwell migration assay, 50,000 cells were placed in a 5.0-μm pore polycarbonate transwell insert (Nalgene Nunc, Naperville, IL, USA). For studies with the neutralizing antibody, supernatants

(Thomas, Starnberg, Germany) and found to be above 94% (data not shown). In total, 3 × 10^6 T cells were plated into six-well plates (Becton Dickinson, Le Pont De Claix, France) in 3 mL X-VIVO 15 serum-free medium (Lonza BioWhittaker, Verviers, Belgium) supplemented with 2 mM glutamine and 50 μg/mL gentamicin (both from Gibco). T cells were activated by using Dynabeads CD3/CD28 T-Cell Expander (Invitrogen, Oslo, Norway) for 64 hours at 37°C and 5% CO2. The TCM from each individual donor was spun down, aliquoted separately, and immediately frozen at ~80°C.

In total, 3 × 10^6 T cells were plated into six-well plates (Becton Dickinson, Le Pont De Claix, France) in 3 mL X-VIVO 15 serum-free medium (Lonza BioWhittaker, Verviers, Belgium) supplemented with 2 mM glutamine and 50 μg/mL gentamicin (both from Gibco). T cells were activated by using Dynabeads CD3/CD28 T-Cell Expander (Invitrogen, Oslo, Norway) for 64 hours at 37°C and 5% CO2. The TCM from each individual donor was spun down, aliquoted separately, and immediately frozen at ~80°C.

Stimulation of HCMs

For the experiments, HCMs from 13 donors (Table) were used at an early passage, which did not exceed p14. A total of 100,000 cells were plated into six-well plates and grown for 14 to 21 days in HIC medium. The culture medium was then changed to the serum-free Melanocyte Growth Medium M2 (PromoCell, Heidelberg, Germany) and the cells cultured for another 12 to 25 days, at which time they became highly pigmented and developed a three-dimensional structure. Cell culture medium was changed two to three times a week. The cells were stimulated for 48 hours at 37°C and 5% CO2, with 1.5 mL TCM and 1.5 mL fresh M2 medium, or 27 ng/mL IFN-γ and 10 ng/mL TNF-α (both from R&D Systems, Minneapolis, MN, USA) in a total volume of 3 mL. The concentration of these two cytokines was chosen from the highest concentration in the TCM of the different T-cell donors. These two cytokines were chosen in order to determine to which extent the differential regulation of the genes induced by activated T cells could be attributed to these cytokines. After stimulation, supernatant was collected and immediately frozen at ~80°C for downstream applications. RNA was purified from the HCMs by using the NucleoSpin RNA II Kit (Macherey-Nagel, Düren, Germany) in accordance with the manufacturer’s instructions.

Microarrays

RNA was labeled and hybridized to a genome-wide microarray Human Gene 2.0 ST (Affymetrix, Santa Clara, CA, USA) at the Copenhagen University Hospital Microarray Center according to Affymetrix protocols. The microarray data were used as screening tool and performed for eight HCM donors. Upregulated genes were functionally annotated with Gene Ontology terms using DAVID Bioinformatics Resources 6.7 (david.abcc.ncifcrf.gov; provided in the public domain by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA); all microarray data are Minimum Information About a Microarray Experiment (MIAME) compliant, and raw data have been submitted to the Gene Expression Omnibus (GEO, accession number GSE70762).

Multiplex Protein Determination

Protein concentration of CXCL8, CXCL9, CXCL10, CXCL11, CCL2, CCL5, vascular endothelial growth factor (VEGF), and intercellular adhesion molecule (ICAM)-1 was quantified by using the BD Cytometric Bead Array (CBA; BD Biosciences) according to the manufacturer’s instructions. Briefly, we first performed a preliminary experiment to determine the optimal dilutions for each sample type. Supernatants from untreated HCMs were assayed at 1X, 4X, and 20X dilutions, stimulated HCM supernatants at 5X, 30X, 75X, 150X, and 300X dilutions, and TCM at 1X and 4X dilutions. For each protein, the dilution that yielded a concentration within the standard curve was used. The data were analyzed by using CBA software supplied by BD Biosciences. The assay was performed in singles for all 13 donors.

Monocyte Purification and Migration Assay

Monocytes were purified from peripheral blood mononuclear cells from healthy young volunteers by CD14+ selection by using magnetic activated cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocyte purity was checked with an FITC-labeled CD14 antibody (BioLegend, San Diego, CA, USA) and found to be above 98% (data not shown). For the transwell migration assay, 50,000 cells were placed in a 5.0-μm pore polycarbonate transwell insert (Nalgene Nunc, Naperville, IL, USA). For studies with the neutralizing antibody, supernatants
Figure 1. Photomicrographs of cultured HCMs from donor 015 in FIC medium and M2 medium; HCMs became highly pigmented in M2 medium and developed a three-dimensional structure. Figures are shown at \( \times5 \), \( \times10 \), and \( \times20 \) magnification.

Monocyte Migration Inhibitory Factor (MIF) ELISA

Migration inhibitory factor was quantified in the HCM supernatant by sandwich ELISA. The standard curve was generated from recombinant MIF (BioLegend). A Maxisorp ELISA plate (Nunc, Rochester, NY, USA) was coated with an MIF capture antibody at a concentration of 2 \( \mu \)g/mL and incubated overnight at 4°C. The plate was washed and blocked for 1 hour at room temperature (RT) with PBS containing 10% FCS. Standards and samples were added in triplicate and incubated overnight at 4°C. After washing, the wells were incubated for 1 hour at RT with the MIF biotinylated detection antibody (BioLegend) at a concentration of 0.5 \( \mu \)g/mL. The plate was washed and incubated for 30 minutes at RT with horseradish peroxidase (HRP)-conjugated streptavidin (BD Biosciences) diluted 1:1000, followed by addition of the \( \alpha \)-Phenylenediamine dihydrochloride (OPD) substrate (Dako, Roskilde, Denmark), according to the manufacturer’s recommendation. The reaction was stopped with 0.5 M H\(_2\)SO\(_4\) and absorbance was measured at 490 nm on a microplate reader (Discovery HTR; BioTek Instruments, Inc., Winooski, VT, USA).

Proliferation of T Cells in Coculture With HCMs

A total of 3800 cells were plated into 96-well flat bottom plates (VWR, Leuven, Belgium) and grown in FIC medium for 16 to 17 days followed by the serum-free medium M2 for 5 to 12 days. For the experiment, the medium was changed to 50% M2 medium and 50% X-VIVO 15 supplemented with 2 mM glutamine and 50 \( \mu \)g/mL gentamicin. A total of 100,000 frozen T cells from freshly drawn blood were added directly to the HCMs, which corresponds to a ratio of HCMs to T cells of 1:1. For this experiment HCMs from donor 001, 013, 015, and 020 were used. For the T-cell titration experiments with HCM donor 015, 1 \( \times \) 10\(^5\), 2 \( \times \) 10\(^5\), and 4 \( \times \) 10\(^5\) frozen purified T cells from buffy coat were used, which corresponds to a ratio of HCMs to T cells of 1:1, 1:2, and 1:4. T cells were activated by using Dynabeads CD3/CD28 T-Cell Expander. After coculture for 48 hours at 37°C with 5% CO\(_2\), [\(^3\)H]-thymidine (0.025 \( \mu \)Ci/\( \mu \)L; Perkin Elmer, Boston, MA, USA) was added to the T cells and incubated for 18 hours. Cells were harvested on a Unifilter (Perkin Elmer, Shelton, CT, USA) and [\(^3\)H]-thymidine incorporation was measured with a TopCount scintillation counter (PerkinElmer, Waltham, MA, USA). The experiments were performed in duplicate or triplicate.

Statistics

Statistical analysis was performed by using the graphic and statistical software (GraphPad Prism version 4.05; GraphPad Software, La Jolla, CA, USA), and \( P \) values less than 0.05 were considered significant. The migration assays, the multiplex protein determination, ELISA, and the T-cell proliferation assay were analyzed with 1-way ANOVA and Bonferroni’s multiple comparison test.

Results

Cultures of HCMs

We aimed to obtain HCM cultures with a morphology comparable to the one observed in the choroid with respect to pigmentation and sheet-like structure\(^2\) (Fig. 1). This morphology was obtained when we grew the HCMs first in FIC medium and subsequently in serum-free melanocyte growth medium M2. It was noticed that in the two oldest donors (donor 009 and donor 020), aged 79 and 73 years, the sheet-like structure was only partially achieved. Supplementary Figure S1 shows the pictures from all donors when grown in FIC and M2 medium.

All HCM cultures that we used were tested by flow cytometry analysis for purity, which in all cases was above 97%. Figure 2A shows a representative dot plot, in which 99.5% of HCMs expressed S-100. Expression of the RPE-specific antigens cytokeratin 8 and 18 was examined by using a pan cytokeratin antibody against the cytokeratins 4, 5, 6, 8, 10, 13, and 18, and found to be negative (Fig. 2D).
Upregulation of Chemokine Gene Expression in HCM Cultures Upon Inflammatory Stimuli

We tested whether soluble factors derived from activated T cells would influence gene expression of the HCMs and therefore exposed the cultured HCM cells from donor 001, 003, 007, 014, 015, 016, 019, and 020 to TCM. After exposure for 48 hours to TCM, gene expression of the eight HCMs was analyzed with a whole-transcription microarray. By using the average of the examined HCM donors, a >2-fold criterion, and an expression level above the median, 642 genes were found to be upregulated when exposed to TCM. Since IFN-γ and TNF-α are the main inflammatory cytokines derived from activated T cells, and the HCMs express the corresponding receptors, a fixed concentration of each was added to the individual HCM cultures in order to compare to what extent the effect by TCM can be observed by the use of these cytokines. It was found that 423 genes were upregulated when exposed to IFN-γ and TNF-α, with a concordance of 388 genes when stimulated with TCM compared to IFN-γ and TNF-α. This indicates that most of the effect caused by TCM might be due to the presence of IFN-γ and TNF-α in the TCM. The upregulated genes were functionally annotated by using Gene Ontology terms. This revealed gene functions related to immune and inflammatory responses, cell death/apoptosis, proliferation, proteolysis, and cell adhesion (Fig. 3).

Owing to the close proximity of blood vessels to HCMs, inflammatory factors present in blood could easily access the HCMs. This might be the case in relation to inflammatory events such as infections or chronic inflammation. Aging has been associated with increased levels of proinflammatory cytokines such as TNF-α in the blood. Therefore, we focused on genes in HCMs encoding for chemokines as well as some other genes involved in leukocyte migration, such as VEGF and ICAM-1.

A heat map of genes encoding for chemokines, ICAM-1, and VEGFA-A is presented in Figure 4. VEGFA-A was not upregulated.
Chemokine Secretion and Monocyte Migration by HCMs

Increased Protein Expression of Chemokines After Stimulation of HCMs

To examine whether selected genes are also translated into their corresponding proteins, we used a CBA to quantify the protein concentration for CXCL8, CXCL9, CXCL10, CXCL11, CCL2, CCL5, VEGF, and ICAM-1 in culture supernatants. Pooling the data from 13 donors (Fig. 5), we noticed that CXCL8, VEGF, ICAM-1, and CCL2 (slightly) were constitutively expressed, whereas the secretion of all the measured proteins was induced when stimulating HCMs with IFN-γ and TNF-α or TCM, with an exception for VEGF, which was slightly downregulated. The concentrations of CXCL9, CXCL10, CXCL11, and CCL5 were significantly lower when HCMs were stimulated with TCM compared to stimulation with IFN-γ and TNF-α. T-cell–conditioned media secreted CCL5, ICAM-1, and some CXCL9. The protein data for all individual samples can be found in Supplementary Figure S2.

Donor-Dependent Effect on Monocyte Migration

Since exposure of HCMs to TCM or IFN-γ and TNF-α induced expression of CXCL8, CXCL9, CXCL10, CXCL11, CCL2, CCL5, VEGF, and ICAM-1 in these cells, we wondered whether the production of these cytokines would influence local inflammation, for example, influx of monocytes. We tested the different supernatants’ ability to attract monocytes in a transwell migration assay. We first determined the effect of the supernatant from unstimulated HCM cultures, then after stimulation with IFN-γ and TNF-α, and finally after exposure to TCM. From their influence on monocyte migration, we identified two groups of HCMs: group 1 had a >1 fold-change (bold) in monocyte migration toward supernatants between stimulated HCMs and unstimulated HCMs, while group 2 had a fold-change of ≤1 (italic), as shown in the Table.

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor 001, 014, and 016</td>
<td>Donor 003, 006, 007, 009, 011, 013, 015, 017, 019, and 020</td>
</tr>
<tr>
<td>Unstimulated</td>
<td>Unstimulated</td>
</tr>
<tr>
<td>CXCL1</td>
<td>2.12 (1.07–4.20)</td>
</tr>
<tr>
<td>CXCL2</td>
<td>2.12 (1.07–4.20)</td>
</tr>
<tr>
<td>CXCL5</td>
<td>2.12 (1.07–4.20)</td>
</tr>
<tr>
<td>CXCL8</td>
<td>2.12 (1.07–4.20)</td>
</tr>
<tr>
<td>CXCL9</td>
<td>2.12 (1.07–4.20)</td>
</tr>
<tr>
<td>CXCL10</td>
<td>2.12 (1.07–4.20)</td>
</tr>
<tr>
<td>CCL2</td>
<td>2.12 (1.07–4.20)</td>
</tr>
<tr>
<td>CCL5</td>
<td>2.12 (1.07–4.20)</td>
</tr>
<tr>
<td>VEGF</td>
<td>2.12 (1.07–4.20)</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>2.12 (1.07–4.20)</td>
</tr>
</tbody>
</table>

On the basis of previous experiments where we and others have shown that IFN-γ has an inhibitory effect on monocyte migration, we neutralized this cytokine in the supernatants from HCMs exposed to IFN-γ and TNF-α before use in the migration assay. This led to a significant increase in monocyte migration in both HCM donor groups (Fig. 6A).

In a previous study, we have shown that supernatants from uveal melanoma cell lines cocultured with activated T cells are able to attract more monocytes than supernatants from unstimulated cells. In the current study we stimulated the HCMs with recombinant IFN-γ and TNF-α. However, to
Figure 5. Determination of CXCL8, CXCL9, CXCL10, CXCL11, CCL2, CCL5, VEGF, and ICAM-1 proteins in supernatants from cultures of HCMs, unstimulated (black squares), or stimulated with 27 ng/mL IFN-γ and 10 ng/mL TNF-α (black triangles) or with TCM (white triangles). The same proteins were also measured in TCM (black circle). All the measured proteins were upregulated when stimulating HCMs with IFN-γ and TNF-α or TCM except for VEGF, which was slightly downregulated. *P < 0.05; **P < 0.01; and ***P < 0.001 (n = 13).
compare the results with the uveal melanoma, we cultured the melanoma cell lines Mel 270 and Mel 290 for the experiments by using identical conditions as for the HCMs. These supernatants also showed an increased attraction of monocytes, compared to unstimulated cultures (data not shown). Therefore, the capability to attract monocytes is not media dependent or stimulation dependent.

Donor-Specific Effect of HCMs on Monocyte Migration Does Not Correlate to Chemokine Secretion or to MIF Levels

We could not establish any relationship between the different migratory behavior of group 1 and group 2 and the donors’ age, sex, cause of death, and postmortem times. Further, we examined whether the different migration pattern of the two groups could be explained by their chemokine expression or secretion. When we compared the chemokine gene expression between the two groups, we did not see a difference as shown in Figure 4: the first three HCM donors are from group 1 and the other five donors are from group 2.

Next, we measured the chemokine secretion, but this did not fully explain the difference seen in the migration assay either, except for CCL2, which was significantly higher in supernatants from HCMs stimulated with IFN-γ and TNF-α in group 2, and for CCL5, which was significantly lower in group 2 (Supplementary Fig. S4).

Since we could not explain the difference in monocyte migration of the two groups by their chemokine expression and secretion, we analyzed whether any difference in the levels of MIF could be observed in the supernatants between the groups. Migration inhibitory factor has been reported to inhibit random migration of monocytes as well as the migration toward a chemotactic gradient.15 However, different from what would be expected, the MIF secretion was significantly decreased from HCM cultures from group 2–TCM-stimulated cultures compared to group 1–TCM-stimulated HCM cultures. There was no significant difference between unstimulated and stimulated HCMs (Fig. 7).

Human Choroidal Melanocytes Inhibit the Proliferation of Activated T Cells

We further tested whether the eye-derived melanocytes could modify T-cell responses and we therefore determined whether they were able to suppress the proliferation of activated T cells. Human choroidal melanocyte cultures from donor 001 (group 1) and donor 013, 015, and 020 (group 2) were all able to almost completely inhibit the proliferation of activated T cells in a direct manner in a ratio of 1:1. Furthermore, titrations with different numbers of T cells to HCMs from donor 015 in ratio 1:1, 2:1, and 4:1 (which corresponded to 1 × 10⁵, 2 × 10⁵, and 4 × 10⁵ T cells) showed that the HCMs were able to inhibit T-cell proliferation at the highest ratio of T cells to HCMs (Fig. 8).

FIGURE 6. (A) Monocyte migration toward supernatant from unstimulated HCMs and from HCMs stimulated with IFN-γ and TNF-α. The 13 HCM donors were grouped according to their ability to attract monocytes (see Table). Blocking IFN-γ resulted in increased monocyte migration in both groups. (B) The same migration pattern of monocyte toward supernatants from HCMs exposed to TCM then exposed to IFN-γ and TNF-α was observed. Three HCM donors showed an increase in monocyte migration when stimulated with TCM. Ten HCM donors showed a decrease in monocyte migration toward supernatants from HCMs exposed to TCM compared to unstimulated monocytes. Each dot represents one HCM donor for one monocyte donor: group 1 (001, 014, and 016); group 2 (003, 006, 007, 009, 011, 013, 015, 017, 019, 020). The results are shown as percentage of migration where 100% FCS was set to 100%. *P < 0.05; ***P < 0.001.

FIGURE 7. Determination of monocyte MIF in supernatants from unstimulated HCM cultures (squares), stimulated with TCM (triangles), or stimulated with IFN-γ and TNF-α (circles). The HCM donors were grouped according to the migration assay. Group 1 contains donors 001, 014, and 016; and group 2 contains donors 003, 006, 007, 009, 011, 013, 015, 017, 019, 020. Migration inhibitory factor was constitutively expressed and did not show any significant change when HCMs were stimulated. *P < 0.05.
cyte-derived malignant cells are able to influence monocyte simulation of HCMs with TCM or IFN-α and the adhesion molecule ICAM-1 (Figs. 4, 5) in response to chemokines CXCL8, CXCL9, CXCL10, CXCL11, CCL2, CCL5, HCMs differentially upregulated 642 genes when exposed to IFN-α and 423 genes when exposed to TCM and 423 genes when exposed to IFN-γ. Part of this finding is supported by Hu et al.5 who have demonstrated that supernatants from CM donors 013 and 015.

**DISCUSSION**

The uveal melanocytes constitute the major part of the cells in the choroid. Besides containing melanin, which acts as an antioxidant, their function is not well characterized.2 As studies in uveal melanoma have shown that those melanocyte-derived malignant cells are able to influence monocyte migration,32 we investigated whether this was part of their malignant behavior, or already a characteristic of normal melanocytes. The purpose of this study was therefore to investigate whether TCM or IFN-γ and TNF-α influenced the secretion of chemokines by HCMs and their effect on monocyte migration. Stimulating the HCMs with TCM or IFN-γ and TNF-α should simulate the effect of proinflammatory factors derived from T cells. Since the HCMs are located in an area of the body that has the highest blood flow per gram of tissue of any other organ, they have easy access to these blood-borne factors.46

In the present study we established primary HCM cultures from 13 individual donors and managed to obtain HCM cultures that lasted over 3 weeks and resembled the normal melanocyte arrangement in the choroid with respect to pigmentation and sheet-like structures22 (Fig. 1). Our methodology differed from most studies where the HCMs used for experiments have been cultured only for a few days.1,3-5 However, in the study from Lai et al.,47 HCMs are cultured, similarly to ours, for up to 4 weeks before their use in experiments.

Microarray analysis of 8 of the 13 donors showed that the HCMs differentially upregulated 642 genes when exposed to TCM and 423 genes when exposed to IFN-γ and TNF-α.

We found an increased expression and production of the chemokines CXCL8, CXCL9, CXCL10, CXCL11, CCL2, CCL5, and the adhesion molecule ICAM-1 (Figs. 4, 5) in response to simulation of HCMs with TCM or IFN-γ and TNF-α. CXCL8, CCL2, ICAM-1, and VEGF were found to be constitutively expressed and VEGF did not change in response to proinflammatory cytokines (Fig. 5). Part of this finding is supported by Hu et al.5 who have demonstrated that supernatants from HCMs display a low basal secretion of CXCL8 and CCL2. To our knowledge, our study is the first to demonstrate that cultured HCMs respond to TCM as well as to the inflammatory cytokines IFN-γ and TNF-α by the secretion of multiple proteins involved in leukocyte attraction.

Murine studies have shown that aging is associated with an increased expression of the transcripts of chemokines such as CXCL9, CXCL16, CCL3, CCL5, CXCL8, CCL2, and the adhesion molecule ICAM-1 in the RPE/choroid.48 Our data did not show any correlation between age of donor (15–76 years, mean 59 years) and the secretion of chemokines (data not shown).

So far, only a few studies have addressed the HCMs' response to inflammation. Hu et al.5 have shown that the secretion of IL-6 is induced when HCMs are stimulated with IL-1β,1 and the secretion of CCL2 and CXCL8 is induced upon stimulation with lipid polysaccharide.

From in vitro studies, it is known that RPE cells can be a source of chemokines in the eye: human RPE cells constitutively secrete CCL2 and CXCL8,49-52 both of which are upregulated in response to oxidative or inflammatory stress in addition to other chemokines.49-51,55-58 Our study showed that HCMs can also contribute to the pool of chemokines in the posterior part of the eye. Of note, stimulation of polarized RPE cells led to very low basolateral secretion of CXCL9, CXCL10, and CXCL11,51 suggesting that HCMs must be the major source of these chemokines outside the outer blood-retinal barrier.

When we compared the HCMs with uveal melanoma cell lines, we found the same genes upregulated, namely CXCL8, CXCL9, CXCL10, CXCL11, CCL2, CCL5, and ICAM-1 in response to proinflammatory cytokines. This was confirmed on the protein level.52 We demonstrated that malignant transformed uveal melanomas still display a similarity to HCMs in terms of gene expression and protein secretion as shown in Supplementary Table S1, comparing the gene expression profile of unstimulated and stimulated melanoma cell lines with primary HCM cultures.

Besides chemokines, macrophages are important players in the inflammatory reaction in the uvea. Resident macrophages are present in the choroid and appear to be of importance in providing homeostatic functions such as immunosurveillance and prevention of retinal/choroidal inflammation by removal of waste material.10,13,55 With age, increased numbers of leukocytes, and in particular macrophages, are found in the choroid and associated with Bruch's membrane.12,13,48 with a higher proportion of the anti-inflammatory M2 macrophages compared to proinflammatory M1 macrophages.14,15

Therefore, we investigated whether supernatants from the HCMs were able to attract monocytes. Analysis of supernatants from unstimulated HCM cultures showed that 10 of 13 donors showed a high initial level of monocyte migration, which decreased upon stimulation with IFN-γ and TNF-α or TCM. The other three donors showed the opposite effect, as their supernatants induced an increased monocyte attraction in response to IFN-γ and TNF-α (Fig. 6A) or TCM (Fig. 6B). The supernatant of the HCMs from donor 001 showed the highest proportion of the anti-inflammatory M2 macrophages compared to proinflammatory M1 macrophages.14,15

Therefore, we investigated whether supernatants from the HCMs were able to attract monocytes. Analysis of supernatants from unstimulated HCM cultures showed that 10 of 13 donors showed a high initial level of monocyte migration, which decreased upon stimulation with IFN-γ and TNF-α or TCM. The other three donors showed the opposite effect, as their supernatants induced an increased monocyte attraction in response to IFN-γ and TNF-α (Fig. 6A) or TCM (Fig. 6B). The supernatant of the HCMs from donor 001 showed the highest monocyte migration when stimulated with IFN-γ and TNF-α; we were able to test the HCMs of the other eye from the same
donor as well and observed a comparable monocyte migration pattern (data not shown). These data suggest that the migration of monocytes is donor dependent and not eye dependent. When comparing the two groups on the basis of measured chemokine concentrations, no obvious difference was found. CCL2, which is a highly potent chemotactant for monocytes, was different from what would be expected. significantly increased in group 2 compared to group 1 when stimulated with IFN-γ and TNF-α (Supplementary Fig. S4). Since it has been shown that MIF can inhibit monocyte migration,\textsuperscript{15} we examined the protein concentration of MIF in the supernatants (Fig. 7). However, these results also did not explain the difference in monocyte migration toward HCM supernatants between the two groups. Therefore, the mechanism behind this needs further elaboration. Neutralization studies could help to identify the chemokine(s) that is(are) responsible for the attraction of monocytes in the migration assay.

Human choroidal melanocytes are also considered as antigen-presenting cells in experimental melanin-induced uveitis and in human Vogt-Koyanagi-Harada disease and thus might play a role in the inflammatory eye conditions.\textsuperscript{1} We could show that the HCMs upregulated a number of the major histocompatibility complex genes when exposed to inflammatory stimuli (data not shown). However, this upregulation indicates that HCMs on the one hand secrete proinflammatory chemokines, but on the other hand are also likely to protect the choroid from inflammatory insults by inhibiting T-cell proliferation.

CXCL8, CXCL9, CXCL10, CCL2, CCL5, and ICAM-1. Ten of the remaining three HCM cultures. These data suggest that HCMs constitutively secrete chemokines into the posterior part of the eye, may respond to systemically increased levels of proinflammatory cytokines, and are capable to attract monocytes. With this in mind, it is likely that these HCMs contribute to the immune-pathogenesis of uveal melanoma, AMD, or uveitis.

Acknowledgments
The authors thank Julie Friis Weber and Tina Maria Axen for their excellent technical assistance, Monika Valtink for sharing her knowledge in establishing HCM cultures, Sumia Ali and Ayse Latif for their advice and help with generating HCM cultures and the “Core Facility for Flow Cytometry, Faculty of Health and Medical Sciences, University of Copenhagen.” Supported by Fight for Sight Denmark, Synoptik-Fonden and the Graduate School of the Faculty of Health and Medical Sciences, University of Copenhagen.

Disclosure: T. Jøhs, None; C. Faber, None; M.S. Udsen, None; M.J. Jager, None; S.J. Clark, None; M.H. Nissen, None

References
17. Dobbs RJ, Charlett A, Purkiss AG, Dobbs SM, Weller C, Peterson DW. Association of circulating TNF-alpha and IL-6


50. Berbik DJ, Murray TG, Tran JM, Ksander BR. Melanomas that develop within the eye inhibit lymphocyte proliferation. *Int J Cancer.* 1979;77:470–478.

51. Yang W, Chen PW, Li H, Alizadeh H, Niedernkovt JY. PD-1: PD-1 interaction contributes to the functional suppression of T-

