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

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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. Supernatants of HCMs were assessed for the capability to attract monocytes in a transwell plate. Proliferation of activated T cells was assessed in a direct coculture with HCMs by a [3H]-thymidine incorporation assay.

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
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, CCL5, CXCL8, 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{55,56} they display a number of different features: In contrast to epidermal melanocytes, uveal melanocytes do not transfer melanin to neighboring cells\textsuperscript{56} and do not express the receptor for the α-melanocyte-stimulating hormone. Therefore, uveal melanocytes do not respond to this hormone, which has an effect on proliferation and melanogenesis.\textsuperscript{57}

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.

**Materials and Methods**

**Ethics Statement**

Ethical approval was waived by the local ethics committee “Den Videnskabsretiske 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 approval from the Manchester University Research Ethics Committee. Our research adhered to the tenets of the Declaration of Helsinki. In all cases, there was prior consent for the eye tissue to be used for research, and guidelines established in the Human Tissue Act of 2004 (United Kingdom) were followed.

**Isolation of HCMs**

Human choroidal melanocytes were isolated from donor eyes (donors were 15–76 years old, mean 59 years) with an average postmortem time of 37 hours (25.5–58 hours). The eyes were stored in sterile saline solution at 4°C before their use. Human choroidal melanocytes were isolated by combining the isolation methods from Hu et al.\textsuperscript{58} and Valtink and Engelmann.\textsuperscript{59} In detail, the eye was cut into halves approximately 8 mm behind the limbus. The anterior segment, vitreous fluid, and neurosensory retina were removed. The posterior segment was washed in phosphate-buffered saline (PBS) + 1% penicillin/streptomycin (Pen/Strep; Gibco, Paisley, UK). To release the retinal pigment epithelial (RPE) cells, the posterior segment was covered in 0.125% trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Gibco) and incubated for 1 hour at 37°C at 5% CO\textsubscript{2}. The RPE cells were flushed off with PBS + 1% Pen/Strep. With forceps, the choroid was carefully removed from the sclera and placed into a tissue culture dish (60 mm; BD Biosciences, Franklin Lakes, NJ, USA). The choroid was kept whole and HCMs were released by incubation in a mix of 0.5 mg/mL collagenase 1A and 0.5 mg/mL collagenase IV (both from Sigma-Aldrich Corp., St. Louis, MO, USA) for 1 hour at 37°C at 5% CO\textsubscript{2} without any agitation. The enzymatic reaction was stopped by adding a protease inhibitor cocktail 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 ng/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.

**Flow Cytometry**

The purity of HCMs from each donor was checked with flow cytometry. Human choroidal melanocytes express S-100 but no cytoketamins, whereas RPE cells express both. Fibroblasts express none of these.\textsuperscript{58} 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 cytoketatin peptides 4, 5, 6, 8, 10, 13, and 18 (pan cytoketatin) (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 Fab′\textsubscript{2} specific secondary antibody (Sigma-Aldrich Corp.) that was diluted 1:100. Cells were analyzed on a LSR II flow cytometer (BD Biosciences). Data were analyzed with FlowJo version 7.6.5 for Windows (Tree Star, Ashland, OR, USA).

**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.

After stimulation, supernatant was collected and immediately frozen at

The highest concentration in the TCM of the different T-cell donors. The concentration of these two cytokines was chosen from the

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

cells from healthy young volunteers by CD14+ Monocytes were purified from peripheral blood mononuclear

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<th>% Migration IFN-γ + TNF-α</th>
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Male (M) and female (F); age; postmortem time in hours; cause of death (SAH, ICH, PMP); percentage of migrated monocytes toward supernatant from unstimulated HCMs; percentage of migrated monocytes toward supernatant from HCMs stimulated with 27 ng/mL IFN-γ and 10 ng/mL TNF-α; percentage of migrated monocytes toward supernatant from HCMs stimulated with 27 ng/mL IFN-γ and 10 ng/mL TNF-α where IFN-γ was neutralized; fold-change of migrated monocytes from IFN-γ- and TNF-α-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.

(Dako, Glostrup, Denmark) and found to be above 94% (data not shown). In total, 3 × 10⁶ T cells were plated into six-well plates (Becton Dickinson, Le Pont de Clai, 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 p4. A total of 100,000 cells were plated into six-well plates (Becton Dickinson, 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 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 plated in a 5.0-µm pore polycarbonate transwell insert (Nalgene Nunc, Naperville, IL, USA). For studies with the neutralizing antibody, supernatants
were preincubated for 3.5 hours at room temperature with 2.4 µg/mL mouse IgG2a anti-human IFN-γ antibodies (R&D System, Minneapolis, MN, USA) before adding to the lower chamber. The plates were incubated for 2.5 hours at 37°C. The filters were removed and the migrated monocytes were detached by using PBS containing 20 mM EDTA and 2% bovine serum albumin (Sigma-Aldrich Corp.). The number of migrated cells was counted on an LSR II flow cytometer and analyzed with FlowJo version 7.6.5 for Windows. For the positive control, we used 100% FCS. To assess the effect of IFN-γ and TNF-α on cell migration, we added 27 ng/mL recombinant IFN-γ and 10 ng/mL recombinant TNF-α to the culture medium with 10% FCS. The assay was performed in duplicate on two to four independent monocyte donors.

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 µg/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 duplicate, respectively, 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 µg/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 o-Phenylenediamine dihydrochloride (OPD) substrate (Dako, Roskilde, Denmark), according to the manufacturer’s recommendation. The reaction was stopped with 0.5 M H2SO4 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 µg/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 × 10⁵, 2 × 10⁵, and 4 × 10⁵ 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% CO₂, [³H]-thymidine (0.025 µCi/µL; 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 [³H]-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 4.03; 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 (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.45 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.18 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 VEGF-A is presented in Figure 4. VEGF-A was not upregulated.
upon inflammatory stimuli but it has a basal expression level. The eight individual HCM donors showed a high degree of similarity of upregulated genes when exposed to TCM or IFN-γ and TNF-α. Furthermore, the chemokines CXCL2, CXCL6, CCL8, and CCL20 were only upregulated when exposed to TCM, which had a rather low gene expression value. Exposure of HCMs to TCM or IFN-γ and TNF-α, especially induced CXCL9, CXCL10, CXCL11, CCL2, CCL5, and ICAM-1.

**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 (Fig. 6A) or TCM (Fig. 6B). 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.

Group 1 comprised three cultures (donor 001, 014, and 016) and had a low basal capacity to attract monocytes. However, stimulation with IFN-γ and TNF-α (Fig. 6A) or TCM (Fig. 6B) increased monocyte migration.

Group 2 comprised 10 cultures (donor 003, 006, 007, 009, 011, 013, 015, 017, 019, 020), which attracted significantly more monocytes under unstimulated conditions, compared to group 1 (Fig. 6A). Supernatants from stimulated HCMs from group 2 attracted significantly fewer monocytes than supernatants from unstimulated HCMs (Figs. 6A, 6B). Supplementary Figure S3 shows the migration of monocytes toward supernatants from all the individual HCM donors when stimulated with IFN-γ and TNF-α or TCM.

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 × 10^5, 2 × 10^5, and 4 × 10^5 T cells) showed that the HCMs were able to inhibit T-cell proliferation at the highest ratio of T cells to HCMs (Fig. 8).

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**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 migration. Studies in uveal melanoma have shown that those melanocyte-derived malignant cells are able to influence monocyte migration,

and the adhesion molecule ICAM-1 (Figs. 4, 5) in response to chemokines CXCL8, CXCL9, CXCL10, CXCL11, CCL2, CCL5, and ICAM-1 in the RPE/choroid.48 Our data did not show increased expression of the transcripts of chemokines such as CXCL9, CXCL10, 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).

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 were able to inhibit T-cell proliferation at the highest T-cell number. Results are shown as counts per minute: \( n = 5 \) for CM donor 001 and 020, and \( n = 2 \) for CM donors 013 and 015. Error bars are SEM. *\( P < 0.05 \) and ***\( P < 0.001 \).

### 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,\(^2\) 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-\(\gamma\) and TNF-\(\alpha\) influenced the secretion of chemokines by HCMs and their effect on monocyte migration. Stimulating the HCMs with TCM or IFN-\(\gamma\) and TNF-\(\alpha\) 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 pigmentary and sheet-like structures\(^42\) (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-\(\gamma\) and TNF-\(\alpha\).

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 stimulation of HCMs with TCM or IFN-\(\gamma\) and TNF-\(\alpha\). 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-\(\gamma\) and TNF-\(\alpha\) 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, CCL8, 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-\(\beta\),\(^3\) 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.\(^49-51,53,54\) 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-\(\gamma\) and TNF-\(\alpha\) or TCM. The other three donors showed the opposite effect, as their supernatants induced an increased monocyte attraction in response to IFN-\(\gamma\) and TNF-\(\alpha\) (Fig. 6A) or TCM (Fig. 6B). The supernatant of the HCMs from donor 001 showed the highest monocyte migration when stimulated with IFN-\(\gamma\) and TNF-\(\alpha\); 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 chemokine attractant 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, 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 make up a large part of the immune system. 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 needs to be confirmed on the protein level and further experiments are necessary to determine whether HCMs can function as professional antigen-presenting cells.

It is known that uveal melanoma cells and RPE cells are able to inhibit T-cell proliferation. In this study we showed that HCMs from group 1 (donor 001) as well as from group 2 (donor 015, 015, and 020) also had the ability to exert a strong inhibitory effect on T-cell proliferation in a direct coculture (Fig. 8). We could not observe any difference between the two groups in their ability to inhibit T-cell proliferation, as HCMs from both groups almost completely inhibited T-cell proliferation. The eye is an immune-privileged organ meaning that multiple mechanisms protect the eye from inflammatory insults, which is of importance since the eye is a rather sensitive organ with poor regenerative capacity. Our results indicate 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, and therefore take part in the establishment of the immune privilege in the posterior part of the eye. To our knowledge this is the first proof of the ability of nonmalignant melanocytes to inhibit T-cell proliferation.

In conclusion, the present study showed that HCMs are immunologically active. They have the capacity to constitutively secrete CXCL8, CCL2, VEGF, and ICAM-1 at a low level. Exposure of inflammatory stimuli induced the secretion of CXCL8, CXCL9, CXCL10, CCL2, CCL5, and ICAM-1. Ten of the HCM donors had a high basal capability to attract monocytes, which was significantly decreased upon stimulation with TCM or IFN-γ and TNF-α. An opposite pattern was observed in the remaining three HCM cultures. These data suggest that HCMs constitutively secrete chemokines into the posterior part of the eye, which 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.

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