Inflammation and immunomodulation in uveal melanoma

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Chapter 5

Inflammatory cytokines in eyes with uveal melanoma and relation with macrophage infiltration

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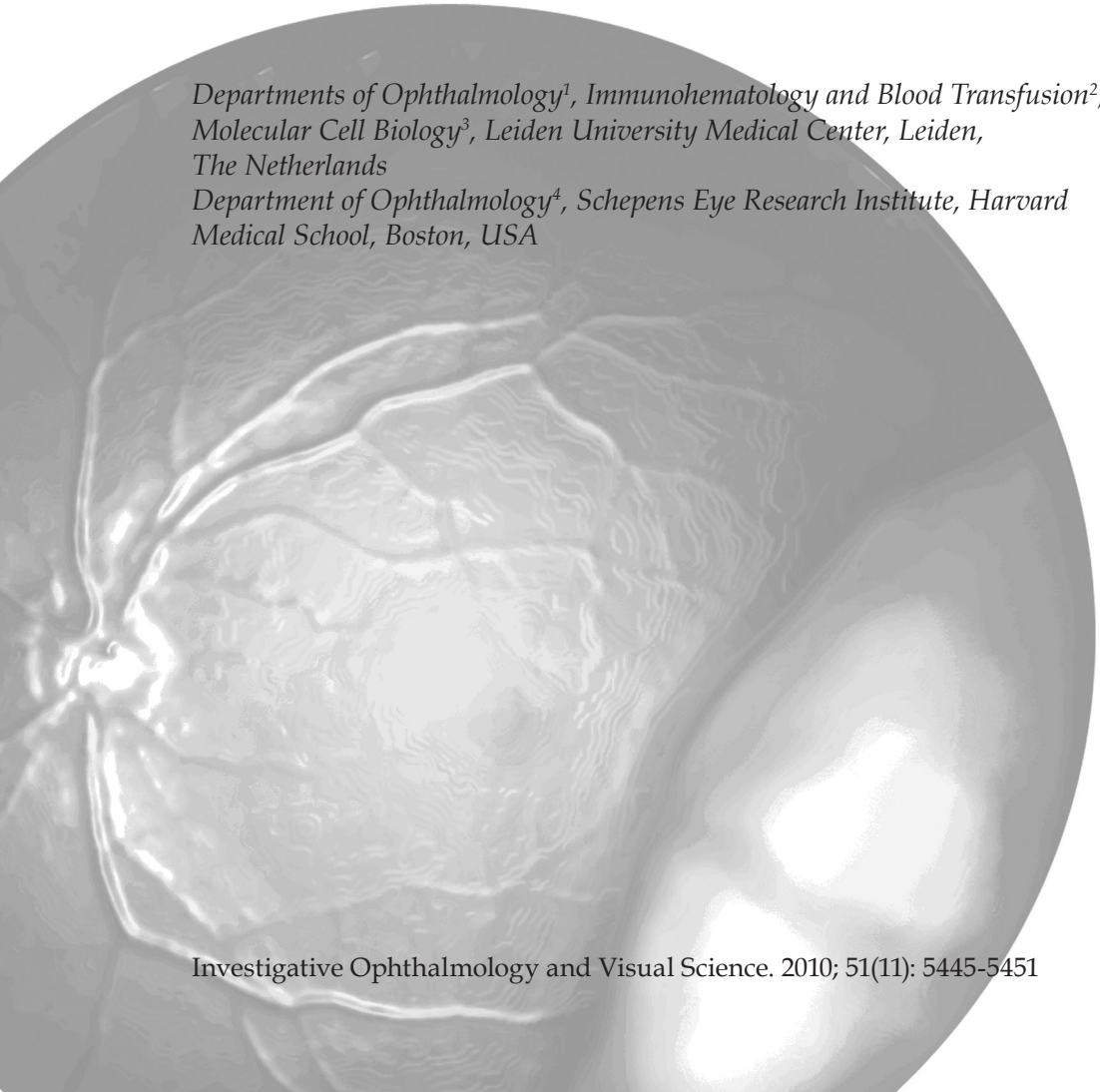

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Abstract

Purpose: The presence of an inflammatory phenotype, characterized by an increased expression of HLA antigens and an immunologic infiltrate, carries a bad prognosis in uveal melanoma. We wondered whether the aqueous humor (AqH) from uveal melanoma-containing eyes would contain inflammatory cytokines, and whether their presence was associated with inflammation.
Methods: Directly following enucleation, AqH was obtained from 37 eyes containing a uveal melanoma. Samples were stored at -80 °C till use. Using a multiplex bead array, 15 different cytokines were measured. Intratumoral macrophages were analysed by immunohistochemistry and immunofluorescence staining. The presence of specific cytokines was compared to histopathological, genetic and clinical tumor characteristics, as well as patient survival.
Results: Several cytokines showed a significantly higher expression in the AqH from uveal melanoma-containing eyes compared to the AqH from eyes undergoing cataract surgery. MCP-3 was associated with the presence of CD68+ macrophages. Correlations were found between some cytokine levels and a few known prognostic factors for uveal melanoma, but cytokine levels were not of predictive value for survival.
Conclusion: Uveal melanoma-containing eyes often carry increased levels of inflammation-related cytokines in their AqH. However, the presence of most specific cytokines was not related to the presence of macrophages, clinical or histopathological parameters, or prognosis.

Introduction

Uveal melanoma constitutes a malignancy in the eye, which may give rise to metastases. Once metastases develop, survival is poor [1]. Categories of patients can be identified that differ in prognosis: when enucleation is indicated as treatment, survival is worse than when local treatment can be applied [2]. Many clinical and histopathological parameters are known as indicators of prognosis, including tumor size, the presence of epithelioid cells, loss of one copy of chromosome 3, a specific mRNA footprint as well as immunological markers [3-5]. The presence of an inflammatory infiltrate carries a poor prognosis, and is part of an "inflammatory phenotype" [6,7]. Inflammatory infiltrates are characterized by increased numbers of CD3+ and CD4+ lymphocytes as well as CD11b+ macrophages, and correspond with an increased expression of HLA Class I and II expression on the tumor cells [8-11]. We described this combination as an inflammatory phenotype, which was associated with the presence of epithelioid cells as well as with monosomy of chromosome 3 [7].

Macrophages can be found at variable numbers in uveal melanoma. Mäkitie [12] classified them into three categories: low, medium, and high, and observed that a high density of Tumor-Associated Macrophages (TAM) was correlated with a decreased survival. The presence of macrophages was associated with hot spots of microvascular density [13]. Vascular endothelial growth factor (VEGF) is often produced by macrophages and it is possible that this production of VEGF by the infiltrating macrophages plays a role in the formation of blood vessels [14]. It may be, that this is also the case in uveal melanoma. VEGF has been shown to be present in the tumor, and also in the aqueous humor (AqH) and the vitreous of eyes containing a uveal melanoma [15,16]. Missotten [16] showed that both the tumor as well as the surrounding retinal tissue are sources of VEGF.

Macrophages can be divided according to the so-called M1 and M2 paradigm, in which the M1-type macrophages appear to be more immunostimulatory, while M2-type macrophages are involved in tissue repair, angiogenesis and immunoregulation [17,17,18] . These M2-type macrophages can be identified by their enhanced expression of the CD163 scavenger receptor [19]. TAM have been described to be mainly M2-type macrophages, which have a tumor-promoting role, especially thanks to their pro-angiogenic and immunosuppressive capacity [17].

Specific molecules (cytokines) are known to stimulate the influx of macrophages, and determining the presence of such cytokines in the eye may help us to understand why some tumors have many and others have few macrophages. While Fine Needle Aspiration Biopsies (FNAB) are currently being employed to discriminate between tumors with and without loss of one chromosome 3 [20,21], hypothetically, studying a sample of AqH from a uveal melanoma-containing eye might provide a profile of cytokines that may also help to differentiate between "good" and "bad" tumors. Furthermore, if identified, such a set of known inflammatory cytokines could predict survival, since the inflammatory phenotype is associated with decreased survival. If AqH would be a good method to predict prognosis of a certain tumor, treatment strategies could be customized for each patient.

We determined the presence of 15 cytokines related to inflammation in 37 eyes with uveal melanoma, and compared the concentration of different cytokines to standard prognostic parameters in uveal melanoma and survival of the patients, as well as with the presence of macrophage subtypes in 30 uveal melanoma samples.

Materials and methods

Patients and controls

AqH samples were collected from 37 eyes that had been removed for a uveal melanoma. Immediately following enucleation, approximately 150 µl of AqH was obtained by using a 1 ml insulin syringe with a 23G needle. AqH was directly stored in a -80 °C freezer. AqH samples from cataract patients were collected after the first paracentesis. Cataract patients were only included as controls, when there was no history of ophthalmic disease or ongoing systemic disease influencing the ocular status, such as diabetes mellitus or hypertension. In July 2009, patient data and survival were updated from the patients' charts and from the database of the Integral Cancer Center West, which records national death data. Death events were also obtained from the Central Bureau of Statistics, the Netherlands. The research protocol followed the tenets of the Declaration of Helsinki and had been approved by the local Medical Ethical Committee.

Pathologic analysis

Histologic sections were prepared from tissues fixed in 4% neutral-buffered formalin for 48 hours and embedded in paraffin. Hematoxylin- and eosin-stained 4 µm sections were reviewed by an ocular pathologist for confirmation of diagnosis, intraocular localization, cell type, largest basal diameter, prominence and scleral invasion. Data regarding a group of 50 patients were previously published [7]. From 37 of these cases, AqH was available for testing.

Cytokine measurement in AqH

In AqH samples of 50 µl, we determined the cytokines IL-6, IL-10, bFGF, GM-CSF, IP-10, MCP-1, MIP-1a, RANTES, VEGF, MCP-3, MIF, TNF-b, TRAIL, ICAM-1 and VCAM-1 using the Bio-Plex Human Cytokine multiplex panel (Bio-Rad Laboratories, Veenendaal, The Netherlands), following the manufacturer's instructions. Samples were analyzed using a Bio-Plex Array Reader equipped with Bio-Plex software.

Histological analysis of uveal melanoma

Immunohistochemical (IHC) staining with mAb CD68 was performed by the alkaline phosphatase monoclonal anti-alkaline phosphatase (APAAP) method[7,22]. For immunofluorescence (IF) double staining, slides were incubated

overnight at room temperature; mouse anti-human CD68 mAb (1:50, clone 514H12; ab49777; Abcam, Cambridge, UK) was used to stain macrophages and mouse anti-human CD163 mAb (1:100, clone 10D6, Novocastra NCL-CD163, Newcastle upon Tyne, UK.) for staining M2-type macrophages. IHC sections were counterstained with Mayer's hematoxylin (Klinipath, Duiven, The Netherlands) and embedded in Kaiser's glycerin.
As a negative control, the primary antibody was replaced by Phosphate Buffered Saline/Bovine Serum Albumin 1%.

Immunofluorescence staining analysis

Pictures of the slides were taken on a confocal Laser Scanning Microscope (Zeiss LSM510; Zeiss, Jena, Germany) in a multitrack setting in which the slide is scanned multiple times, using a fixed laser-filter pair. Alexa-488 (staining CD68) was excited at 488 nm and detected using a 505-530 bandpass filter. Alexa-546 (staining CD163) was excited at 543 nm and detected using a 560-615 nm bandpass filter, leading to a two colour signal, green (CD68) and red (CD163). All pictures contained 512 x 512 pixels; 8-bit depth; stack size 368,5 µm x 368,5 µm; scaling 0,72 µm x 0,72 µm. A PH2 Plan-NEOFluar 25X/0.80 Imm Korr objective (Zeiss) was used. Images obtained were viewed and saved as overlay and as a set of two separate panels in LSM files.

Assessment of staining

Scoring of the slides stained with IHC and IF was performed blinded with respect to the clinical outcome. IHC evaluation was described previously [7,12]. For IF, the 10 most representative high-power scans (250x magnification) per slide were manually selected, in order to assess repeatability and validity; each scan represents one square optical field (OF: area 0,137 mm2). Images of the sections were exported as JPG files using an image-converting software program (Zeiss LSM Data Server, version 3.2.0.70, Carl Zeiss GmbH 1997-2002).
For digital analysis of the sections, we used a software image analysis program, called Stacks (Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands). The images with the green and red signals of CD68 and CD163, respectively, were first thresholded and in the resulting binary images, the presence of the subtype of macrophages was calculated by the number of pixels expressing green, red or both colours.

Chromosome analysis

Chromosome analysis was performed by karyotyping as well as by Interphase FISH on nuclei isolated from 50 µm paraffin-embedded tissue sections, as described before [23].

Statistics

A Student T-test was performed to compare two groups for differences in

expression of cytokine levels. Linear regression was applied to observe which factors exactly determined differences in cytokine levels between the uveal melanoma and cataract patients. ANOVA Trend testing was performed for comparing the mean expression among more ordinal groups. Correlations with Spearman's Rho coefficient were performed to analyze correlations between two numerical variables. Cox regression was performed to observe whether cytokine levels were associated with decreased survival. A *p*-value of less than 0.05 was considered to be statistically significant.

Results

Cytokine levels in AqH

We determined whether eyes containing a uveal melanoma contained more or other inflammatory cytokines in their AqH than normal eyes. We selected 15 cytokines and chemokines that could be related to the presence of inflammation and macrophage infiltration.
AqH was obtained from 37 eyes with uveal melanoma and 37 with cataract; the average age of the controls was 68.7 ±12.4 years (Mean ± SEM) and of the uveal melanoma patients 58.5 ±14.9 years ($p = 0.002$). The distribution of female and male patients was not significantly different ($p = 0.16$). In the control group, 40.5% were men, while 59.5% were women, in the patient group, these numbers were 56.8% and 43.2%, respectively. Mean follow-up time of the enucleated patients was 4.8 years (± 2.3). 46% of the patients were still alive at the end of follow-up, and metastasis and subsequent death occurred in 41% of the patients (Table 1).
In comparison to eyes with cataract, eyes containing a uveal melanoma showed increased levels of almost all cytokines tested: IL-6 ($p < 0.01$), MCP-1 ($p < 0.001$), MIF ($p < 0.01$), bFGF ($p = 0.01$), RANTES ($p < 0.01$), GM-CSF ($p < 0.01$), VEGF ($p < 0.01$), ICAM-1($p < 0.01$), VCAM-1($p < 0.01$), and IP-10 ($p < 0.01$). Expression of TRAIL ($p = 0.46$), MCP-3 ($p = 0.63$), and MIP-1a ($p = 0.11$) was not significantly decreased in eyes with a uveal melanoma compared to the controls (Figure 1). TNF-b was only expressed in two samples of the control patients.
Since there is a significant difference in age between the patients with a uveal melanoma and the control group, we applied a linear regression to study whether only having a uveal melanoma or the difference in age between both groups was contributing to the observed significant differences in expression of cytokine levels. After analysis, only the presence of a tumor in the eye was found to determine the higher level of expression of cytokines and not age (data not shown).

Mutual correlation of cytokines

Some cytokines were correlated with each other, i.e. patients who have a high level of a certain cytokine in their AqH, often also had high levels of other cytokines and vice versa. GM-CSF, RANTES, VCAM-1, VEGF and IL-6 constituted one group with correlations, as did MCP-1, MIP-1a and MCP-3. This

indicates that in uveal melanoma patients, an inflammatory state can exist with a co-expression of a cluster of cytokines (supplementary Table 1).

Table 1. Patient and tumor characteristics, and metastasis and survival data

		Number of cases or mean with SD	
Age	Mean ± SD (in years)	58.5	± 14.9
Gender	Male	21	56.8%
	Female	16	43.2%
Eye	Right	21	56.8%
	Left	16	43.2%
Ciliary Body Involvement	Not present	21	56.8%
	Present	16	43.2%
Largest Basal Diameter	Mean ± SD (in mm)	13.4	± 3.0
Prominence	Mean ± SD (in mm)	7.1	± 2.7
Tumor size according pTNM (7th edition)	T1	4	11.1%
	T2	11	30.6%
	T3	20	55.6%
	T4	1	2.8%
Cell type	Spindle	9	24.3%
	Mixed + Epithelioid	28	75.7%
Macrophage Density (IHC)	Low	12	32.4%
	Medium	15	40.5%
	High	10	27.0%
Microvascular Density	First quartile	10	27.0%
	Second quartile	9	24.3%
	Third quartile	7	18.9%
	Fourth quartile	11	29.7%
Chromosome 3 Status	Disomy	13	35.1%
	Monosomy	24	64.9%
HC10	Mean ± SD (in % of tumor cells)	44.2	± 32.8
HCA2	Mean ± SD (in % of tumor cells)	46.5	± 31.7
HLA-DR	Mean ± SD (in % of tumor cells)	20.3	± 21.3
Follow-up in years	Mean ± SD	4.8	± 2.3
Metastasis	Not present	22	59.5%
	Present	15	40.5%
Survival Status	Alive	17	45.9%
	Dead due to metastasis	15	40.5%
	Dead due to other causes	5	13.5%

Comparison of cytokine concentrations with clinical and histopathological characteristics

We hypothesised that eyes with a uveal melanoma showing the so-called inflammatory phenotype [7] would have higher levels of inflammatory cytokines in the AqH than uveal-melanoma containing eyes without this phenotype. Since we had already studied this set of uveal melanomas previously [7], we know that these tumors have different characteristics based on known prognostic factors. Therefore, we compared cytokine levels with a series of markers that either determined the inflammatory phenotype, such as the presence of macrophages and HLA expression (HCA2, HC10 and HLA-DR), or variables that specifically determine prognosis, such as the presence of ciliary body ingrowth, microvascular density, tumor size according to the pTNM classification (7th edition) [24], epithelioid cells and monosomy of chromosome 3.

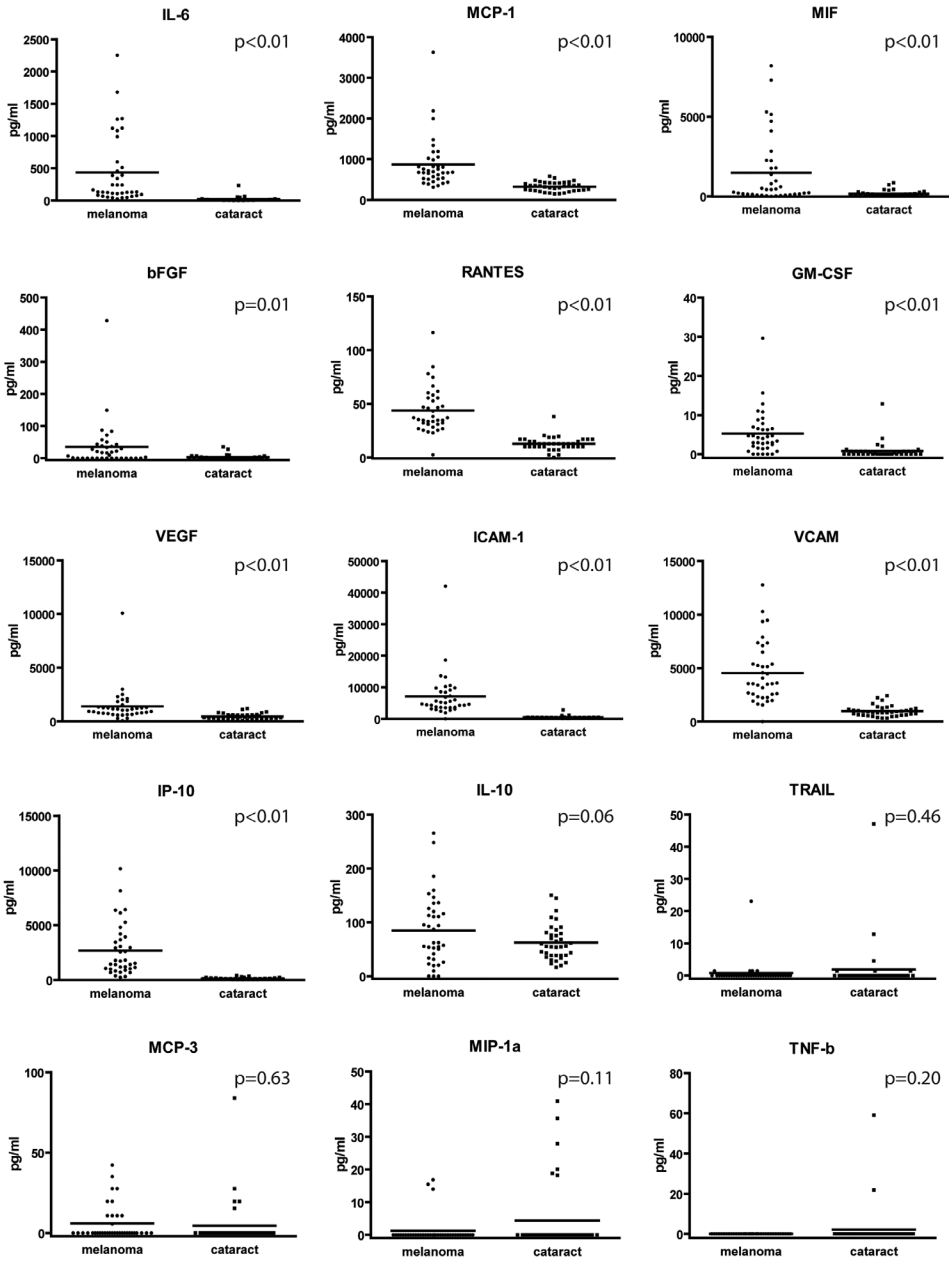

Figure 1. Expression of 15 different cytokines in Aqueous humor (AqH) of eyes with uveal melanoma and control eyes with cataract. For most cytokines, the concentration of the cytokine was significantly higher in uveal melanoma patients. The mean in each group is represented by the horizontal line.

Macrophages in uveal melanoma

The presence of macrophages was determined with standard CD68 IHC staining on paraffin sections in all 37 patients: 32% of the tumors had a low amount of macrophages according to the classification of Makitie [12], 41% a medium amount and 27% a high amount (Table 1). From 30 of the 37 patients, sufficient material was available to perform IF on the markers CD68 and CD163 in order to determine the number of M2 macrophages, which express both antigens. This was performed by analyzing the sections with image calculation software, after visualization with confocal microscopy. The results on CD68+ cells obtained by binary analysis with the image calculation software of the IF staining were in accordance with the CD68 IHC staining (ANOVA test, $p < 0.01$).
The density of CD163+ M2 macrophages was associated with the quantity of CD68+ macrophages, as determined by IHC staining (ANOVA test, $p < 0.01$) and correlated also significantly with the CD68+ staining with IF (Spearman's rho = 0.91, $p < 0.01$).
Cytokine levels were compared to the densities of CD68+, CD163+ and CD68+/CD163+ macrophages. A correlation was observed between CD68 density and the level of the cytokine MCP-3 ($p = 0.03$ and $p = 0.02$, respectively, see Table 2 and Figure 2). None of the other cytokines or chemokines showed a correlation with the intratumoral density of M2-type macrophages.

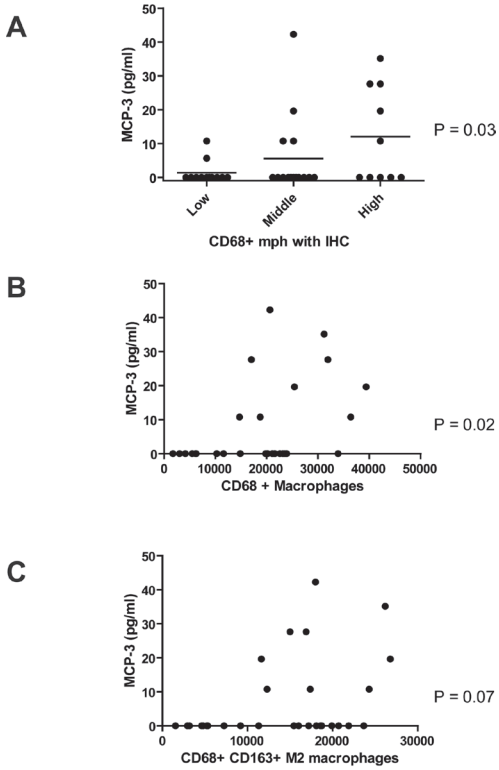

Figure 2. Expression of the cytokine Monocyte Chemotactic Protein-3 versus the three groups of CD68 IHC staining **(A)**, and correlation with CD68 IF **(B)** and CD68, CD163 IF **(C)** staining. Significant associations or correlations were observed between both types of CD68 staining and MCP-3. In Figure A, the data are shown as mean values with SEM for each group.

Table 2. P-values of correlation between cytokines and CD68+ macrophages (performed with IHC in 37 cases or IF staining in 30 cases) or CD68+ CD163+ M2-type macrophages. Macrophages determined by IHC were categorized into low, medium and high concentrations, while with IF, the macrophages were scored as the area (pixels) that stained positive.

	CYTOKINES														Test applied
	IL-6	**IL-10**	**bFGF**	**GM-CSF**	**IP-10**	**MCP-1**	**MIP-1a**	**RANTES**	**VEGF**	**MCP-3**	**MIF**	**TRAIL**	**ICAM-1**	**VCAM-1**	
CD68+ Mph (IHC)	0.33	0.73	0.60	0.22	0.32	0.23	0.41	0.06	0.25	***0.03***	0.54	0.11	0.41	0.61	ANOVA
CD68+ Mph (IF)	0.80	0.09	0.99	0.08	0.40	0.68	0.26	0.41	0.14	***0.02***	0.97	0.42	0.08	0.33	Spearman's correlation
CD68+ CD163+ M2 type Mph (IF)	0.77	0.13	0.67	0.13	0.22	0.79	0.68	0.48	0.14	0.07	0.73	0.62	0.18	0.61	Spearman's correlation

IHC = Immunohistochemistry
IF = Immunofluorescence
Mph = macrophages

Table 3. P-values of correlation and associations between cytokines and other clinical and histopathological parameters, including the association with survival.

	CYTOKINES														Test applied
	IL-6	**IL-10**	**bFGF**	**GM-CSF**	**IP-10**	**MCP-1**	**MIP-1a**	**RANTES**	**VEGF**	**MCP-3**	**MIF**	**TRAIL**	**ICAM-1**	**VCAM-1**	
Ciliary body ingrowth	***0.04***	0.60	0.13	0.05	0.81	0.26	0.08	0.05	0.21	0.30	***0.04***	0.22	0.16	0.11	T-test
Largest basal diameter	0.13	0.39	0.31	0.06	0.44	0.43	0.32	***0.03***	0.91	0.91	0.40	0.84	0.78	0.86	Spearman's correlation
Prominence	0.67	0.63	0.38	0.55	0.94	0.43	0.74	0.81	***0.02***	0.58	0.26	0.73	0.74	0.83	Spearman's correlation
Tumor size pTNM (7^{th} edition)	0.71	0.40	0.10	0.98	0.48	0.54	0.88	0.50	0.77	0.99	0.31	0.92	0.99	0.51	ANOVA
Presence of epithelioid cell type	0.77	0.47	0.89	0.28	0.47	0.91	0.69	0.96	0.80	***0.02***	0.86	0.22	0.36	0.96	T-test
Microvascular density	0.21	0.69	0.42	0.82	0.42	0.33	0.15	0.08	0.81	0.08	0.79	0.39	0.33	0.70	ANOVA
Presence of monosomy 3	0.48	0.40	0.63	0.52	0.46	0.50	0.08	0.31	0.77	0.13	0.94	0.22	0.11	0.30	T-Test
HLA class I with HC10 staining	0.72	0.40	0.13	0.97	0.08	0.93	0.19	0.64	0.56	0.59	0.20	0.18	0.15	0.59	Spearman's correlation
HLA class I with HCA2 staining	0.91	0.39	0.74	0.78	0.56	0.39	0.85	0.60	0.15	1.00	0.49	0.13	0.12	0.08	Spearman's correlation
HLA Class II with HLA-DR staining	0.26	0.97	0.23	***0.03***	0.80	0.55	0.46	0.11	0.75	***0.03***	0.95	***0.01***	0.27	0.78	Spearman's correlation
Survival analysis	0.21	0.92	0.47	0.99	0.16	0.30	0.40	0.06	0.40	0.71	0.31	0.65	0.39	0.94	Cox regression

Other prognostic parameters in uveal melanoma

We wondered whether there was a correlation between the presence of any of the cytokines and specific prognostic parameters (Table 3). Since TNF-b was only expressed in two patients, no analysis was performed for this cytokine. Correlations were observed between some specific cytokines and a few prognostic factors: an increased IL-6 as well as MIF expression were found to be correlated with ciliary body involvement (both $p = 0.04$) and the presence of the epithelioid cell type ($p = 0.03$). The presence of TRAIL and GM-CSF was associated with a higher HLA Class II expression (HLA-DR staining), and, with regard to tumor size, a higher RANTES expression correlated with a larger basal diameter ($p = 0.03$), and the presence of measurable VEGF with a higher prominence ($p = 0.02$).

Predictive value of cytokines for survival

We also studied whether cytokine levels could predict survival. With cox regression, we determined the Hazard Ratio for a decreased survival of the increased expression of any of the cytokines. No predictive value of any of the cytokines for survival was found (Table 3).

Discussion

Previous studies have already indicated alterations in the blood-aqueous barrier in eyes containing a uveal melanoma. Küchle et al.[25], using a laser flare-cell meter, showed that eyes containing a uveal melanoma had an increased aqueous flare compared to eyes containing a benign nevus or to normal eyes. An increased flare correlated with higher tumor height, serous retinal detachment, tumor necrosis and a lymphocytic tumor infiltration. Proteomic analysis of AqH showed a difference in protein constitution between AqH from melanoma-containing eyes and eyes undergoing surgery for cataract [26], but the identity of the precise proteins is as yet unknown. Therefore, changes in the AqH take place when a uveal melanoma develops in the eye. We demonstrate here that in uveal melanoma-containing eyes, several chemotactic cytokines are highly expressed in the AqH compared to controls.
Many studies elaborated on the expression of cytokines in the AqH of eyes with uveitis, which primarily is an inflammatory disease: in idiopathic acute anterior uveitis, AqH shows increased levels of various pro-inflammatory cytokines such as IL-6, MCP-1 (CCL2) and IFN-gamma [27]. Other cytokines such as TGFb2 and CXCL12 were seen to be decreased in cases with severe inflammation [28]. In a study using a multiplex immunoassay, van Kooij et al. [29] not only observed increased levels of IL-6 and IL-8 in the AqH of uveitis patients, but also of soluble vascular cell adhesion molecule (sVCAM), IP-10, and RANTES. Several leukocyte-attracting chemokines have been found specifically in acute inflammation, i.e. IL-8 (CXCL8, a recruiter of neutrophils), MCP-1 (CCL2) and MIP-1beta (CCL4), both of which are known to be able to attract monocytes [30].

We now show that many of these uveitis-related cytokines are also increased in the AqH of uveal melanoma eyes.
The presence of an intraocular lymphoma is associated with specific cytokine levels which differ in concentration compared to uveitis: in case of a lymphoma, the ratio of IL-6/IL-10 is lower than 1.0, while in uveitis it is vice versa. In our cases of uveal melanoma, on average, IL-6 was higher than IL-10, indicating that the ratio observed with lymphoma is not a general rule for malignancies. The cytokine pattern in uveal melanoma is an indicator of inflammation, but it is not specific.

While in general the AqH of uveal melanoma-containing eyes shows a pattern corresponding to inflammation, quite a lot of variation in the cytokine levels was observed. When correlating individual cytokine levels with clinical parameters and prognostic parameters, including those that are part of the inflammatory phenotype, we noticed that almost no cytokine was associated with any of those. The occurrence of some sporadic associations can be caused by the fact that we put $p = 0.05$ as the probability for finding significant associations. Since we tested 15 cytokines in our set, it is likely that sometimes a significant association is found, without a biologically relevant explanation. Similar to Missotten [16], we found a correlation of the cytokine VEGF with tumor prominence. We recently showed that in uveal melanoma, VEGF is upregulated by ischemia [31]. It may be that thicker tumors have less oxygenation, leading to an increased VEGF production. One could doubt whether the levels of cytokines in the AqH are a good representation of the presence of cytokines in the posterior eye. However, Boyd [15] had shown a good correlation between VEGF levels in AqH and vitreous.

Nevertheless, some remarkable associations found in our cytokine set have also been previously described for other cell types. We found that increased levels of GM-CSF and TRAIL were correlated to HLA-DR expression. GM-CSF is one of the stimulating cytokines for myeloid cells, such as macrophages, causing them to express MHC Class II (HLA-DR) at a high level [32]. This helps to stimulate antigen presentation to CD4+ T helper cells and TRAIL secretion, killing hostile cells by apoptosis [33].
Furthermore, we found that the presence of the pro-inflammatory cytokine IL-6 was correlated with GM-CSF. Recently, an article has been published on GM-CSF production by activated T cells, leading to recruitment of macrophages producing IL-6 [34]. In the presence of this pro-inflammatory cytokine, more inflammatory cells are attracted, leading to a self-perpetuating macrophage-T cell loop in inflammatory environments. We know from a previous study from our laboratory that macrophage infiltration, HLA expression and CD3+ T cell lymphocytes are associated with a decreased survival [11]. We found that the cytokines are not associated with the HLA expression and macrophage infiltration. It is known that T lymphocytes are capable in producing cytokines [35,36]; therefore the levels of cytokines could be representative for T lymphocyte infiltration.

As chemotaxis of immune cells into the eye is thus mediated by cytokines, we expected that the level of infiltrating macrophages and the specific tumor-promoting subtype of M2 macrophages would be associated with a higher expression of the chemotactic cytokines in the AqH. We found no correlation between the amount of cytokines expressed in the AqH and the number and subtype of macrophages. Apparently, the inflammatory cytokines found in the AqH are not determining the macrophage infiltration in a tumor, nor the other way around.

An explanation for our finding that cytokines are not representative for the presence of (M2-type) macrophages and the inflammatory phenotype of the tumor, can be that not the tumor, but surrounding tissue secretes the cytokines in the AqH, e.g the retinal pigment cells or also leukocytes [37,38], due to the inflammatory process. We observed previously with VEGF, that both tumor cells and the surrounding tissue can produce this cytokine, leading to high amounts in the eye [16]. Apparently, the presence of a melanoma leads to the production of a lot of proinflammatory cytokines, beneficial to the tumor's survival by creating a new microenvironment, which clearly differs between a diseased eye, and a "healthy" cataract eye. This would be a point of interest from a therapeutical view, since targeting the production of cytokines could then become a treatment for the tumor in the eye.
Another explanation for the absence of correlations between cytokines and leukocytes could be that macrophages and other immune cells do not require cytokines to be recruited into the eye. Macrophages and other cells of the innate system may also respond to ischemic or necrotic tumor cells, leading to infiltration of e.g. macrophages by HIF-1a triggering [39].

When studying whether a higher expression of the cytokines is associated with a better or worse survival, we observed no prognostic value of individual cytokines in this dataset. We were not able to create a specific set of cytokines to determine the bad or good prognosis of a tumor. We will therefore continue to rely on obtaining tumor tissue in order to determine important prognostic factors as monosomy of chromosome 3, or mRNA gene expression analysis [4,40].

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Supplementary Table 1. Mutual correlation of cytokines in uveal melanoma patients.

		IL-6	IL-10	bFGF	GM-CSF	IP-10	MCP-1	MIP-1a	RANTES	VEGF	MCP-3	MIF	TRAIL	ICAM-1	VCAM-1
IL-6	Correlation Coefficient		-0.09	-0.22	0.59**	0.02	0.04	-0.11	0.78**	0.31	-0.08	0.05	-0.26	0.31	0.49**
	p-value		0.62	0.19	<0.01	0.91	0.84	0.53	<0.01	0.06	0.66	0.80	0.13	0.06	<0.01
IL-10	Correlation Coefficient			0.57**	0.21	0.26	0.40*	0.18	0.16	0.64**	0.30	0.24	0.05	-0.02	-0.17
	p-value			<0.01	0.22	0.13	0.02	0.28	0.36	<0.01	0.07	0.20	0.79	0.89	0.31
bFGF	Correlation Coefficient				-0.12	0.14	0.07	0.16	-0.19	0.33*	-0.18	0.35*	0.02	0.08	-0.09
	p-value				0.50	0.42	0.67	0.36	0.25	0.04	0.28	0.04	0.92	0.65	0.61
GM-CSF	Correlation Coefficient					0.09	0.12	0.26	0.70**	0.38*	0.31	-0.09	0.17	0.13	0.25
	p-value					0.60	0.49	0.12	<0.01	0.02	0.06	0.62	0.31	0.44	0.14
IP-10	Correlation Coefficient						0.25	0.17	0.32	0.22	0.02	-0.01	-0.12	0.15	0.13
	p-value						0.13	0.32	0.06	0.20	0.90	0.95	0.47	0.37	0.45
MCP-1	Correlation Coefficient							0.33*	0.43**	0.26	0.36*	0.15	0.21	0.10	-0.08
	p-value							0.04	0.01	0.13	0.03	0.38	0.21	0.55	0.62
MIP-1a	Correlation Coefficient								0.16	-0.06	0.31	0.17	0.47**	0.02	-0.17
	p-value								0.38	0.75	0.07	0.33	<0.01	0.90	0.30
RANTES	Correlation Coefficient									0.38*	0.21	-0.02	0.06	0.25	0.35*
	p-value									0.02	0.21	0.91	0.73	0.14	0.03
VEGF	Correlation Coefficient										0.07	0.15	-0.22	0.21	0.38*
	p-value										0.67	0.38	0.19	0.21	0.02
MCP-3	Correlation Coefficient											-0.06	0.56**	-0.27	-0.20
	p-value											0.74	<0.01	0.11	0.23
MIF	Correlation Coefficient												0.16	0.09	-0.07
	p-value												0.33	0.60	0.70
TRAIL	Correlation Coefficient													-0.09	-0.36*
	p-value													0.59	0.03
ICAM-1	Correlation Coefficient														0.54**
	p-value														<0.01
VCAM-1	Correlation Coefficient														
	p-value														

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).