Analysis of Inflammatory Cells in Uveal Melanoma after Prior Irradiation

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Purpose. Primary uveal melanomas with a poor prognosis contain high numbers of infiltrating macrophages, especially of the M2 phenotype, as well as lymphocytes. We wondered whether local inflammatory responses were affected by irradiation and therefore determined the presence of inflammatory cells in uveal melanomas enucleated after prior irradiation.

Methods. We analyzed 46 uveal melanoma–containing eyes that had to be enucleated due to nonresponsiveness, tumor recurrence, or complications. Immunofluorescent staining was performed to determine the presence of $CD68^+$ and $CD68^+CD163^+$ macrophages, and of $CD4^+$, $CD8^+$, and $Foxp3^+$ regulatory T lymphocytes. Outcomes were compared with clinical and histologic parameters.

Results. Numbers of $CD68^+$ and $CD68^+CD163^+$ macrophages in secondarily enucleated eyes varied widely, but did not differ from primarily enucleated eyes and were not related to the reason for enucleation. Similarly, the number of $CD4^+$, $CD8^+$, and $Foxp3^+$ T lymphocytes showed great variability. Tumors with epithelioid cells showed significantly more lymphocytes than spindle cell tumors. In the first 2 years after enucleation, previously irradiated tumors showed increased numbers of lymphocytes compared with primarily enucleated eyes.

Conclusions. Numbers of infiltrating T lymphocytes and macrophages varied widely between tumors, but tumors with high numbers of macrophages also contained more lymphocytes. Irradiation had no effect on the number and type of macrophages, but led to an increased amount of T lymphocytes up to 24 months postirradiation. Because the presence of infiltrating cells was related to the tumor cell type, it is conceivable that the presence of an infiltrate is especially a consequence of the primary tumor characteristics before irradiation. (*Invest Ophthalmol Vis Sci.* 2013;54:360–369) DOI:10.1167/iovs.12-9424

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Malignant melanoma of the uvea is the most common primary intraocular neoplasm, with an annual incidence varying from two to eight cases per million per year in European countries.[1] Over the past decades, patient survival has not improved, despite progress in the diagnosis of melanocytic lesions and successful treatment of the intraocular melanoma.[2–4] The 5-year mortality rate after diagnosis of a uveal melanoma is approximately 30% due to metastatic disease.[2,5]

Until the 1970s, the traditional treatment of uveal melanoma was enucleation.[6] However, when eye-preserving techniques became available, and after the Collaborative Ocular Melanoma Study revealed no survival difference in medium-sized melanoma after irradiation versus enucleation,[2–4,6–8] a shift to more eye-saving approaches occurred.[2–5,7,9] Local radiotherapy is often able to preserve the eye and sight, and is less mutilating than enucleation.[6,10] While eye retention following local treatment is usually achieved in more than 80% of cases after 5 years,[8,10,11] secondary enucleation may be required when failure of local tumor control occurs. This may be inadequate tumor regression or local (contiguous and noncontiguous) recurrence.[12–17] In addition, secondary enucleation is sometimes necessary in case of radiation-related ocular side effects, such as neovascular glaucoma, persistent hemorrhage, or exudative retinal detachment.[14]

The histopathologic findings of uveal melanoma previously treated with different eye-conserving therapies are quite similar, regardless of the difference in physical properties of the various radiation sources used.[15–17] These findings include: vacuolization with balloon cell degeneration, tumor cell necrosis surrounding vascular sclerosis, vascular damage, and fibrosis of the tumor stroma with frequent accumulation of pigmented macrophages.[15–17]

Several studies have revealed the presence of tumor-infiltrating macrophages and lymphocytes in primarily enucleated as well as irradiated and secondarily enucleated eyes with uveal melanoma.[18–25] High numbers of tumor-infiltrating macrophages in primarily enucleated eyes are related to an unfavorable prognosis and are associated with the presence of epithelioid cells, increased microvascular density, and monosomy of chromosome 3.[18,21] Such intratumoral macrophages were mainly of the tumor-promoting M2 phenotype, harboring anti-inflammatory and proangiogenic functions.[26] Moreover, tumors containing higher numbers of lymphocytes were associated with a bad prognosis.[25,27] Histologic studies after transpupillary thermotherapy (TTT) or transscleral thermotherapy (TSTT) showed an influx of macrophages after local therapy.[28,29] It is feasible that macrophages play an important scavenger role in removing debris after irradiation. Based on these findings, we hypothesize that the combination of irradiation and thermotherapy will induce an influx of macrophages that should be noticeable in uveal melanoma enucleated after prior radiotherapy. In addition, because primarily enucleated uveal melanomas that have monosomy 3

contain more inflammatory infiltrate,[20,21,27] and tumor recurrence may perhaps especially occur in tumors that have similarly lost one copy of chromosome 3, irradiated uveal melanomas enucleated due to failure of local tumor control can be expected to contain more inflammatory cells; therefore, we determined the number and subtype of tumor-infiltrating macrophages and lymphocytes in uveal melanoma–containing eyes enucleated after irradiation and compared results to prior studies on primarily enucleated eyes.

Materials and Methods

Eye-Preserving Radiotherapy

The primary choice of conservative treatment at the Department of Ophthalmology of the Leiden University Medical Center (LUMC), The Netherlands, is brachytherapy, delivered with ruthenium-106 applicators. Between 1995 and 2008, plaque treatment was frequently combined with adjuvant TTT.[30] Proton beam radiotherapy was indicated for large and highly prominent melanomas, or in tumors located close to or in contact with the optic disc.[6,11] For this treatment, patients were referred to the Hôpital Ophthalmique Jules Gonin, Lausanne, Switzerland.

Patients and Specimens

Tissue specimens were obtained from 69 consecutive eyes with uveal melanomas that had undergone a secondary enucleation after prior irradiation between 1996 and 2010 at the LUMC. Only formalin-fixed paraffin-embedded specimens from which enough tumor material was present for histopathologic analysis were selected from the archives of the Pathology and Ophthalmology Departments, leaving 46 eligible specimens for inclusion. Reasons for enucleation included the following: failure of local tumor control, defined as (1) contiguous tumor progression after partial regression due to nonresponsiveness to prior treatment; (2) intraocular tumor recurrence after total tumor regression; or (3) radiation-related ocular complications, such as persistent hemorrhage, exudative retinal detachment, or a blind painful eye due to neovascular glaucoma. Clinical histories were analyzed by a clinician, using fluorescein angiographic and ultrasonographic (including A and B scans) images, to accurately divide eyes into three subgroups based on the reason for enucleation. Clinical data from the 46 enrolled cases were collected from the clinical records.

Clinical, histopathologic, and inflammatory parameters on a set of 43 primarily enucleated nonirradiated tumors have been described previously[20,21,27] and were used for the comparison with irradiated, secondarily enucleated eyes.

All patients were informed regarding the use of their eye for research purposes and signed an informed consent form. The use of tumor material for research followed the current revision of the tenets of the Declaration of Helsinki (World Medical Association Declaration of Helsinki 1964; Ethical principles for medical research involving human subjects).

Histopathologic Data

After enucleation, eyes were fixed in 4% neutral-buffered formalin for 48 hours and embedded in paraffin. Two ocular pathologists analyzed the hematoxylin and eosin–stained (H&E) 4-μm sections for the pathologic diagnosis, tumor location, ciliary body involvement (presence or absence), tumor cell type (spindle or nonspindle), largest basal diameter (LBD, in millimeters), prominence (in millimeters), and the presence of necrosis.

Immunostaining Protocol for Tumor-Infiltrating Macrophages and Lymphocytes

Immunostaining was performed using double- and triple-immunofluorescence (IF) staining as described earlier,[20,31] to identify the different subsets of tumor-infiltrating leucocytes. In short, 4-μm sections were cut from paraffin-embedded tumor blocks and deparaffinized. Antigen retrieval was performed by a 10-minute incubation in boiling Tris-EDTA buffer, at pH 9.0.

The primary antibodies used to characterize the macrophage phenotype were mouse anti-human CD68 mAb (1:50, clone 514H12, ab49777; Abcam, Cambridge, UK) as a marker for total macrophages, and mouse anti-human CD163 mAb (1:100, clone 10D6, NCL-CD163; Novocastra, Newcastle upon Tyne, UK) as a marker for M2 macrophages.

The rabbit anti-human CD3 pAb (1:100, IgG, clone ab828; Abcam) was used to label $CD3^+$ T cells, and the mouse anti-human CD8 mAb (1:100, IgG2b, clone 4B11; Novocastra) was used to label $CD8^+$ T cells. The mouse anti-human Foxp3 (forkhead box p3) mAb (1:200, IgG1, clone 236A/E7; Abcam) was used as a nuclear marker for regulatory T cells (Tregs).

Secondary antibodies were IgG2a (AlexaFluor 488) goat-anti-mouse for CD68, IgG1 (546) goat-anti-mouse for CD163, IgG (546) goat-anti-rabbit for CD3, IgG2b (647) goat-anti-mouse for CD8, and IgG1 (488) goat-anti-mouse for Foxp3 (all antibodies from Invitrogen/Molecular Probes, Eugene, OR).

Detection of Immunostaining

Images of the stained sections were captured with a confocal laser-scanning microscope (LSM510; Carl Zeiss Meditec, Jena, Germany) in a multitrack setting and H&E-stained sections were used for orientation and location of the scans. Each scan represented one square optical field (area, 0.137 mm^2).

All images for the tumor-infiltrating lymphocytes were 1024×1024 pixels and for the macrophages 512×512 pixels with stack size 368.5×368.5 μm. A PH2 Plan-NEOFluar 25×/0.80 Imm Korr objective (Carl Zeiss Meditec) was used.

The fluorochrome signals in the slides were visualized with an artificial color: red for both AlexaFluor 546 antibodies (CD163, CD3), blue for AlexaFluor 647 (CD8), and green for both AlexaFluor 488 antibodies (CD68, Foxp3). Images were viewed as an overlay, as a set of two (for macrophages) or three divided panels (for lymphocytes).

Assessment of Immunostaining

For characterization of the macrophage subtypes, five representative high-power fields (×250 magnification) per slide were randomly selected. Because of the polymorphic appearance of macrophages, it was difficult to count them. Instead, we calculated the amount of staining in pixels per mm^2 by using an image-analysis software program (Stacks; Department of Molecular Cell Biology, LUMC, Leiden, The Netherlands). In the resulting binary images, the green and red pixels corresponded to the anti-CD68 and anti-CD163 staining, respectively. The overlay of both colors was expressed as yellow pixels (Fig. 1).

$CD3^+CD8^+$ cells (purple signal) were considered to be $CD8^+$ T cells, $CD3^+CD8^-$ cells (red signal) $CD4^+$ helper T cells, and $CD3^+CD8^-FoxP3^+$ cells (red signal with a green center) $Foxp3^+$ Tregs (Fig. 1). Positive cells were counted in 10 randomly taken high-power fields (×250 magnification) by two independent observers masked to the clinical outcome and the reason for enucleation. The mean of the two observers was calculated for each tumor and tumor-infiltrating lymphocyte cell counts were presented as the number of cells per mm^2. Necrotic areas were not analyzed.

Statistical Analyses

All analyses were performed with a statistical software program (SPSS for Windows, version 17.0; SPSS Inc., Chicago, IL). The performed tests were two-sided and a value of $P < 0.05$ was considered as statistically significant. For the comparison of categorical data between two or more independent groups, the χ^2 test was performed, and for numerical data the nonparametric Wilcoxon rank-sum test and the Kruskal–Wallis test were performed. Spearman's rank correlation analysis (two-sided)

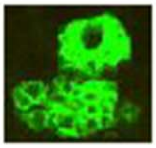
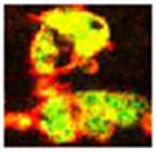
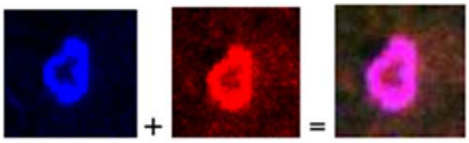
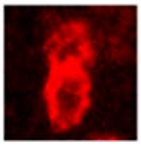
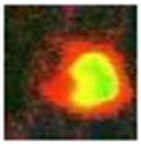
Cell type	*Epitopes*	*Phenotype*
Double-IF staining		
Macrophages	$CD68^+$	**Macrophages** 
	$CD68^+CD163^+$	**M2-type macrophages** 
Triple-IF staining		
Lymphocytes	$CD3^+CD8^+$	**Cytotoxic T cells** 
	$CD3^+CD8^-Foxp3^-$	**Helper T cells** 
	$CD3^+CD8^-Foxp3^+$	**Regulatory T cells** 

Figure 1. Detection of macrophages and lymphocytes in uveal melanomas by double- and triple-IF staining with antibodies directed against specific immune cells.

was performed to assess correlations between the different types of immune cells, and with time between irradiation and enucleation.

Results

The numbers of infiltrating immune cells were determined in 46 uveal melanomas that had been irradiated previously, and often treated by TTT. We compared the cell counts with demographic and histopathologic patient and tumor characteristics, as summarized in Table 1.

Of the 46 irradiated eyes with uveal melanoma, 12 had been treated with proton beam radiotherapy, 4 with ruthenium monotherapy, and 30 with sandwich therapy (ruthenium-106 brachytherapy with TTT). Given the fact that only four eyes received ruthenium-106 as monotherapy and no conclusion

Table 1. Comparison of Clinical and Histologic Data of Nonirradiated and Irradiated Eyes with Uveal Melanoma

		Irradiated Eyes			
	Nonirradiated Eyes	**Total**	**Nonresponsiveness**	**Recurrence**	**Complications**
Subjects, *n*	43	46	15	14	17
Sex, male/female	23/20	31/15	8/7	9/5	14/3
Eye, right/left	23/20	25/21	8/7	8/6	9/8
Prognostic groups					
Stage I	3	17*	6	3	8
Stage IIA	9	18	7	4	7
Stage IIB	13	4	1	1	2
Stage IIIA	15	5	1	4	0
Stage IIIB	3	2	0	2	0
Cell type					
Spindle	11	10	5	1	4
Mixed + epithelioid	32	36	10	13	13
CB involvement					
Not present	25	41*	15	10	16
Present	18	5	0	4	1†
Bruch's membrane					
Broken	33	23*	6	5	12
Intact	4	18	9	5	4
Not clear	6	5	0	4	1
Necrosis					
Not present	36	22*	8	9	5
Present	7	22	7	3	12
Unclear	0	2	0	2	0
Median prominence, mm (range) 8 (2–12)	8 (2–12)	4 (1–14)*	3 (1–8)	4 (1–14)	4 (1–11)
Median LBD, mm (range)	13 (8–18)	12 (4–21)*	12 (6–20)	12 (4–21)	10 (5–15)
Median age at irradiation (range)	-	60 (32–84)	56 (42–84)	61 (32–78)	60 (42–70)
Median age at enucleation (range)	63 (27–88)	63 (38–85)	57 (42–85)	69 (38–80)	61 (43–77)

Prominence and LBD measurements were obtained from histologic examinations. Prognostic groups based on the 7th edition AJCC–UICC criteria for T staging, including the anatomic extent of the tumor based on involvement of the ciliary body and extrascleral tissues. *P* values for categorical parameters were obtained by the χ^2 test, for the numerical data by Wilcoxon rank-sum test and Kruskal–Wallis test; all statistical tests were two-tailed.

* Significant at $P \leq 0.05$ between irradiated and nonirradiated eyes.

† Significant at $P \leq 0.05$ between secondarily enucleated eyes: nonresponsiveness, recurrence, and complications.

can be drawn from such a small sample size, these cases were combined with the sandwich therapy group for statistical analysis. The median interval between irradiation and enucleation was 14 months (range: 4–146 months) for patients who received proton beam irradiation, and 22 months (range: 3–125 months) for ruthenium-106 brachytherapy (with or without TTT).

Causes for enucleation were: nonresponsiveness to prior irradiation in 15 (33%) cases, tumor recurrence after prior total regression in 14 (30%) cases, and radiation-related ocular side effects in 17 (37%) cases. Of the 12 eyes that had to be enucleated following proton beam irradiation, one (8%) was due to nonresponsiveness, two (17%) showed a tumor recurrence, and nine (75%) had radiation-related complications (especially neovascular glaucoma). Of the 34 eyes treated with ruthenium-106 brachytherapy, 14 (41%) had to be enucleated due to nonresponsiveness, 12 (35%) due to tumor recurrence, and 8 (24%) due to complications.

At the time of irradiation, the median age of the patients (31 males, 15 females) was 60 years (range: 32–84 years), and by the time of enucleation it was 63 years (range: 38–85 years).

The primarily enucleated eyes and the irradiated eyes differed significantly with regard to the American Joint Committee on Cancer–Union Internationale Contre le Cancer (AJCC–UICC) prognostic stage groups ($P < 0.001$), tumor prominence ($P < 0.001$), LBD ($P = 0.014$), involvement of the ciliary body ($P = 0.001$), break through Bruch's membrane ($P = 0.005$), and the presence of necrosis ($P = 0.001$): these variables, except for necrosis, were seen more often in nonirradiated eyes.

Tumor-Infiltrating Macrophages

Double-IF was performed on tumor sections of 46 irradiated eyes to analyze the amount and phenotype of tumor-infiltrating macrophages. In cases of severe tumor pigmentation, positive cells were easily recognized by IF staining with the confocal microscope.

Because most of the CD68-positive cells were also CD163-positive, macrophages in irradiated uveal melanoma belonged mainly to the M2 phenotype. The amount of $CD68^+$ ($P = 0.80$) and $CD68^+CD163^+$ ($P = 0.44$) staining was similar in irradiated and primarily enucleated uveal melanoma (Table 2, Fig. 2A). In addition, there was no relation between the amount of $CD68^+$ ($P = 0.16$) and $CD68^+CD163^+$ ($P = 0.34$) staining and the cause of enucleation in irradiated eyes (Table 2, Fig. 2A) or with the type of irradiation (data not shown).

However, the frequency of macrophages in secondarily enucleated uveal melanomas seemed to decrease slightly with increase in time interval between irradiation and enucleation (r: < -0.402, $P < 0.03$; Fig. 2B). Because the time between irradiation and enucleation varied broadly between patients, the data were normalized to account for time after irradiation by dividing the secondarily enucleated eyes into four time-interval categories: 0 to 12, 13 to 24, 25 to 36, and >36 months, and then to compare these categories with the

Table 2. Tumor-Infiltrating Lymphocytes and Macrophages in Nonirradiated and Irradiated Eyes, and in Relationship with the Different Reasons of Secondary Enucleation

Immunofluorescence	Nonirradiated Eyes ($n = 43$)		Irradiated Eyes ($n = 46$)			Non responsiveness ($n = 15$)		Recurrence ($n = 14$)		Complications ($n = 17$)		
Staining	Median	Range	Median	Range	*P* Value	Median	Range	Median	Range	Median	Range	*P* Value
$CD3^+$ total cells	31	1–1834	164	6–2526	*0.001*	127	23–1684	152	13–1988	247	6–2526	0.26
$CD8^+$ T cells	16	1–1566	99	2–1956	*0.002*	59	15–1336	62	11–1017	179	2–1956	0.12
$CD3^+CD8^-(CD4^+)$ T cells	14	0–268	64	1–971	*<0.001*	49	8–530	69	1–971	65	4–570	0.60
$CD3^+CD8^-FoxP3^+$ Tregs	3	0–158	9	0–226	*0.02*	14	0–75	9	0–226	8	0–63	0.74
$CD3^+CD8^-FoxP3^-$ Th cells	10	0–151	55	1–745	*<0.001*	40	4–467	65	1–745	60	4–523	0.36
$CD68^+$ macrophages	125	13–290	119	13–301	0.80	119	21–248	94	36–222	149	13–301	0.16
$CD68^+CD163^+$ macrophages	86	11–211	82	9–230	0.44	90	11–225	55	22–162	83	9–230	0.34

The amount of intratumoral lymphocytes is represented as the number of cells per mm^2. The amount of staining for tumor-infiltrating macrophages is represented as pixels per mm^2. For the numerical parameters, the median and range are shown. For the comparison between nonirradiated and irradiated eyes, *P* values were obtained by the Wilcoxon rank-sum test (Mann–Whitney *U* test). For the comparison between the three secondary enucleation reasons, *P* values were obtained by the Kruskal–Wallis test. Values of $P \leq 0.05$ are shown in italics.

primarily enucleated eyes. The amount of $CD68^+$ ($P = 0.11$, $P = 0.71$, $P = 0.91$, $P = 0.12$) and $CD68^+CD163^+$ staining ($P = 0.37$, $P = 0.66$, $P = 0.49$, $P = 0.06$) was not significantly different between the four categories compared with nonirradiated uveal melanoma (Figs. 2C, 2D). Moreover, the phenotype did not change over time, because the M2 macrophages stayed dominant.

Subtypes of Tumor-Infiltrating Lymphocytes

Triple-IF staining was performed on tumor sections of 46 irradiated eyes to assess the number of intratumoral $CD8^+$ cytotoxic T cells, $CD4^+$ helper T cells, and $Foxp3^+$ Tregs. $CD8^+Foxp3^+$ T cells were seldom observed and therefore excluded from analysis. Generally, all tumors displayed the whole spectrum of different subtypes of lymphocytes, but the number varied widely between the nonirradiated and irradiated uveal melanomas, and also between the tumors of each category (Table 2).

Irradiated uveal melanomas contained more lymphocytes of all subtypes than uveal melanomas from primarily enucleated eyes (Table 2, values of $P < 0.02$; Fig. 3A), but this was not related to the reason for secondary enucleation (Table 2, Fig. 3A) or the type of irradiation (data not shown).

However, the total number of lymphocytes decreased slightly with increasing time between irradiation and enucleation (r: < -0.422, $P < 0.055$; Fig. 3B). To normalize the data to account for time after irradiation, the secondarily enucleated eyes were divided into four categories: 0 to 12, 13 to 24, 25 to 36, and >36 months, and were then compared with primarily enucleated eyes. Significant differences were observed in the numbers of total intratumoral $CD3^+$ T cells between nonirradiated and irradiated eyes enucleated 0 to 12 months ($P = 0.001$) and 13 to 24 months ($P = 0.014$) after radiotherapy, but not at 25 to 36 months and >36 months (Fig. 3C).

Correlations between Subtypes of Immune Cells

Infiltration of the tumor by one subtype of immune cell was accompanied by other subtypes. Spearman rank analysis showed significant correlations between the numbers of $CD8^+$ T cells, $CD4^+$ T cells, $Foxp3^+$ Tregs, and the amount of $CD68^+$ and $CD68^+CD163^+$ macrophages (Table 3; Spearman correlation coefficient [r] range = 0.379–0.983, $P < 0.009$). These observations are similar to the situation in nonirradiated uveal melanomas where tumors with high numbers of Tregs contained more of any other type of tumor-infiltrating immune cell[27]; however, the effect and correlations are less strong in irradiated eyes.

Relation with Clinical and Histological Parameters

The amount of tumor-infiltrating immune cells (macrophages or lymphocytes) in irradiated uveal melanomas was not

Table 3. Correlations between Different Infiltrating Immune Cells (Lymphocytes and Macrophages) in Irradiated, Secondarily Enucleated Eyes

Immunofluorescence Staining	CD8	CD4 Total	CD4 Th	CD4FoxP3	CD68	CD68CD163
CD3 *r*	0.980 *<0.001*	0.939 *<0.001*	0.916 *<0.001*	0.818 *<0.001*	0.607 *<0.001*	0.497 *<0.001*
CD8 *r*		0.873 *<0.001*	0.851 *<0.001*	0.766 *<0.001*	0.616 *<0.001*	0.501 *<0.001*
CD4 total *r*			0.983 *<0.001*	0.833 *<0.001*	0.470 *0.001*	0.379 *0.009*
CD4 Th *r*				0.746 *<0.001*	0.467 *0.001*	0.380 *0.009*
CD4FoxP3 *r*					0.450 *0.002*	0.387 *0.008*
CD68 *r*						0.956 *<0.001*

r = rho, two-tailed Spearman correlation coefficient. Values of $P \leq 0.05$ are shown in italic.

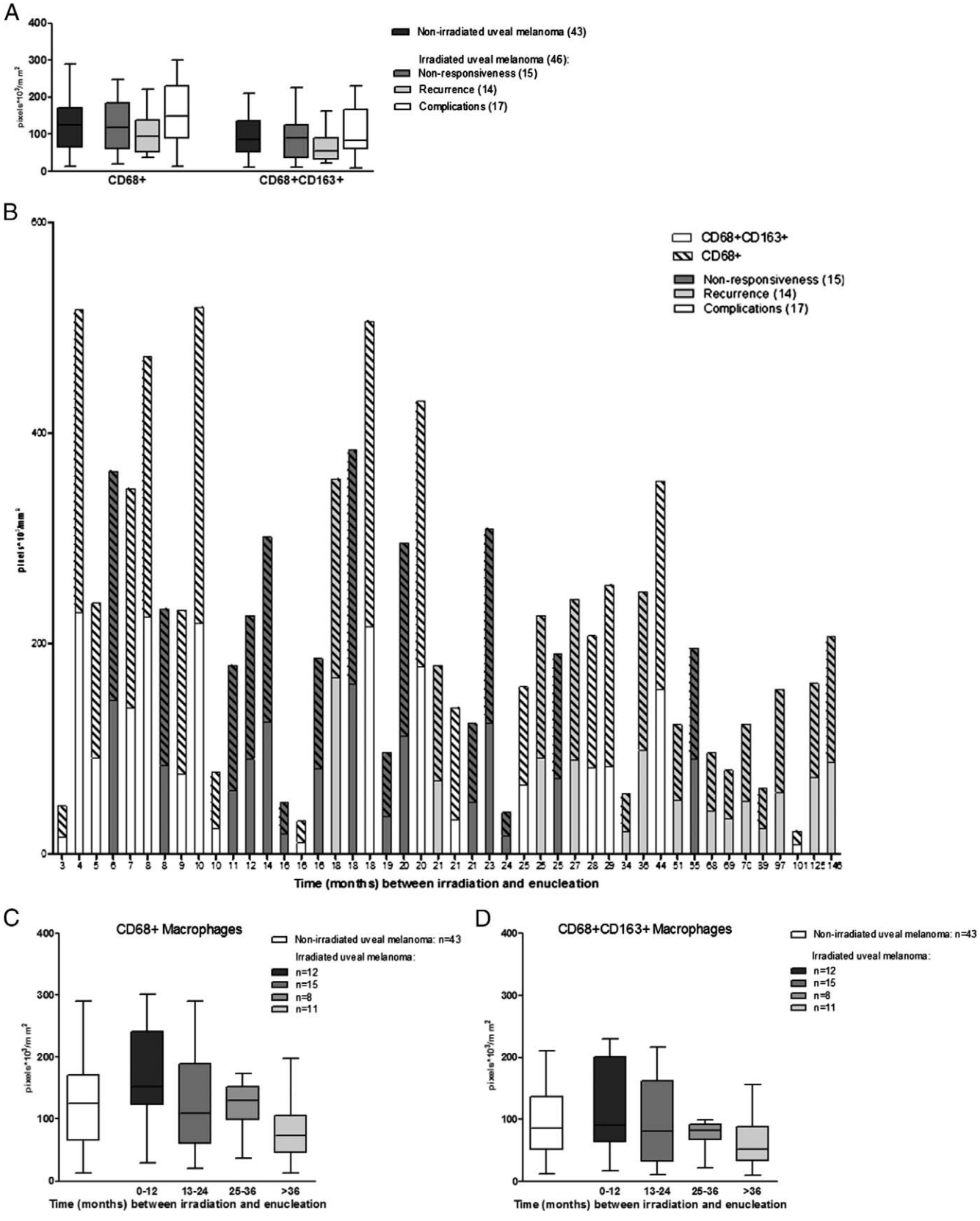


Figure 2. (**A**) Subtypes of tumor-infiltrating macrophages in uveal melanomas. Comparison in the amount of $CD68^+$ and $CD68CD163^+$ staining in nonirradiated and irradiated uveal melanomas, in which the latter group is divided into three subgroups based on the cause of secondary enucleation. The *box-and-whisker plots* represent the 25th and 75th percentiles with the median and the minimum and maximum values. No significant differences were observed between the irradiated and nonirradiated uveal melanomas or subgroups of secondary enucleation. (**B**) Tumor-infiltrating macrophages in uveal melanomas postirradiation. Variation in the amount of $CD68^+$ and $CD68^+CD163^+$ staining in irradiated uveal melanomas with time. The *stacked bars* represent the total amount of $CD68^+$ and $CD68^+CD163^+$ staining at a specific time point. The 46 time

points represent the time interval between irradiation and enucleation for each of the 46 irradiated, secondarily enucleated eyes. (**C**) Tumor-infiltrating $CD68^+$ macrophages in uveal melanomas postirradiation. Comparison in the amount of $CD68^+$ staining in nonirradiated and irradiated uveal melanomas, in which the latter group is divided into four subgroups based on the time (in months) between irradiation and enucleation. The *box-and-whisker plots* represent the 25th and 75th percentiles with the median and the minimum and maximum values. No significant differences were observed in the amount of $CD68^+$ staining in irradiated uveal melanomas at different time periods after irradiation and the nonirradiated uveal melanomas. (**D**) Tumor-infiltrating $CD68^+CD163^+$ macrophages in uveal melanomas postirradiation. Comparison in the amount of $CD68^+CD163^+$ staining in nonirradiated and irradiated uveal melanomas, in which the latter group is divided into four subgroups based on the time (in months) between irradiation and enucleation. The *box-and-whisker plots* represent the 25th and 75th percentiles with the median and the minimum and maximum values. No significant differences were observed in the amount of $CD68^+CD163^+$ staining in irradiated uveal melanomas at different time periods after irradiation and nonirradiated uveal melanomas.

associated with sex, AJCC–UICC prognostic groups, or ciliary body involvement. Tumors with necrosis showed a trend toward more IF-$CD68^+$ and $CD68^+CD163^+$ staining (data not shown). Irradiated uveal melanomas with epithelioid cells contained significantly more intratumoral $CD3^+$ lymphocytes ($P = 0.011$), $CD8^+$ T cells ($P = 0.009$), $CD4^+$ T cells ($P = 0.018$), and $Foxp3^+$ Tregs ($P = 0.036$) than pure spindle cell tumors (Fig. 4). Previously, for nonirradiated uveal melanoma, it had been shown that tumors with an increased LBD contained more $CD68^+$ and $CD68^+CD163^+$ macrophages.[20] In irradiated eyes, however, no correlation was found between the amount of tumor-infiltrating leucocytes and LBD or tumor prominence (data not shown).

Discussion

Because the local immune response may play a role in removing tumor debris or in either stimulating or suppressing antitumor immune responses, we determined the quantity of infiltrating leucocytes in uveal melanomas previously treated with ruthenium-106 brachytherapy (with or without adjuvant TTT) or proton beam radiotherapy. These eyes had been enucleated after failure of local tumor control or radiation-related complications, and were studied by IF-staining of intratumoral immune cells. We compared the characteristics of local inflammation in these uveal melanomas with primarily enucleated eyes. We found that prior irradiation had no effect on the number and type of tumor-infiltrating macrophages, which showed a similar variability as previously observed in primarily enucleated eyes,[20] but led to an increased T lymphocytic infiltrate up to 24 months postirradiation. The reason to perform a secondary enucleation was not related to the amount and type of tumor-infiltrating leucocytes.

In a previous study, significantly more necrosis and a lower microvascular density were present in previously irradiated eyes, but the number of macrophages was not affected.[19] In our study, we were able to assess the macrophage phenotype by determining the presence of the alternatively activated (M2) macrophage. M2 macrophages show more phagocytic activity; promote tissue remodeling, tumor progression, and angiogenesis; and have immunoregulatory functions.[26] In both of our study populations, $CD68^+CD163^+$ immunopositive cells constituted the majority of $CD68^+$ cells, showing that in irradiated as well as in nonirradiated uveal melanoma, the tumor-promoting M2 macrophage is the main type. In addition, we observed no increase in the amount of macrophages in nonnecrotic areas of irradiated compared with primarily enucleated eyes, even when normalized for time after irradiation, despite more extensive necrosis in the former group. An explanation may be that obliteration of the vascular supply prevented influx of leucocytes to the tumor and clearance of necrotic tumor debris by macrophages, leading to more necrosis.[15–17,19] Toivonen et al.[19] described that along with melanoma cells, resident macrophages may have been sterilized by irradiation. This is supported by the finding that necrotic, irradiated macrophages lacking vimentin filaments were immunopositive for CD68; therefore, they may not be functional.

Interestingly, in contrast with our findings, previous studies with TTT and TSTT demonstrated an increased amount of tumor-infiltrating macrophages, especially at the borders of the TSTT-treated areas, with predominantly M2 macrophages.[28,29] These tumors had been treated only one or several weeks before enucleation. Their presence may reflect direct phagocytosis (i.e., removal of dead tumor cells) by a macrophage-mediated repair mechanism.[28,29] Infiltration of the tumor by M2 macrophages instead of M1 macrophages would be unfavorable for the stimulation of a specific immune response, but it may be that in this posttreatment stage, macrophages may function only in an innate immune response, clearing tumor debris, instead of exploiting their tumor-promoting functions.[26,29]

After irradiation, increased numbers of tumor-infiltrating lymphocytes were observed up to 24 months postirradiation, which included the whole spectrum of $CD8^+$ T cells, $CD4^+$ T cells, and $Foxp3^+$ Tregs. However, a previous study of nine enucleated eyes with uveal melanomas managed by pre-enucleation electron beam radiation showed a lower expression of HLA class II antigens and a lack of lymphocytic infiltrate in comparison with nonirradiated tumors.[32] It is uncertain why we observed an increased influx of lymphocytes after irradiation. The differences in the time and type of treatment may be important, because in the aforementioned study, irradiation took place one week prior to enucleation, in otherwise quiet eyes, whereas the cases studied here had to be enucleated following failure of local tumor control or complications, at a median time after irradiation of 21 months (range: 3–146 months). Moreover, radiotherapy may induce intratumoral expression of chemokines that favors the recruitment of T cells, in the same manner as chemotherapy-induced chemokines correlate with T cell infiltration in mouse and human melanoma tumors.[33] This effect might dampen, given that we could not see any difference between irradiated eyes enucleated after >24 months postirradiation and primarily enucleated eyes.

Because most of the irradiated eyes were enucleated due to failure of local tumor control, the presence of M2 macrophages and Tregs in these eyes would be detrimental for the induction of an effective immunologic antitumor response, thereby promoting tumor growth in an immunosuppressive environment.[26] Unfortunately, we were not able to assess the functional activity of these tumor-infiltrating immune cells, in that functional immunologic studies were not possible on our sections. Another limitation of our study is its cross-sectional design, because it provides only a snapshot of the moment of enucleation and does not reflect potential changes in infiltrating leucocytes over time. Thus, it remains unclear whether this inflammatory infiltrate is a consequence of the characteristics of the primary tumor before irradiation or is due to irradiation. Besides, eyes that had to be enucleated following radiation represent biased material and therefore do not allow us to draw definite conclusions regarding the effect of therapy.

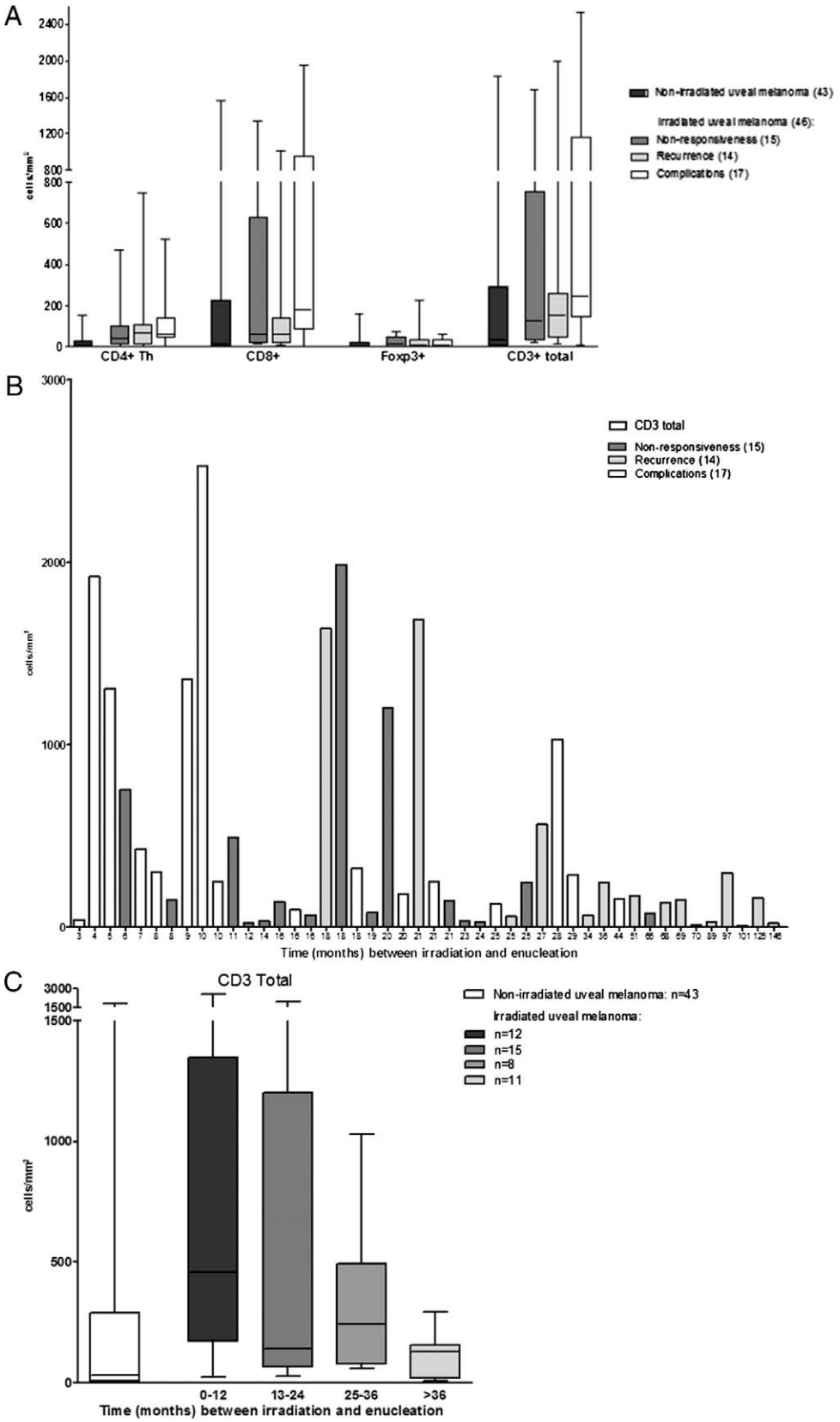


Figure 3. (**A**) Subtypes of tumor-infiltrating lymphocytes in uveal melanomas. Comparison of the number of intratumoral $CD4^+$ helper T cells, $CD8^+$ T cells, $Foxp3^+$ Tregs, and total T cells ($CD3^+$) in nonirradiated and irradiated uveal melanomas, in which the latter group is divided into three subgroups based on the cause of secondary enucleation. The *box-and-whisker plots* represent the 25th and 75th percentiles with the median and the minimum and maximum values. Significant differences were observed between nonirradiated and irradiated eyes for $CD4^+$ helper T cells ($P < 0.001$), $CD8^+$ T cells ($P = 0.002$), $Foxp3^+$ Tregs ($P = 0.02$), and the total amount of $CD3^+$ T cells ($P = 0.001$). No significant differences were observed between the subgroups of secondary enucleation. (**B**) Tumor-infiltrating lymphoyctes in uveal melanomas postirradiation. Variation in the

total number of intratumoral T cells in irradiated uveal melanomas with time. The columns represent the total number of $CD3^+$ T cells at a specific time point. The 46 time points represent the time interval between irradiation and enucleation for each of the 46 irradiated, secondarily enucleated eyes. (**C**) Tumor-infiltrating $CD3^+$ lymphocytes in uveal melanomas postirradiation. Comparison in the number of total T cells ($CD3^+$) in irradiated uveal melanomas, in which the latter group is divided into four subgroups based on the time (in months) between irradiation and enucleation. The *box-and-whisker plots* represent the 25th and 75th percentiles with the median and the minimum and maximum values. Significant differences were observed in the total number of intratumoral $CD3^+$ T cells between nonirradiated and irradiated eyes enucleated 0 to 12 months ($P = 0.001$) and 13 to 24 months ($P = 0.014$) after irradiation, but not at 25 to 36 months and >36 months.

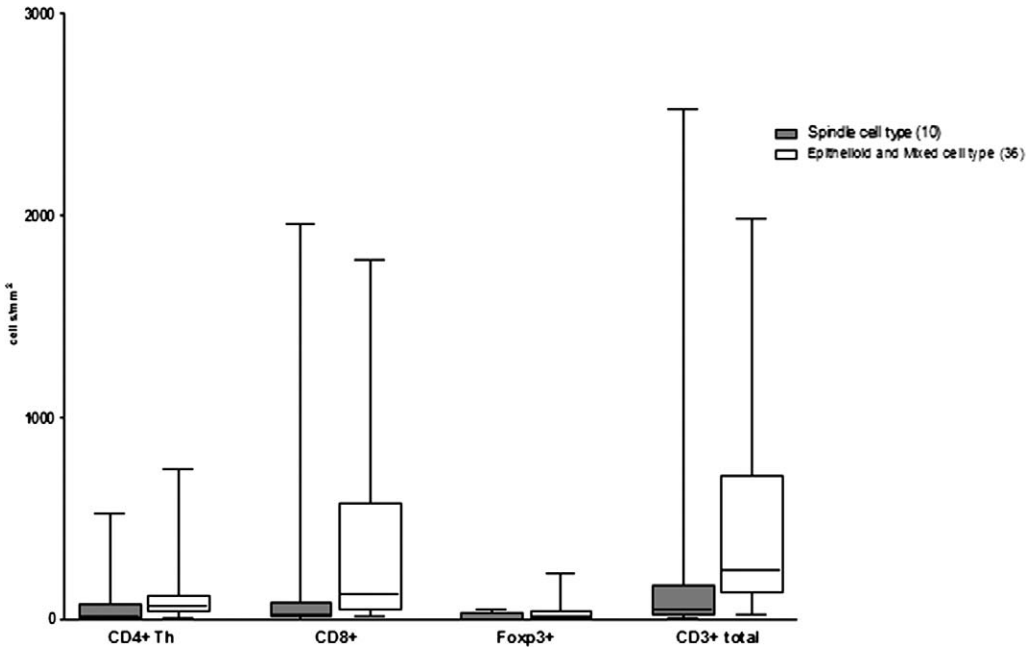


Figure 4. Subtypes of tumor-infiltrating lymphocytes in uveal melanomas and tumor cell types. Comparison of the number of intratumoral $CD4^+$ helper T cells, $CD8^+$ T cells, $Foxp3^+$ Tregs, and total T cells ($CD3^+$) in irradiated uveal melanomas containing spindle or epithelioid (mixed) cells. The *box-and-whisker plots* represent the 25th and 75th percentiles with the median and the minimum and maximum values. Significantly more intratumoral total $CD3^+$ T cells ($P = 0.011$), $CD8^+$ T cells ($P = 0.009$), $CD4^+$ helper T cells ($P = 0.018$), and $Foxp3^+$ Tregs ($P = 0.036$) were seen in tumors containing epithelioid cells compared with spindle cells.

A logical limitation is the fact that the largest group of irradiated tumors, that is, those that were successfully managed without local tumor control failure or complication, were not available for analysis, given that such eyes are not enucleated.

An association exists between the presence of epithelioid cells and increased numbers of intratumoral lymphocytes in irradiated eyes. Epithelioid cells may be responsible for providing a tumor microenvironment with different chemokines and cytokines, thereby attracting a different composition of immune cells. A previous study at our department showed that prognostically bad tumors (i.e., those with loss of one chromosome 3) in primarily enucleated eyes contained high numbers of tumor-infiltrating macrophages and lymphocytes.[20,21,27] One might thus expect that uveal melanomas enucleated due to local recurrence and nonresponsiveness would contain more inflammatory cells than those enucleated due to complications. Nonetheless, no significant differences were found between the subgroups of secondary enucleation. This is quite surprising, because several studies showed an increased tumor cell proliferative activity in uveal melanomas in the case of recurrent tumor growth, in comparison with irradiated uveal melanomas enucleated due to complications.[34–36] However, radiosensitive uveal melanomas, displaying monosomy of chromosome 3, greater tumor height, and an epithelioid cell type, regressed more rapidly postirradiation and were associated with a worse prognosis.[15,37,38] It may be that especially these melanomas led to locally uncontrolled tumors and ended up in our study. Therefore, we will in the future analyze tumor intrinsic properties such as monosomy of chromosome 3, to evaluate whether such tumors are especially present in our secondary-enucleation group, and are associated with an infiltrate.

In conclusion, the present study showed that prior irradiation leads to an increased amount of T lymphocytes, but not of macrophages. Moreover, the inflammatory infiltrate was associated with tumor characteristics, but not with the cause of secondary enucleation. Future studies involving information on biopsies obtained prior to irradiation and analysis of tissues after secondary enucleation may help to understand the changes in tumor behavior in locally uncontrolled uveal melanomas.

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