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Cytotoxic T Cells and their Activation Status are Independent Prognostic Markers in Meningiomas

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Abstract

Purpose: Clinically aggressive meningiomas (MGMs) are rare but treatment-resistant tumors in need for more effective therapies. Because tumor-infiltrating T lymphocytes (TILs) are essential for successful immunotherapy, we assessed TIL numbers and their activation status in primary (p-) and recurrent (r-) meningiomas and their impact on survival.

Experimental Design: Presence of TILs was analyzed in 202 clinically well-annotated cases ($n = 123$ pMGMs and $n = 79$ rMGMs) focusing on higher-grade meningiomas [$n = 97$ World Health Organization (WHO) °II, $n = 62$ WHO°III]. TILs were quantified by a semiautomated analysis on whole-tissue sections stained by multicolor immunofluorescence for CD3, CD8, FOXP3, and programmed cell death protein 1 (PD-1).

Results: Median T-cell infiltration accounted for 0.59% TILs per total cell count. Although there were no significant WHO°-dependent changes regarding helper ($CD3^+CD8^-FOXP3^-$) and cytotoxic ($CD3^+CD8^+FOXP3^-$) TILs in pMGMs, higher number of cytotoxic TILs were associated with an improved progression-free survival (PFS) independent of prognostic confounders. rMGMs were characterized by lower numbers of TILs in general, helper, and cytotoxic TILs. The additional analysis of their activation status revealed that a proportion of PD-1^+CD8^+ TILs within the TIL population was significantly decreased with higher WHO grade and in rMGMs. Furthermore, lower proportions of PD-1^+CD8^+ TILs were associated with inferior PFS in multivariate analyses, arguing for PD-1 as activation rather than exhaustion marker.

Conclusions: We identified higher numbers of $CD3^+CD8^+FOXP3^-$ TILs and proportions of PD-1–expressing $CD3^+CD8^+FOXP3^-$ TILs as novel biomarkers for better survival. These findings might facilitate the selection of patients who may benefit from immunotherapy and argue in favor of an intervention in primary rather than recurrent tumors.

Introduction

Meningiomas (MGMs) are primary tumors of the central nervous system (CNS), originating from the meningeal coverings of the spinal cord and the brain (1). With an incidence rate of 8.14/100,000 in the United States, meningiomas constitute 36.8% of all primary CNS neoplasms. Meningiomas are commonly observed in patients with advanced age, while women appear to be more frequently (2.3-fold) affected than men (2). Classification and grading of meningiomas is histologically defined by the World Health Organization (WHO; ref. 1). With a slow and often well-demarcated growth, the majority of

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Translational Relevance

Clinically aggressive meningiomas are comparably rare tumors and therefore are still underinvestigated. Therapeutic options are limited, resulting in a poor survival time of less than 2 years in patients suffering from anaplastic meningiomas. Hence, it is important to explore novel treatment modalities such as immunotherapy. For that, intratumoral T-cell infiltration is a prerequisite. However, studies quantifying intratumoral T cells in meaningful numbers of clinically aggressive meningiomas, at the onset of the disease and their changes after reoccurrence are largely missing. We addressed this need in a multicenter study using a large study sample of 202 primary and recurrent meningiomas including considerable numbers of rare higher grade meningiomas. Altogether, we identified higher numbers of cytotoxic and proportion of programmed cell death protein 1–expressing cytotoxic T cells to be prognostic for better progression-free survival independent of clinical confounders. Decrease of both T-cell populations in recurrent meningiomas mandates for the application of immunotherapy at an early timepoint of the disease.

meningiomas are benign WHO°I tumors (80%) and mostly curable by complete surgical resection. However, up to 5% of WHO°I meningiomas exhibit a more aggressive behavior with a tendency for local recurrence (2, 3). In contrast, less frequent, atypical WHO°II meningiomas (15%–20%) and anaplastic WHO°III meningiomas (1%–3%) feature an average 5-year recurrence rate of up to 40% in WHO°II and 50%–80% in WHO°III, respectively (2, 3). Accordingly, prognosis of patients with higher grade meningiomas remains poor with less than 2 years median survival in patients suffering from WHO°III tumors (4). Usually, treatment of WHO°I meningiomas consists of a radical tumor resection, whereas higher-grade lesions often require a combination of surgery and radiotherapy (5). Although successful in other tumor entities, chemotherapy in patients with meningioma has not shown convincing efficacy (6). Therefore, it is important to explore novel treatment modalities such as immunotherapy, which already has shown promising advances in other tumors (7–9). Generally, this treatment type relies on a tumor-specific infiltration of immune cells, which often is associated with a survival benefit (10–13). Moreover, in colorectal cancer, levels of tumor-infiltrating T lymphocytes (TILs) were shown to allow an even more precise survival prediction than the conventional UICC tumor–node–metastasis classification (11). In addition, TIL levels can also predict response to therapy (14). Thus, many studies focused on TILs in different tumor entities such as glioblastoma, but only few studies addressed their amount in meningiomas (13, 15, 16). Furthermore, due to the higher prevalence of WHO°I meningiomas, most groups predominantly investigated TILs in lower-grade meningiomas, whereas higher grade meningiomas usually are underrepresented and detailed analyses of changes in recurrent tumors are missing so far (17–19).

However, not only TIL quantities are crucial, but also their phenotype and functional state are important (17). To control T-cell activation, binding of tumor antigens to the T-cell receptor (TCR) initiates the upregulation of costimulatory (CD28) and coinhibitory molecules such as programmed cell death protein 1 (PD-1) or cytotoxic T-lymphocyte–associated protein 4 (CTLA-4) (20, 21). In addition, tumor cells can directly impair T-cell immune responses by secreting IL10 or TGF-β or through a high expression of the ligands of coinhibitory molecules such as B7-1/B7-2 (ligand for CTLA-4) and PD-L1 (ligand for PD-1; ref. 22). For example, PD-1/PD-L1 interaction can transmit inhibitory signals and induce anergy, exhaustion, and apoptosis of T cells (23). Because of their key role in the regulation of T-cell responses, PD-1 and CTLA-4 are known as immune checkpoint molecules and as such are already used for targeted therapy of several tumors to maintain the activation status of TILs and to enhance immune responses against tumor cells (15, 24, 25). Thereby, it has also been shown that response rate to PD-1 antibodies was associated with expression levels of PD-L1 (26).

Initially, PD-1 and CTLA-4 were regarded as markers for exhausted T cells (27, 28). However, findings in melanoma suggest a dual role of PD-1 in malignancies because the expression of PD-1 has not been solely affiliated to hypo-responsive (exhausted) T cells with an impaired functionality, but also mirrored the repertoire of clonally expanded autologous tumor-reactive $CD8^+$ T cells (29, 30). Up to now, phenotypical and functional diversity of TILs in meningiomas and their influence on patient outcome are poorly explored (18, 31). In this multicenter study, we compiled a large cohort of 202 primary (pMGM) and recurrent (rMGM) meningioma tissues, including a considerable number of higher grade meningiomas. Through a comprehensive analysis of frequency, phenotype, and functional status of TILs in meningiomas, we demonstrate higher numbers and activation of $CD3^+CD8^+FOXP3^-$ cytotoxic T cells as independent prognostic factors. Furthermore, our analysis revealed a significant decrease of effector T cells in recurrent tumors, which may reflect an increased immunosuppression. Interestingly, due to a positive impact on survival, expression of PD-1 on cytotoxic T cells seems to reflect an activated rather than an exhausted T-cell phenotype in meningiomas.

Materials and Methods

Samples and patient characteristics

A total of 202 meningioma specimens were obtained from patients undergoing surgical resection in the Departments of Neurosurgery at University Hospitals Heidelberg, Bonn, Hamburg, Homburg, Frankfurt, and Würzburg, Germany as part of the FORAMEN initiative (32). Institutional review boards at each medical facility approved this study in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients. This multicenter approach enabled the analysis of an extensive study sample covering all WHO grades (WHO°I $n = 43$; WHO°II $n = 97$; and WHO°III $n = 62$; Supplementary Fig. S1). Samples used for immunofluorescence staining were immediately snap-frozen after surgery and stored at −80 °C until processing. Tumor cell content ≥60% was confirmed for all samples by experienced neuropathologists (A. von Deimling and F. Sahm). Clinical data were collected using a detailed questionnaire and are summarized in Table 1.

Staining

Multicolor immunofluorescence staining was performed on acetone-fixed cryosections (5–7 μm). To quantify T-cell subpopulations, a combination of primary antibodies specific for CD3 (rabbit, 1:100, A0452, Dako), CD8 (rat, 1:100, ab60076, Abcam),

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Table 1. Clinical data overview of primary and recurrent meningiomas

n Total variable	pMGMs, 123 tumors			rMGMs, 79 tumors		
	n	**Patients (%)**	**Median (range)**	***n***	**Patients (%)**	**Median (range)**
Sex						
Male	48	39.02		44	55.70	
Female	75	60.98		35	44.30	
Age at 1st diagnosis (y)			60.7 (24.0–87.6)			55.0 (18.0–86.5)
WHO grade + clinical subgroup						
WHO°I	33	26.83		10	12.66	
WHO°II	64	52.03		33	41.77	
WHO°III	26	21.14		36	45.57	
Subtype						
WHO°I						
Transitional	12	36.36		9	90.00	
Fibroblastic	8	24.24				
Meningothelial	8	24.24				
Angiomatous	1	3.03				
Secretory	1	3.03				
NA	3	9.09		1	10.00	
WHO°II						
Atypical	53	82.81		26	78.79	
NA	11	17.19		7	21.21	
WHO°III						
Anaplastic	13	50.00		30	83.33	
Rhabdoid	1	3.85		1	2.78	
Papillary	2	7.69				
NA	10	38.46		5	13.89	
Localization						
Convexity	52	42.28		22	27.85	
Cranial base	29	23.58		17	21.52	
Falx	14	11.38		15	18.99	
Parasagittal	18	14.63		15	18.99	
Tentorial	4	3.25		4	5.06	
Other multiple or NA	6	4.88		6	7.59	
Resection grade						
Simpson 1	69	56.10		30	37.97	
Simpson 2	33	26.83		20	25.32	
Simpson 3	16	13.01		13	16.46	
Simpson 4	4	3.25		13	16.46	
Simpson 5	0	0.00		1	1.27	
NA	1	0.81		2	2.53	
Further treatment after resection postoperative radiotherapy						
Yes	35	28.46		34	43.04	
No	82	66.67		42	53.16	
NA	6	4.88		3	3.80	
Postoperative chemotherapy						
Yes	0	0.00		9	11.39	
No	120	97.56		66	83.54	
NA	3	2.44		4	5.06	
PFS (months)						
WHO°I	33		100.4 (0.2–169.1)			
WHO°II	64		68.2 (0.0–213.6)			
WHO°III	26		19.0 (0.0–427.1)			
OS (months)						
WHO°I	33		142.4 (23.9–182.2)			
WHO°II	64		86.7 (0.0–213.6)			
WHO°III	26		28.4 (0.0–427.1)			
Follow-up time (months)						
WHO°I	30		130.0 (0.2–182.2)	9		149.6 (117.0–195.8)
WHO°II	59		87.3 (0.0–213.6)	28		134.5 (26.0–346.5)
WHO°III	25		27.3 (0.5–427.1)	35		87.9 (24.3–538.9)

Abbreviation: NA, not available.

and FOXP3 (mouse, 1:20, ab20034, Abcam) were used as described previously (ref. 13; Supplementary Fig. S1). Briefly, primary antibodies were diluted with Antibody Diluent (Dako). To determine T-cell activation, we used a combination of CD3, CD8, and PD-1 (mouse, 1:200, ab52587, Abcam) antibodies. Secondary antibodies were used as follows: anti-rabbit AlexaFluor647 (1:200, Thermo Fisher Scientific), anti-rat AlexaFluor488 (1:600, Thermo Fisher Scientific), and anti-mouse AlexaFluor555 (1:400, Thermo Fisher Scientific) for staining of CD3, CD8, and FOXP3 and anti-rabbit AlexaFluor555 (1:200, Thermo Fisher Scientific), anti-rat AlexaFluor488 (1:200, Thermo Fisher Scientific), and anti-mouse AlexaFluor647 (1:200, Thermo

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Fisher Scientific) for staining of CD3, CD8, and PD-1. Antibodies were incubated for 1 hour. Secondary antibodies were diluted with DPBS-containing DAPI (Invitrogen) at 1:1,000 to stain nuclei. Human tonsil tissue and isotype-matched antibodies (rabbit IgG, x0936, Dako; rat IgG2b, 14-4031, eBioscience; and mouse IgG1, ab91353, Abcam) served as positive and negative controls, respectively.

Image analysis

High-resolution automated multiple image alignments of whole-tissue sections were acquired using a 20× objective on an Olympus IX51 microscope equipped with a XM10 Camera (Olympus). The Olympus cellSens Dimension Software (version 1.9) was used for image acquisition. Automatic detection and context-based quantification of T-cell infiltration by immunofluorescence markers was performed by the StrataQuest Software (version 5.0.1, TissueGnostics GmbH). Regions of interest (ROI) were manually defined depending on histology and quality of the section to exclude adjacent normal brain or necrotic areas. ROIs were drawn in the slide overview using software-based mark-up tools. Qualification and quantification was solely performed in areas with high tumor cell content (≥60%).

Automatically detected cells were visualized in scattergrams and gated according to defined gating schemes for the expression of nucleic and cell surface markers (Supplementary Fig. S2). Cutoff between positive and negative gated cells was manually validated by backward gating. To enable robust and reliable cell quantification, strict parameters by means of nuclear size, staining intensity, and background threshold were defined. Cell nuclei were detected on the basis of DAPI staining and used as origin to generate a growing mask over the cytoplasm to the cell membrane. On the basis of this mask, T cells were analyzed regarding cell surface expression of CD3 and CD8 and a nuclear colocalization of FOXP3 and DAPI (Supplementary Fig. S2A) or the cell surface expression of CD3, CD8, and PD-1 (Supplementary Fig. S2B). For statistical analysis, number of cells in percent of total cell count (% TCC, defined as total number of $DAPI^+$ nuclei without further distinction of cell types), as well as proportion of cells in percent of $CD3^+$ T cells (% of $CD3^+$) were calculated.

Statistical analysis

Data were analyzed by R (Ver.3.4.1). Differences between two groups (WHO°I vs. °II; WHO°II vs. °III; WHO°I vs. °III; pMGMs vs. rMGMs; high vs. low expression, divided by the median) were calculated using the nonparametric Mann–Whitney–Wilcoxon test. $P < 0.05$ was considered significant. Data presented in boxplots are based on median values (outliers not drawn), unless otherwise specified. Kaplan–Meier plots were used to visualize survival estimates, whereas comparison of survival differences was done by log-rank test. Progression-free and overall survival analyses (PFS and OS) were only performed on patients with pMGM without any prior radio- or chemotherapy experiencing a future tumor recurrence, tumor-related death, or with a follow-up time of at least 60 months. Variables reaching significance in univariate analyses were further included in a multivariate model to assess the independence of clinical covariates. To further select unsupervised variables with the highest power in survival prediction, we used the "Least Absolute Shrinkage and Selection Operator" (lasso) regression implemented in the R package glmnet (33). The categorical variables sex, Simpson grade, and WHO grade were transformed into numeric values. These representations of the categorical variables and the numeric infiltration variables were normalized by the scale function. To optimize the model for lowest partial likelihood deviance, we used leave-one-out cross validation (loo-cv). To prevent overfitting, we selected Lambda within one SD lower than the minimum of the partial likelihood deviance (Lamda.1se) to define the optimal set of covariates (33).

Results

Higher numbers of cytotoxic T cells are an independent prognostic factor for improved PFS

To study the infiltration of meningiomas by different T-cell phenotypes, we first focused on pMGMs (n = 123; 33 WHO°I, 64 WHO°II, and 26 WHO°III tumors; Table 1). Median age of patients was 60.7 years at the time of first diagnosis (range, 24.0–87.6 years) with a female preponderance (75/123 women).

For the simultaneous detection of effector and regulatory T cells (Treg), we used multicolor immunofluorescence staining with a well-established combination of antibodies against CD3, CD8, and FOXP3 (Fig. 1A; Supplementary Fig. S3A) enabling the identification of cytotoxic T cells ($CD3^+CD8^+FOXP3^-$), Th cells ($CD3^+CD8^-FOXP3^-$), and Tregs ($CD3^+CD8^-FOXP3^+$; ref. 13; Supplementary Fig. S1). To achieve reliable and objective results, scanning and quantitative analysis of whole-tissue sections (median size: 12.17 mm^2; range, 2.47–51.77 mm^2) was performed in a semiautomated set-up at a single-cell level with subsequent phenotypic hierarchical clustering in a flow cytometry–like manner (Supplementary Fig. S2A).

Quantitative analysis revealed an extremely heterogeneous T-cell infiltration, ranging from 0.01%–24.88% $CD3^+$ T cells relative to the TCC (median = 0.59% T cells; Fig. 1B). However, within a tumor tissue we found a rather homogeneous distribution of helper and cytotoxic T cells in tumors of all WHO grades with a substantial proportion located around vessels or migrating deeper into the tumor tissue as single cells or as little aggregates (Supplementary Fig. S3A). When assessing general T-cell numbers in different WHO grades, we did not find a significant change of $CD3^+$ TILs in higher WHO grades (Fig. 1C), although this has been described in other brain tumors such as gliomas (13). By subdividing TILs into the common T-cell phenotypes, we did not observe significant differences as for the numbers of helper and cytotoxic T cells in different WHO grades (Fig. 1D and E). When looking at the proportion of both subpopulations within the entire T-cell population, we found that more Th than cytotoxic T cells infiltrate meningioma tissues (WHO°I, 65.7%; WHO°II, 75.5%; and WHO°III, 77.3% Th cells; Supplementary Fig. S3B).

When quantifying Tregs by additional nuclear FOXP3 staining (Fig. 1A), we detected Tregs in the majority of pMGMs (Supplementary Fig. S3F). Although the quantity was comparably low, we observed a significant increase of Tregs from WHO°I to °III ($P = 0.027$; Fig. 1F).

We next explored the impact of TIL numbers in pMGMs on patient outcome. Analyses were performed on patients with a therapy-naïve pMGM, undergoing complete tumor resection (Simpson 1–3). When including all WHO grades, we observed a significantly improved PFS for patients with a higher infiltration of cytotoxic T cells ($P = 0.038$) but no significant impact on OS (Fig. 1G; Supplementary Fig. S4A and S4B). Subsequent multivariate analysis including clinically relevant covariates age, sex, and WHO grade confirmed infiltration of cytotoxic T cells as an

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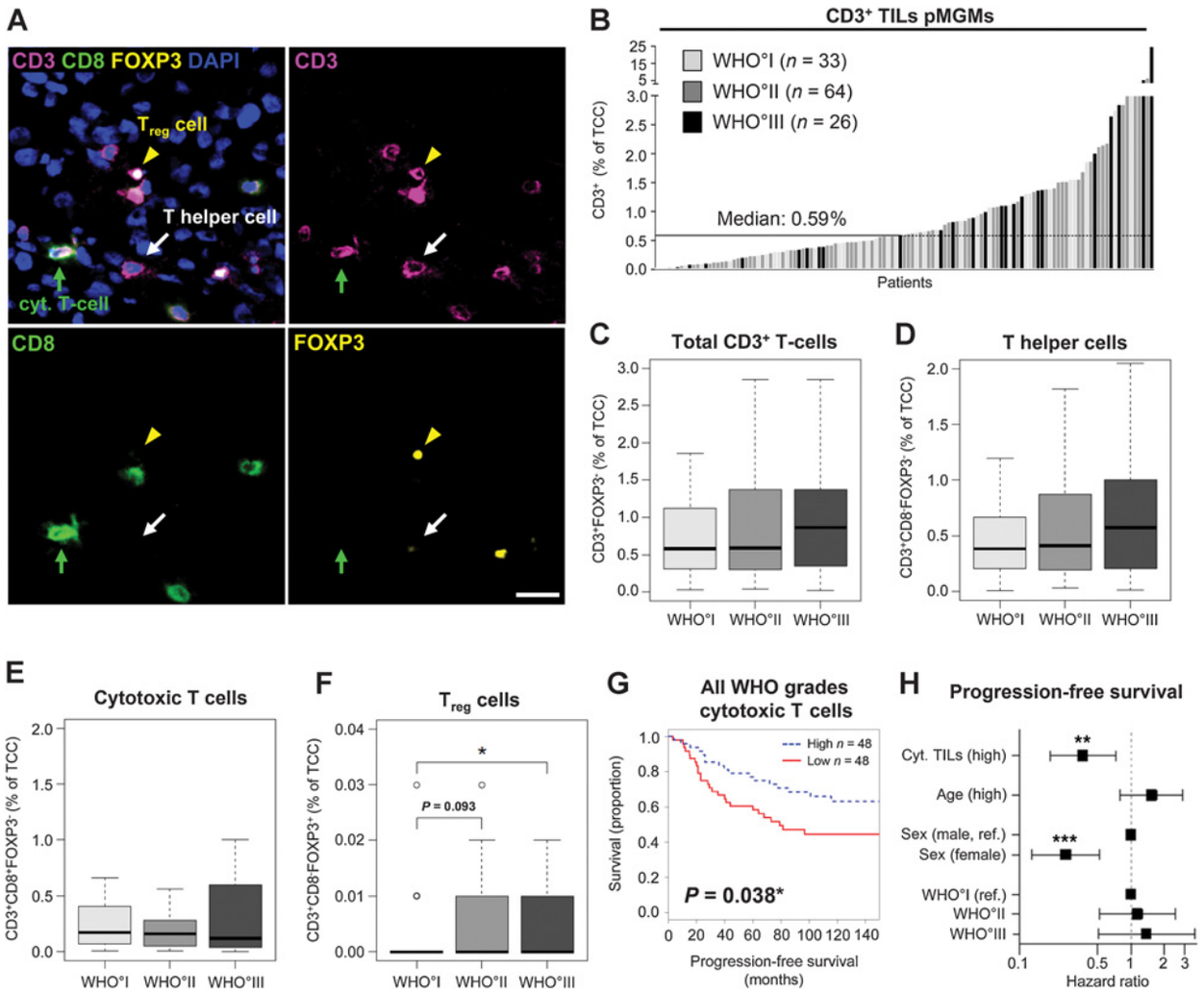

Figure 1.

Quantification of TILs in pMGMs. **A,** Representative fluorescence images and overlay of a pMGM stained for CD3 (purple), CD8 (green), FOXP3 (yellow), and DAPI (blue). Green arrow depicts a cytotoxic T cell while white arrow demarcates a Th cell. Yellow arrowhead points to a $CD4^+$ regulatory T cell. Scale bar, 20 μm. **B** and **C,** Quantification of $CD3^+$ TILs in WHO°I–III pMGMs. Subclassification of TILs into effector (**D** and **E**) and regulatory TILs (**F**). **G,** Kaplan–Meier curve shows PFS of patients with high (blue) and low (red) infiltration of cytotoxic T cells. **H,** Multivariate survival analysis for PFS including high levels of cytotoxic T cells, patient age, sex, and WHO grade. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). cyt., cytotoxic; ref., reference.

independent prognostic factor for PFS ($P = 0.004$; Fig. 1H; Supplementary Table S1).

Taken together, pMGMs of all WHO grades exhibit comparable levels of TILs, a rare infiltration of Tregs, and vary in the composition of effector T cells. Higher numbers of cytotoxic T cells are associated with an improved PFS independent of patient age, sex, and WHO grade.

Significant decrease of TILs in rMGMs

Next, we interrogated whether the recurrence of a tumor leads to changes in TIL composition and analyzed 79 rMGMs (10 WHO°I, 33 WHO°II, and 36 WHO°III tumors; Table 1; Supplementary Fig. S2A). Median area of rMGM tissues was 10.74 mm^2 (range: 0.84–35.27mm^2).

Like pMGMs, rMGMs showed a variable proportion of $CD3^+$ TILs in all WHO grades, ranging from 0.01% to 5.96% TILs of TCC (median = 0.33%; Fig. 2A). We observed decreasing amounts of $CD3^+$ TILs with higher WHO grade especially when comparing TIL numbers of WHO°II with °III tumors ($P = 0.031$; Fig. 2B). This decrease might be mainly due to decreasing numbers of cytotoxic T cells ($P = 0.005$) rather than Th cells ($P = 0.051$; Fig. 2C and D). Like for pMGMs, Th cells were the predominant T-cell population in rMGMs (Supplementary Fig. S3C). We also investigated the role of Tregs in rMGMs, but if at all could only find very few cells reaching a median proportion of not more than 0.01% (WHO°II) of TCC (Fig. 2E).

Notably, comparison of TIL levels in pMGMs and rMGMs demonstrated a significantly reduced infiltration of all effector T-cell phenotypes in rMGMs (Fig. 2F) but not of Tregs, which however, were hardly detectable (Supplementary Fig. S3G). Generally, high-grade rMGMs displayed lowest infiltration of

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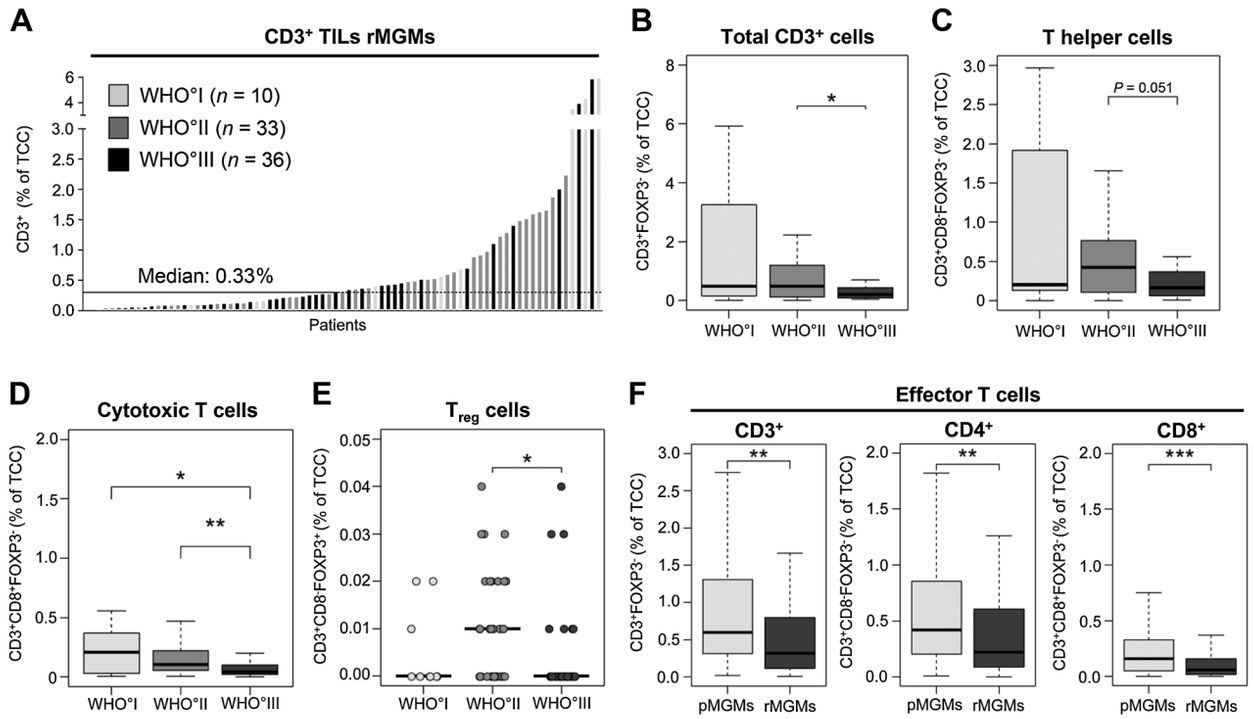

Figure 2.

Quantification of TILs in recurrent meningiomas. **A** and **B,** Analysis of CD3$^+$ TILs in rMGMs of different WHO grades. Subclassification of TILs into effector (**C** and **D**) and regulatory (**E**) TILs. **F,** Comparison of effector TILs in primary ($n = 123$) and recurrent ($n = 79$) meningiomas. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

T cells suggesting profound variances in the tumor microenvironment during tumor progression.

TIL levels are independent of tumor localization

We next asked whether tumor localization has an impact on TIL levels in meningiomas. We observed a higher infiltration of CD3$^+$ T cells ($P = 0.019$) and Th cells (P = 0.037) in pMGMs located in the parasagittal region as compared with pMGMs resected in the cranial base area (Supplementary Fig. S5A). Furthermore, no location-dependent differences were found in pMGMs or rMGMs (Supplementary Fig. S5B), indicating that localization of meningiomas has no major influence on infiltration of T cells.

Higher proportions of PD-1–expressing TILs are associated with an improved PFS

To further increase our knowledge on the functionality of TILs, we performed a costaining for PD-1 known to regulate immune responses and suggested as a biomarker for the detection of tumor-specific T cells (34). Because FOXP3$^+$ Tregs were rarely detected, FOXP3 was replaced by PD-1 in multicolor staining and thus analyzed in addition to the previous T-cell populations (Fig. 3A; Supplementary Fig. S2B).

In pMGMs, we detected PD-1–expressing TILs in almost every tissue (119/121; Fig. 3B). Quantitative analysis revealed a smooth distribution with a median proportion of 28.11% PD-1–expressing T cells within the CD3$^+$ TIL population (range = 0.00%–67.12% of all CD3$^+$ TILs). When looking at PD-1 expression in tumors of different WHO grades, a significant reduction of the proportion of PD-1$^+$ TILs with increasing WHO grade was found (WHO°I vs. °III: $P = 0.007$; WHO°II vs. °III: $P = 0.006$; Fig. 3C). Although the majority of PD-1–expressing TILs belonged to the cytotoxic T-cell phenotype (Supplementary Fig. S3D), decreasing proportions of PD-1–expressing TILs were found for both T-cell subpopulations (Th cells: WHO°I vs. °III, $P = 0.040$ and WHO°II vs. °III, $P = 0.020$; cytotoxic T cells: WHO°I vs. °III, $P = 0.011$ and WHO °II vs. °III, $P = 0.015$; Fig. 3D and E).

When exploring the biological relevance of the proportion of PD-1$^+$ T cells for patient outcome, we found an association of high proportions of tumor-infiltrating PD-1–expressing T cells with a significantly improved PFS ($P = 0.009$; Fig. 3F). Similarly, high proportions of PD-1–expressing cytotoxic T cells were associated with prolonged PFS ($P = 0.029$; Fig. 3G). Regarding OS, with $P = 0.050$, we failed to reach significance (Fig. 3H).

In summary, we identified PD-1–expressing T cells in almost all pMGMs, whereby cytotoxic T cells constitute the majority of PD-1–expressing T cells. High proportions of PD-1–expressing CD3$^+$ and cytotoxic T cells were found to be prognostic for a beneficial PFS of patients with pMGM.

Lower proportions of PD-1$^+$ T cells in rMGMs

Next, we determined the impact of the proportion of PD-1–expressing TILs in rMGMs. They were detected in almost all tissues (median infiltration, 21.34% PD-1–expressing T-cells of all CD3$^+$ T cells; Fig. 4A). As for pMGMs, almost all PD-1–expressing TILs belonged to the cytotoxic T-cell subpopulation (Supplementary Fig. S3E). Therefore, we focused on this T-cell subpopulation. When comparing the proportion of PD-1–expressing TILs

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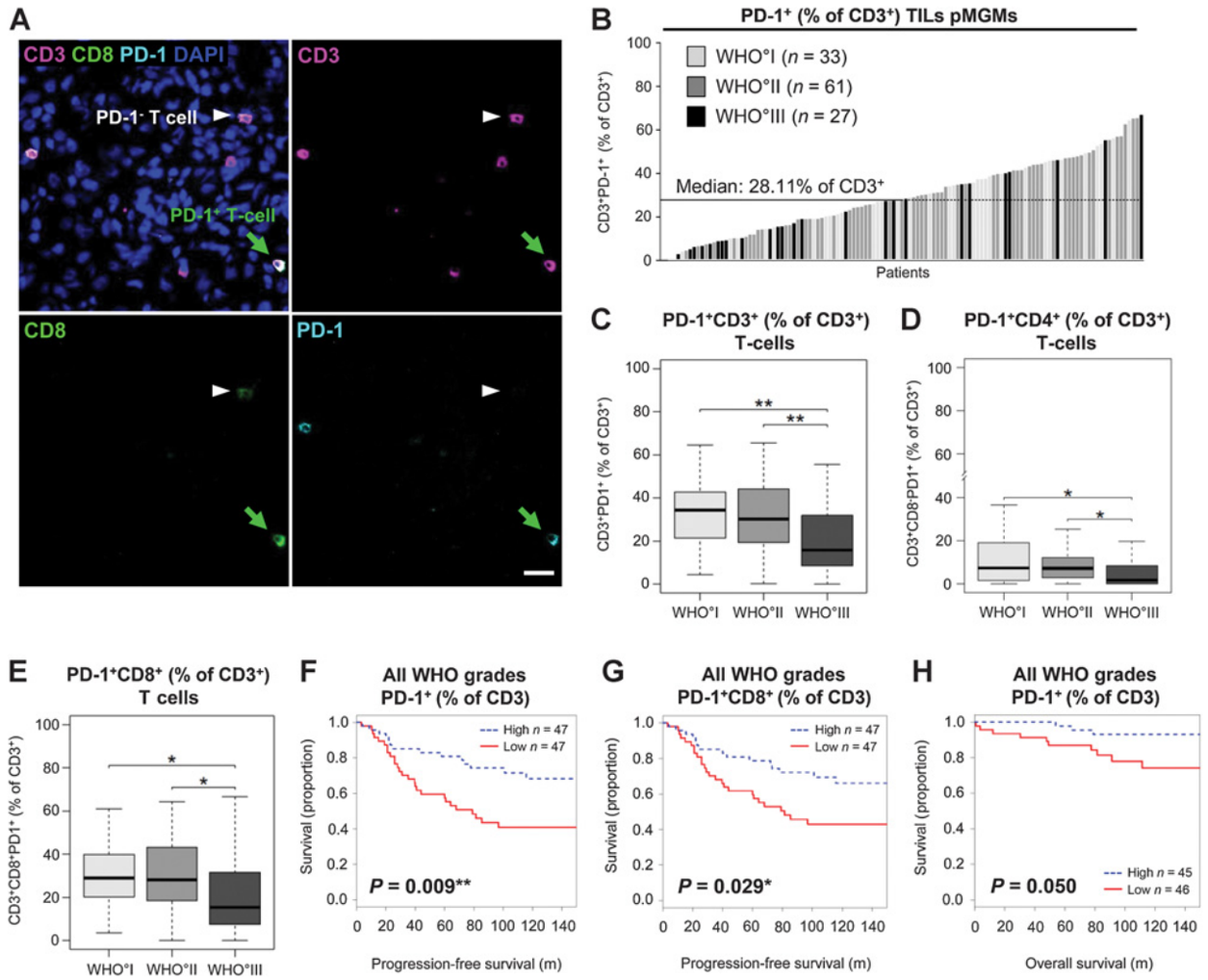

Figure 3.

Proportion of infiltrating PD-1–positive T cells in pMGMs. **A,** Representative fluorescence images and overlay of a pMGM stained for CD3 (purple), CD8 (green), PD-1 (turquoise), and DAPI (blue). Green arrow depicts a PD-1$^+$ cytotoxic T cell while white arrowhead demarcates a PD-1$^-$ cytotoxic T cell. Scale bar, 20 μm. **B** and **C,** Analysis of proportion of CD3$^+$PD-1$^+$ TILs in pMGMs of different WHO grades. Proportion of infiltrating PD-1$^+$ Th cells (**D**) and PD-1$^+$ cytotoxic T cells (**E**) in tumors of different WHO grades. Kaplan–Meier curves show PFS (**F** and **G**) and OS (**H**) of patients with high (blue) and low (red) proportion of infiltration of PD-1$^+$ T cells or PD-1$^+$ cytotoxic T cells separated on the basis of the median. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$; m, months).

between pMGMs and rMGMs, we found significantly reduced proportions of PD-1–expressing cytotoxic T cells during tumor progression ($P = 0.002$; Fig. 4B) and a WHO grade–dependent decrease as in pMGMs (WHO°I vs. °III, $P = 0.034$; WHO°II vs. °III, $P = 0.030$; Fig. 3E, 4C). We further analyzed the infiltration of PD-1–expressing cytotoxic T cells in corresponding pairs of primary and recurrent tumors ($n = 15$). Interestingly, five of six patients with increasing WHO grade in their recurrent tumor showed a marked decrease of the proportion of PD-1$^+$ cytotoxic T cells, whereas in the group of patients with no change in the WHO grade ($n = 9$), approximately the same number of patients showed an increase, as well as a decrease of PD-1$^+$ cytotoxic T cells (Fig. 4D and E).

Altogether, proportion of PD-1–expressing cytotoxic TILs decreased in rMGMs in general and in particular with higher WHO grade. This further substantiates our finding of a positive influence of PD-1–expressing T cells on patient survival.

Proportion of PD-1–expressing T cells is an independent prognostic factor for favorable PFS

Finally, we queried the importance of the proportion of PD-1–expressing TILs in an unsupervised multivariate analysis (Fig. 5). To overcome the problem of often used Cox proportional hazard models in assessing the influence of multiple highly correlated variables, we used lasso linear regression in combination with loo-cv. We selected altogether 13 T-cell variables (amount of T cells, cytotoxic T cells, Th cells, Tregs, PD-1–expressing T cells, PD-1–expressing cytotoxic T cells, PD-1–expressing Th cells, percentage of cytotoxic T cells of all T cells,

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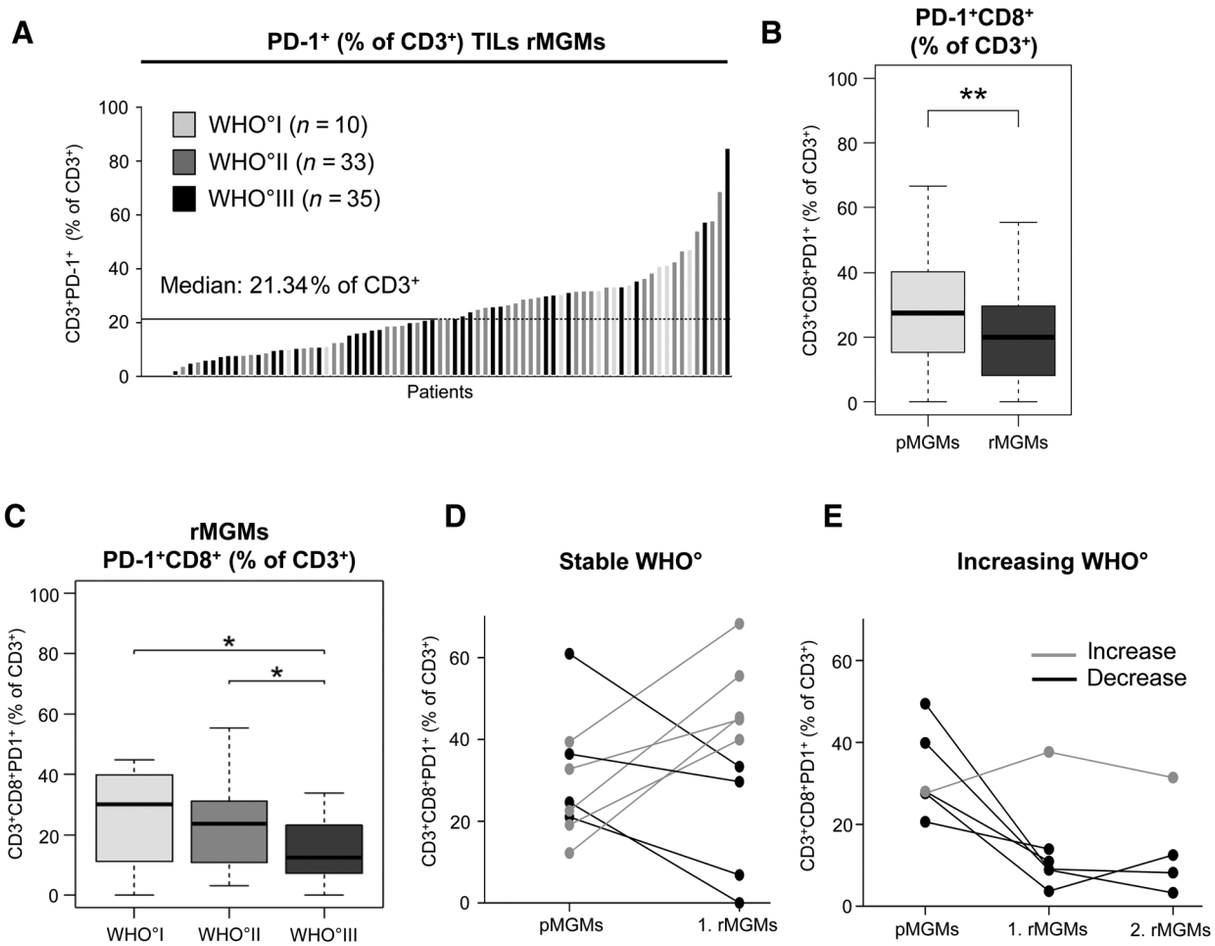

Figure 4.

Proportion of PD-1–expressing T cells decreases in rMGMs and higher WHO grades. **A,** Analysis of proportion of $CD3^+PD\text{-}1^+$ TILs in rMGMs of different WHO grades. **B,** Proportion of infiltrating $PD\text{-}1^+$ cytotoxic T cells in primary ($n = 121$) and recurrent ($n = 78$) meningiomas of all WHO grades. **C,** Proportion of infiltrating $PD\text{-}1^+$ cytotoxic T cells in tumors of different WHO grades. Proportion of infiltrating $PD\text{-}1^+$ cytotoxic T cells in corresponding pairs of primary and recurrent tumors, of which nine pairs showed no change in the WHO° (**D**) and six pairs an increasing WHO° (**E**) at recurrence. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$).

percentage of Th cells of all T cells, percentage of regulatory T cells of all T cells, percentage of PD-1–expressing T cells of all T cells, percentage of PD-1–expressing T cells of all cytotoxic T cells, and percentage of PD-1–expressing T cells of all Th cells) and four clinical variables (age at diagnosis, WHO grade, sex, and Simpson grade) as input for the algorithm. By testing different Lambda values that evaluate variables and thereby stepwise removing unimportant variables, we found a point where the loo-cv–estimated prediction error was minimal (Fig. 5A and D; Supplementary Fig. S4C and S4D; lambda.min). To prevent overfitting toward the analyzed study sample, we used a slightly higher Lambda value (lambda.1se) resulting in a more simplified model with two (PFS) and four (OS) prediction variables (Fig. 5B and E), demonstrating that high percentage of PD-1–expressing $CD3^+$ T cells ($P = 0.008$) and gender ($P < 0.001$) are independent predictors of longer PFS (Fig. 5C; Supplementary Table S2). On the contrary, increased patient age was highly prognostic for shorter OS (Fig. 5F; Supplementary Table S2). With a nonsignificant P value of 0.076, higher $PD\text{-}1^+$ TIL numbers failed to prognosticate better OS.

In summary, high proportion of PD-1–expressing TILs predicts improved PFS independent of known clinical covariates.

Discussion

In tumors other than meningiomas, there is a large body of evidence that T-cell infiltration and their activation status predict survival or response to chemotherapy and immune checkpoint inhibitors (35). Because of the comparably lower frequency of higher grade and clinically aggressive meningiomas and, because primary and recurrent tumors have not been analyzed separately so far, respective data for this tumor entity is largely missing. Moreover, profound knowledge about longitudinal changes in T-cell infiltration and activation is important for the optimal timing of immunotherapy. To address these important questions, we analyzed a comprehensive cohort of 123 pMGMs and 79 rMGMs and identified higher numbers of cytotoxic T cells

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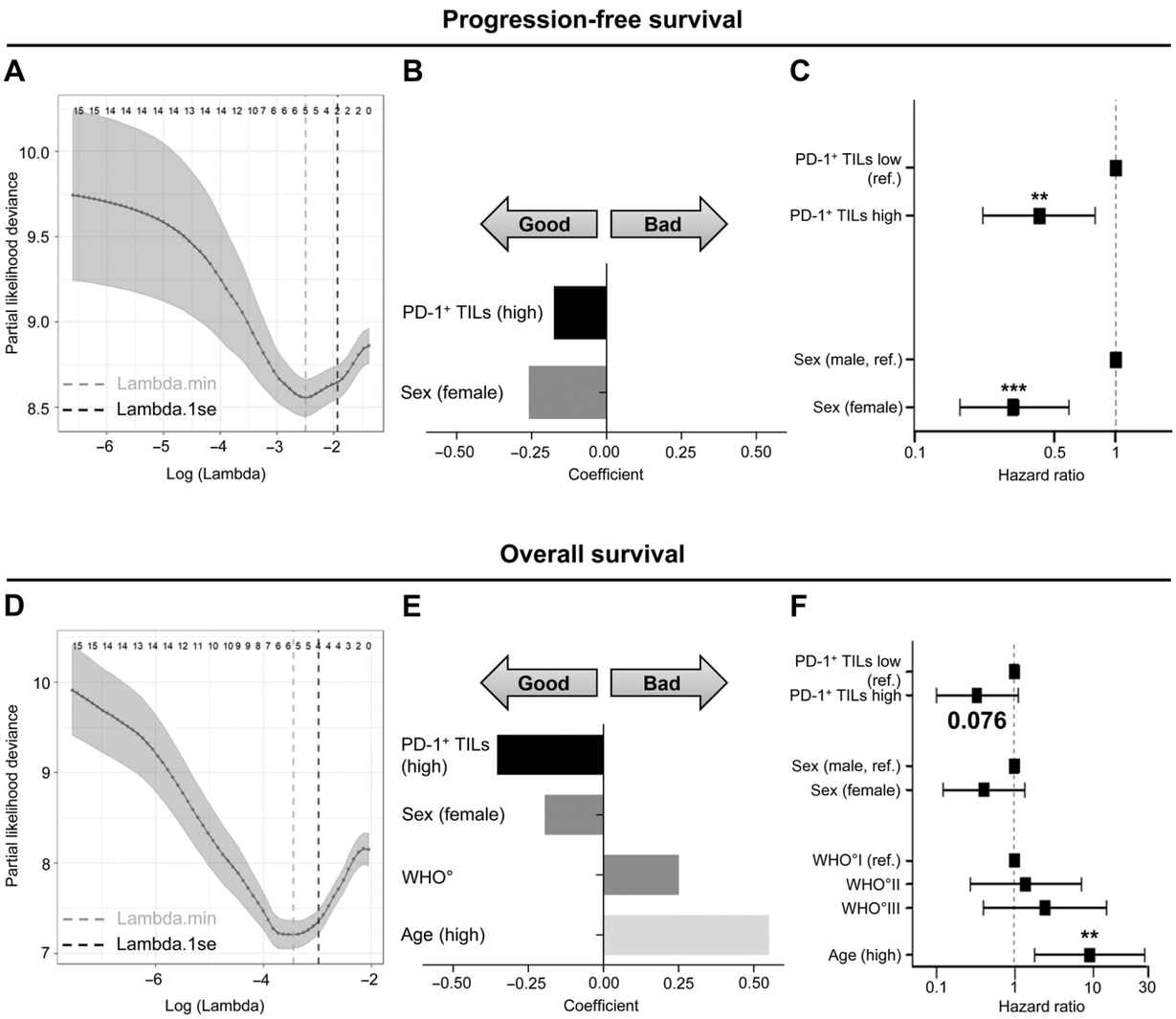

Figure 5.

Proportion of PD-1–expressing T cells as predictor of prolonged PFS. Lasso regression for unsupervised multivariate analysis selecting the most important TIL features and clinical variables for PFS (**A–C**) and OS (**D–F**). Gray shades are the 5%–95% confidence interval determined by leave-one-out cross-validation. Asterisks indicate significant differences (**, $P < 0.01$; ***, $P < 0.001$; ref., = reference).

and higher proportions of PD-1–expressing TILs as independent prognostic factors for better patient survival. As another evidence for the importance of TILs in meningiomas, we discovered that numbers of effector TILs and the proportion of PD-1–expressing TILs are significantly lowered in rMGMs.

The use of a large and clinically well-annotated multicenter study sample enriched for rare higher grade and rMGMs is a major strength of our work, allowing us to interrogate survival associations of different TIL types and longitudinal changes of TIL composition in meningiomas. Accordingly, we present important findings including the identification of tumor-infiltrating cytotoxic T-cells and the proportion of PD-1-expressing cytotoxic T-cells as independent prognostic markers for the survival of meningioma patients. Furthermore, the use of an observer-independent, semiautomated system assessing TIL numbers on whole tissue sections rather than small tissue microarray cores corroborates the robustness of our observations.

Although infiltration with cytotoxic T cells as favorable prognostic marker has already been reported for lung and breast cancer (36, 37), there are only very few reports about TILs in meningiomas. Most of these focused on TIL quantification and some assessed the infiltration of other immune cells such as macrophages (17–19, 38). They conclude that besides the most prevalent tumor-associated macrophages, TILs are one of the most frequent immune cell populations in meningiomas (17). Moreover, most studies focused on WHO°I tumors, while less frequent higher grade meningiomas are notably underrepresented (17–19, 38). Only few studies included meaningful numbers of higher grade meningiomas but primarily focused on PD-L1 expression as important target for immune checkpoint inhibitors (15, 16). Yet, one study

confirms our observation of decreased cytotoxic T cells and PD-1–expressing T cells in higher grade meningiomas, but conflicts with our data, because it claims a similar decline for Th cells (17–19, 38). This contradiction might be explained by a lacking distinction between data obtained from pMGMs and rMGMs because our data revealed such a reduction only in rMGMs. Another reason hindering a direct comparison with our results is the scoring system employed, dividing the samples median based into low or high T-cell infiltration (17–19, 38), which only allows comparison of findings within a given dataset. However, as a further strength, our analysis is based on the absolute numbers of T cells detected per TCC and the quantification of the proportion of PD-1–expressing TILs within the population of infiltrating T cells. These objective parameters do not depend on a distinct study sample and can easily be employed by other investigators. Furthermore, it constitutes an important prerequisite to apply these novel biomarkers in future routine settings with digital imaging increasingly used in modern pathology especially because so far only few have been described in meningiomas. For instance, sequencing studies revealed several mutations in meningiomas including *NF2* and the *TERT* promoter prognosticating higher risk of recurrence and shorter survival (39–41). Furthermore, use of distinct epigenetic patterns might serve as prognostic markers (42). However, such analyses are still rather expensive, can only be performed in few institutions, and require a high sample quality. In contrast, the immunofluorescence detection of cytotoxic T cells and PD-1–expressing cytotoxic TILs as a surrogate for patient outcome is affordable and may be integrated in a timely manner in the routine setting of digital imaging. This might offer additional information on the individual clinical course and help selecting patients needing a more intensified treatment or suitable for immunotherapy. However, future studies are warranted to implement suitable staining protocols for frequently used paraffin-embedded tissues.

Our findings on the prognostic relevance of the proportion of PD-1–expressing TILs might appear surprising because PD-1 has long been regarded exclusively as an exhaustion marker (27). However, there is a controversial discussion about the interpretation of PD-1 as a marker for activation rather than for exhaustion (43). This is based on recently published data demonstrating PD-1–expressing cytotoxic T cells from healthy individuals to feature gene expression profiles resembling those of activated effector memory cells rather than exhausted T cells from human immunodeficiency virus–infected patients (25). Moreover, it is well-known that the transient PD-1 expression on naïve T cells is induced upon TCR stimulation and decreases in the absence of TCR signaling (43). Meanwhile, the dual importance of PD-1 has been strengthened in melanoma where it is a valuable marker to detect activated and tumor-specific TILs exhibiting a high avidity for tumor antigens (29). Even though our findings clearly indicate that PD-1 expression on TILs in meningiomas can be considered as a marker for tumor-reactive T cells, it is important to emphasize that this is not in conflict with the administration of therapeutic antibodies against PD-1 already approved in the United States for immunotherapy of several tumors (44). These immune checkpoint inhibitors solely prevent T-cell exhaustion by tumor cells or other antigen-presenting cells after binding of PD-1 and its ligand PD-L1, but do not interfere with T-cell activation or expression of PD-1.

Finally, our longitudinal analysis of TIL composition in pMGMs and rMGMs provides important information for future immunotherapeutic trials. It clearly demonstrates a significant decline of effector TILs in rMGMs by more than 40%. This does apply to general effector TILs, helper, and cytotoxic T cells and to the proportion of PD-1–expressing cytotoxic T cells, suggesting severe changes in the tumor microenvironment during the course of the disease, which impairs sufficient T-cell infiltration and activation. Accordingly, immunotherapy at an early stage of the disease and especially in patients with proven higher intratumoral TIL numbers might be more successful.

In summary, by analyzing a large study sample enriched for higher grade pMGMs and rMGMs we identified the proportion of cytotoxic TILs and PD-1–expressing cytotoxic TILs as novel biomarkers for better survival. Because efficacy of immunotherapy depends on preexisting immune responses evolving from tumor-specific infiltration of immune cells (45), our analysis facilitates selection of patients who may benefit from immunotherapeutic approaches and thus might guide the optimal timing for such an intervention.

Disclosure of Potential Conflicts of Interest

F. Sahm reports receiving speakers bureau honoraria from Agilent, Illumina, and Medac, and is a consultant/advisory board member for AbbVie. A.F. Kessler reports receiving speakers bureau honoraria from and reports receiving commercial research grants from Novocure. A. Abdollahi is a consultant/advisory board member for BioMedX, and reports receiving commercial research grants from Merck and Fibrogen. No potential conflicts of interest were disclosed by the other authors.

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