Paucity of Tumor-infiltrating Lymphocytes Is an Unfavorable Prognosticator and Predicts Lymph Node Metastases in Cutaneous Melanoma Patients

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Abstract. *Background/Aim: Tumor infiltrating lymphocytes (TILs) have been a subject of growing scientific interest; however, a full picture of their role in cancer pathogenesis is still unclear. The present study aimed to reveal correlations between TIL grade and clinicopathological features, especially 5-year survival parameters in cutaneous malignant melanoma (CMM) patients. Materials and Methods: Presence of TILs was assessed by hematoxylin and eosin staining in routine diagnostic histopathological specimens from 104 patients with cutaneous melanoma. Results: In the entire group of 104 patients, decreasing TIL intensity was a highly negative prognostic factor and indicated considerably shorter overall survival (OS), cancer-specific overall survival (CSOS) and disease-free survival (DFS). We also report on a significant association between decreased TIL intensity and worse prognosis in lymph node-negative patients. Shorter survival (p=0.002 for OS, p=0.038 for CSOS and p=0.011 for DFS) was observed in patients with negative sentinel lymph node biopsies (SLNB), as well as in patients with lack of metastases in regional lymph nodes (p=0.034 for OS, p<0.001 for CSOS and DFS). Conclusion: Our results indicate that TIL grade is a valuable diagnostic parameter that may be helpful in risk stratification in CMM.*

Despite advances in global healthcare, both incidence and mortality among cutaneous malignant melanoma (CMM) patients are rising. Examining cancer's molecular basis is a powerful weapon against it as it helps to develop novel therapies and identify high-risk patients. Factors commonly regarded as the most important prognosticators in melanoma include Breslow thickness, ulceration of the tumor and presence of nodal metastases (1, 2). Mitotic rate (MR) (3, 4), tumoral angiogenesis (5) and tumor-infiltrating lymphocytes (TILs) (6) are other major parameters that have received a lot of attention lately.

TILs are a histopathological manifestation of host immune response against melanoma. Depending on their number and distribution within the tumor, TILs are quantified as absent, non-brisk or brisk. Considering great heterogeneity of TILs, that is the variety of phenotypes and multitude of effector mechanisms, there is no simple answer to the question regarding their clinical significance (7). However, many studies have indicated a distinct correlation between enhanced TIL intensity and better prognosis (6, 8-10). This beneficial effect is attributed to subsets of infiltrating lymphocytes exhibiting melanoma-specific, both direct and indirect, cytolytic activity (7), which has been the basis for successful trials of therapies utilizing adoptive cell transfer techniques (11, 12). Moreover, TIL grade, being a simple parameter, is characterized by good inter-observer reproducibility (13) and seems to increase the accuracy of risk stratification when analyzed together with the current American Joint Committee on Cancer (AJCC) staging recommendations (6). Besides survival, correlation with higher TIL grade has been reported for other clinicopathological parameters including lower Breslow thickness and Clark level, negative sentinel lymph node biopsy (SLNB) and less consistently younger age, lower mitotic rate and absence of ulceration (6, 10, 14).

The present study was undertaken to assess the relationship of TIL grade in CMM with other clinico-pathological features, especially survival parameters, with special focus on the lymph node-negative group of melanoma patients.

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Table I. *Clinicopathological characteristics of patients.*

Clinicopathological characteristics	No (%)	TILs brisk	TILs non-brisk or absent	*p*-Value
All patients	104 (100.0)			
Age in years (21-79)[a] mean, 56.5±15.4; median, 58.5		52	52	0.594
Gender[b]				
Female	60 (57.7)	31	29	0.421
Male	44 (42.3)	21	23	
Primary tumor location[c]				
Head/neck	15 (14.4)	4	11	
Extremities	43 (41.3)	22	21	0.246
Hand/foot	4 (3.8)	2	2	
Trunk	42 (40.4)	24	18	
Primary tumor (pT)[a]				
pT1	34 (32.7)	25	9	
pT2	21 (20.2)	13	8	**<0.001**
pT3	26 (25.0)	7	19	
pT4	23 (22.1)	7	16	
Sentinel lymph node biopsy status (SNLB)[b]	60 (57.7)			
No metastases (SNLB–)	48 (80.0)	29	19	0.198
Metastases present (SNLB+)	12 (20.0)	5	7	
Regional lymph nodes status (pN)[b]				
No metastases (pN–)	86 (82.7)	47	39	**0.034**
Metastases present (pN+)	18 (17.3)	5	13	
Recurrence[b]				
No	87 (83.7)	48	39	**0.016**
Yes	17 (16.3)	4	13	
Distant metastases[b]				
No	99 (95.2)	52	47	**0.028**
Yes	5 (4.8)	0	5	

[a]*p*-value of Mann-Whitney's *U*-test; [b]*p*-value of Fisher's exact test; [c]*p*-value of chi2 test; Statistically significant results ($p<0.05$) are in bold text; TILs, Tumor-infiltrating lymphocytes.

Table II. *Correlations between grades of TILs and histopathological characteristics of primary tumors.*

Histopathological parameters	No (%)	TILs brisk	TILs non-brisk or absent	*p*-Value
Breslow thickness[a]				**<0.001**
<=1 mm	34 (32.7)	25	9	
1.01-2.00 mm	20 (19.2)	13	7	
2.01-4.00 mm	27 (26.0)	7	20	
>4 mm	23 (22.1)	7	16	
Clark thickness[a]				
I	0 (0.0)	0	0	
II	18 (17.3)	15	3	
III	49 (47.1)	24	25	**0.002**
IV	26 (25.0)	10	16	
V	11 (10.6)	3	8	
Histologic type[b]				
Superficial spreading melanoma (SSM)	68 (65.4)	45	23	
Nodular malignant melanoma (NMM)	32 (30.8)	6	26	**<0.001**
Acral-lentiginous melanoma (ALM)	4 (3.8)	1	3	
Mitotic rate[a]				
0	45 (43.3)	32	13	
1-2	26 (25.0)	10	16	**<0.001**
≥3	33 (31.7)	10	23	
Ulceration[c]				
No	55 (52.9)	38	17	**<0.001**
Yes	49 (47.1)	14	35	
Lymphangioinvasion[c]				0.065
No	74 (71.2)	41	33	
Yes	30 (28.8)	11	19	
Growth phase[c]				
Radial	3 (2.9)	2	1	0.5
Vertical	101 (97.1)	50	51	
Microsatellitosis[c]				
No	98 (94.2)	49	49	0.661
Yes	6 (5.8)	3	3	
Tumor regression[c]				
No	96 (92.3)	49	47	0.358
Yes	8 (7.7)	3	5	

[a]*p*-value of Mann-Whitney's *U*-test; [b]*p*-value of chi2 test; [c]*p*-value of Fisher's exact test; Statistically significant results ($p<0.05$) are in bold.

Materials and Methods

Patients. The study group consisted of 104 patients with CMM diagnosed between 2005 and 2010 and treated in the Lower Silesian Oncology Center in Wroclaw, Poland. The group was selected on the basis of the availability of tissue material (paraffin blocks and histopathology slides) and medical documentation. Comprehensive clinical data were obtained from archival medical records. The diagnostic and therapeutic procedures utilized were determined from medical records of the oncology outpatient clinic of the Lower Silesian Oncology Center and data provided by the Lower Silesian Cancer Registry and Civil Register Office. The study was approved by the ethical committee of the Wroclaw Medical University, Poland.

The clinicopathological profile of patients included the following parameters: age and gender, primary tumor location, tumor stratification according to AJCC (pT), presence or absence of nodal (pN) and distant (pM) metastases, information on disease recurrence and SLNB procedures (Table I).

Tumor samples and histopathological evaluation. Tumor specimens were fixed in 10% buffered formalin and embedded in paraffin. All haematoxylin and eosin stained sections were examined by two pathologists. The parameters of the primary tumor recorded in pathology reports were Breslow thickness, Clark level, growth phase, histologic type, mitotic rate (number of mitotic figures per 1 mm^2), presence of ulceration, lymphangioinvasion, micro-satellitosis, intensity of lymphocytic inflammatory infiltrate (TILs, tumor-infiltrating lymphocytes) and microscopic evidence of regression (Table II).

TILs were assessed in a semiquantitative way as defined below. Absence of TILs: no lymphocytes present or lymphocytes are present but they do not infiltrate tumor at all (Figure 1a). Non-brisk TILs: lymphocytes infiltrate melanoma only focally or not along

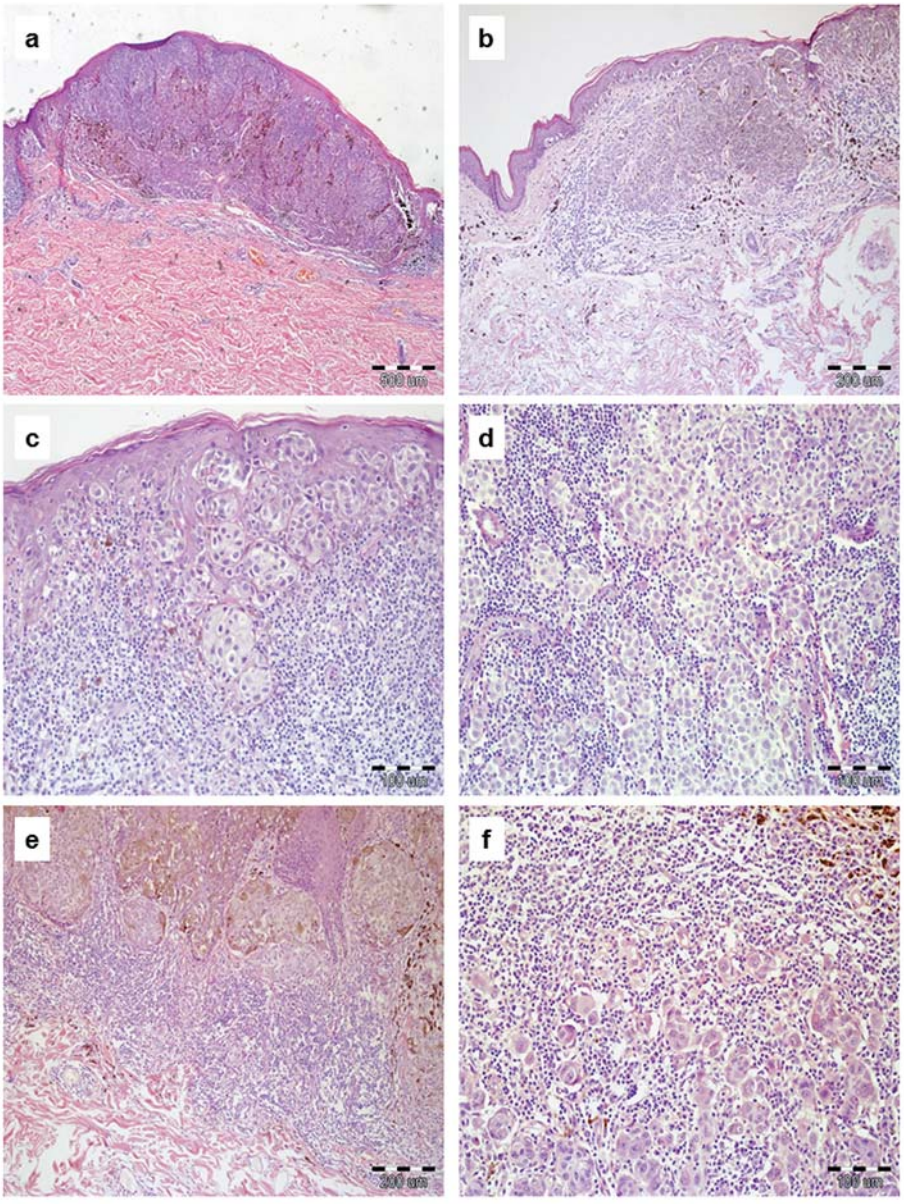

Figure 1. *Grades of TILs in melanoma. Absence of TILs: no lymphocytes present (a: H&E staining; ×40); non-brisk TILs: lymphocytes infiltrate melanoma only focally or not along the entire base of the vertical growth phase (b: H&E staining; ×100); brisk TILs: lymphocytes diffusely infiltrate the entire base of the vertical growth phase or the entire invasive component of the melanoma (c: H&E staining; ×200; d: H&E staining; ×200; e: H&E staining; ×100; F: H&E staining; ×200).*

the entire base of the vertical growth phase (Figure 1b). Brisk TILs: lymphocytes diffusely infiltrate the entire base of the vertical growth phase or the entire invasive component of the melanoma (Figures 1c-f).

Statistical analysis. Statistical analysis was performed using the Statistica 10.0 (StatSoft Inc., Tulsa, Oklahoma, United States) and IBM SPSS 21 software packages (IBM Corporation, Armonk, New York, United States). Overall survival (OS) was defined as the time between the primary surgical treatment and death; OS was censored at last follow-up for patients who were still alive. Disease-free survival (DFS) was defined as the time between the primary surgical treatment and the date of relapse. DFS was censored at the last follow-up for patients who survived without disease recurrence or at the date of non-cancer-associated death. Cancer-specific overall survival (CSOS) was defined as the time between the primary surgical treatment and cancer-associated death and was censored at the last follow-up for surviving patients.

A χ^2 test, exact Fisher test, in the case of 2×2 tables, and Spearman rank correlation were used to analyze associations between mitotic rate and presence of ulceration and clinicopathological parameters. Differences between the means were tested with a non-parametric test (Mann-Whitney *U*-test and Kruskal-Wallis test); the log-rank test was used to compare survival in two groups, the overall survival rate was estimated by the Kaplan-Meier method and the influence of explanatory variables on death risk was analyzed by means of the Cox proportional hazard regression. *p*-Values <0.05 were considered statistically significant.

Results

Grades of TILs in 104 melanoma patients. Intense lymphocytic inflammatory infiltrate (brisk TIL grade) was observed in 52 patients with CMM (50% of study group), whereas no infiltrate was noted in 18 patients (17.3%). Intermediate lymphocytic reaction (non-brisk TIL grade) was observed in 34 patients (32.7%).

Correlations between TILs and clinical parameters. Brisk TIL grade was observed significantly more often in less advanced tumors (infiltration depth <2 mm) ($p<0.001$). Strong lymphocytic reaction was correlated with the absence of regional lymph node metastases ($p=0.034$). Interestingly, absent or non-brisk TIL infiltration was associated with disease recurrence ($p=0.016$). Importantly, brisk TIL grade was not observed in any of the tumors from patients with distant metastases ($p=0.028$). No statistically significant relations between TIL intensity and age at the moment of diagnosis, gender, primary tumor location or SLNB status were observed (Table I).

Correlations between TILs and histopathological parameters. Lower tumor advancement according to Breslow thickness and Clark level significantly correlated with brisk TIL grade ($p<0.001$ and $p=0.002$, respectively). The majority of non-mitogenic tumors (mitotic rate, MR=0) were characterized by the presence of intense lymphocytic inflammatory infiltrate (71%, 32/45), whereas tumors with high mitogenic potential (MR≥3) were characterized by no or non-brisk TIL infiltration ($p<0.001$). A similar relationship regarding the presence of brisk TIL grade was observed in non-ulcerated primary tumors ($p<0.001$). An analysis of TIL grade, in the context of histological type of the primary tumor, revealed that nodular malignant melanoma was characterized by absent or non-brisk lymphocytic reaction ($p<0.001$). No significant correlations were observed between TIL grade and other histopathological features including lymphangioinvasion, microsatellitosis, tumor regression and growth phase (Table II).

Impact of TIL grade on 5-year survival in the whole group of melanoma patients. The Kaplan-Meier analysis revealed that the absence of lymphocytic inflammatory infiltration significantly correlated with shorter OS (Figure 2a, $p=0.007$), CSOS (Figure 2B) ($p<0.001$) and DFS (Figure 2C) ($p<0.001$). Because of the observed differences regarding the prognostic value of individual TIL grades, the study group was divided into two subgroups: one presenting brisk TIL infiltrates and the other with no or scanty lymphocytic infiltrates, which did not fulfil the criteria of brisk TIL grade. Patients with intense lymphocytic reaction were characterized by much better prognosis comparing to the other subgroup, especially in the context of DFS ($p=0.001$) (Figures 2d-f).

Impact of TIL grade on 5-year survival in lymph node-negative melanoma patients. Low intensity and no lymphocytic inflammatory infiltrate were strongly related to unfavorable prognosis defined as shorter OS ($p=0.002$) (Figure 3a), CSOS ($p=0.038$) (Figure 3b) and DFS ($p=0.011$) (Figure 3c) in patients with negative SLNB status. Moreover, in patients who underwent regional lymphadenectomy in which histopathological examination revealed no nodal metastases, paucity of TILs was also a factor indicating poor outcome ($p=0.034$ for OS, $p<0.001$ for CSOS and DFS) (Figures 3d-f).

Discussion

The study confirms previous observations of general prognostic utility of TIL grade in cutaneous melanoma. Moreover, our findings indicate that the determination of lymphocytic infiltrate intensity may be helpful in predicting regional lymph node status and risk stratification in lymph node-negative sub-group of patients. This conclusion seems very important as patients without nodal metastases have relatively good prognosis *a priori*. Nevertheless, this group of patients is very heterogeneous and by subdividing it further by particular tumor characteristics it becomes possible to define factors responsible for differences in survival within the group. The odds of the patients at risk may be then increased by applying more aggressive postoperative adjuvant therapy or simply by intensification of follow-up controls.

Our study supports the well-established view that higher TIL grades are observed more often in thinner, less advanced CMM (8-10). The associations between intense lymphocytic reaction, lower MR and absence of ulceration, though logically coherent with commonly postulated anti-tumoral properties of TILs in melanoma, have only rarely been previously reported (9, 10). Interestingly, like a few other studies (15-17), our results do not support the claim that TIL grade is a reliable prognosticator of SLNB positivity. This discrepancy may be related to the relatively small number of patients in our study and differences regarding TIL categorization for statistical analyses. In this experiment, melanomas presenting no and non-brisk TIL infiltrate were

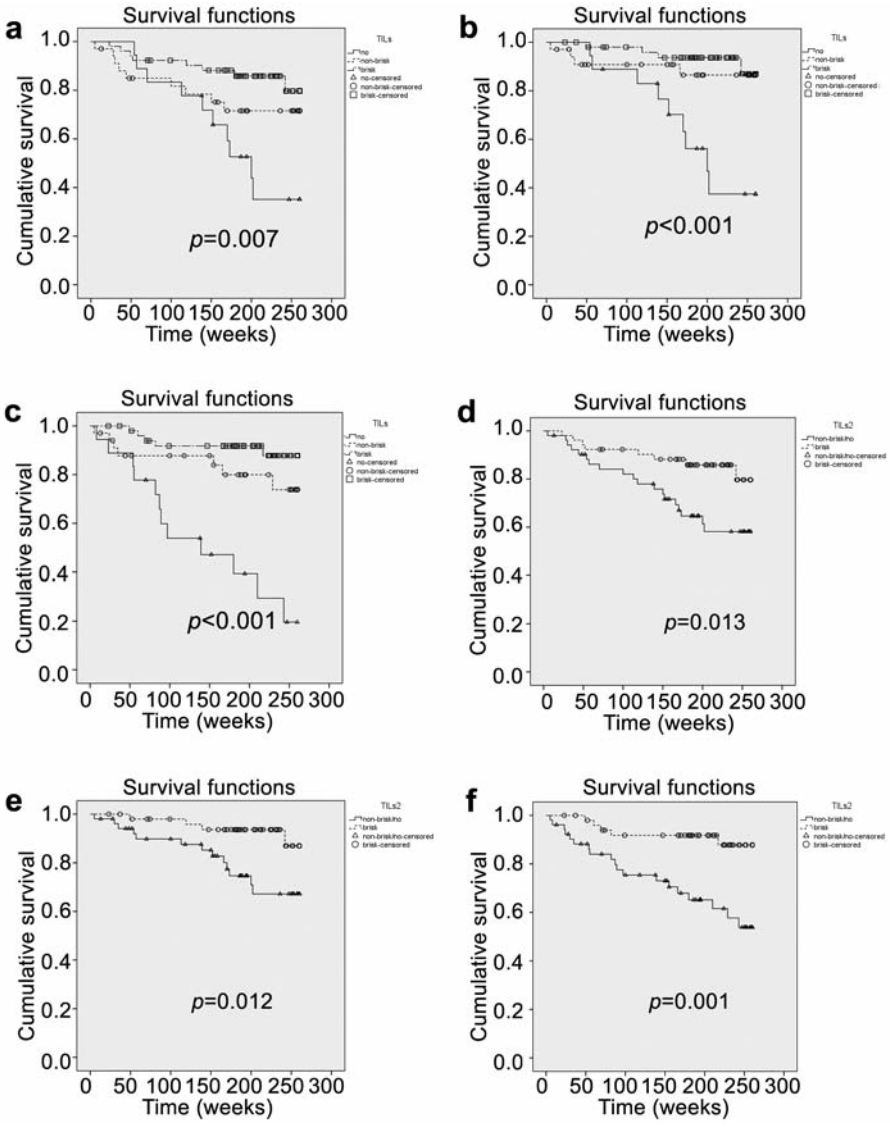

Figure 2. *Impact of TILs on 5-year survival in melanoma patients in the entire study group of 104 patients. Kaplan-Meier analysis revealed that the absence of lymphocytic inflammatory infiltration significantly correlated with shorter overall survival (a), cancer-specific overall survival (b) and disease-free survival (c). Additionally, the study group was divided into two sub-groups: one presenting brisk TIL infiltrates and the other with no or scanty lymphocytic infiltrates, which did not fulfil the criteria of brisk TIL grade: patients with intense lymphocytic reaction were characterized by much better prognosis comparing to the other subgroup (d for OS; e for CSOS and f for DFS).*

grouped together and confronted with brisk grade tumors, whereas other authors compared TIL-positive vs. negative tumors (4, 9, 10).

Based on the available knowledge, TILs are considered a manifestation of host immunity struggling against developing cancer. In our study, the observed correlation between higher TIL grade and decreasing incidence of metastases suggests that lymphocytic infiltrate hampers the disease progress. Moreover, even when melanoma metastasizes, a higher TIL level limits the negative consequences in a 5-year observation.

Another aspect worth keeping in mind is the considerable differences in histopathologic characteristics of study groups between various experiments, making it difficult to directly juxtapose them (14). A good example comes from two articles by Clemente *et al*. (8) and Barnhill *et al*. (18). Although not consistent as regards the influence of TIL grade on patient survival, they have radically different populations: there are 18.2% of melanomas ≤2 mm in the first study and 74.6% <1.7 mm in the latter. That is a disparity that warrants caution.

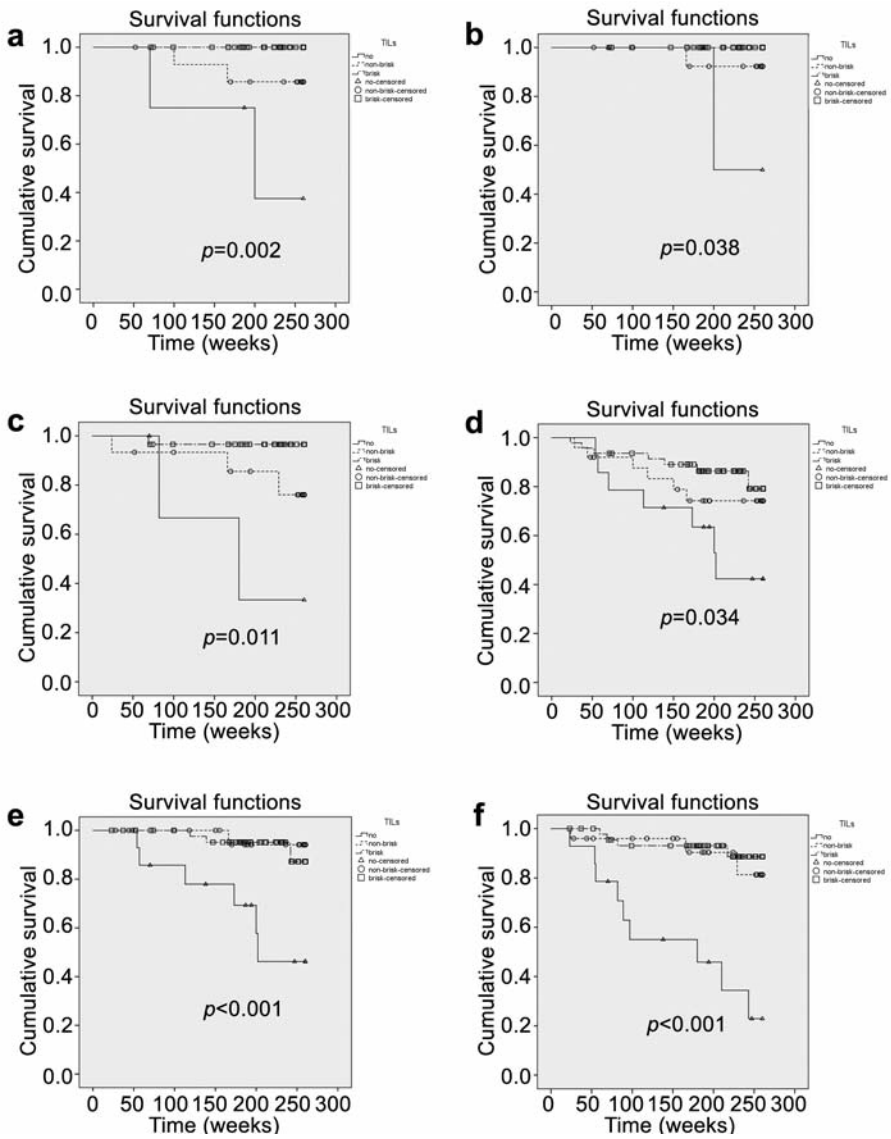

Figure 3. *Impact of TIL grade on 5-year survival in lymph node-negative melanoma patients. Low intensity and no lymphocytic inflammatory infiltrate were strongly related to unfavorable prognosis defined as shorter OS (a), CSOS (b) and DFS (c) in patients with negative SLNB status. Moreover, in patients who underwent regional lymphadenectomy, in which histopathological examination revealed no nodal metastases, paucity of TILs was also a factor indicating poor outcome (d for OS, e for CSOS and f for DFS).*

The relation between higher overall intensity of TILs or prevalence of particular TIL sub-populations and better prognosis has been also observed in other cancers. Loi *et al*. have demonstrated that robust lymphocytic infiltrate was associated with decreased distant recurrence rate in triple-negative breast cancer (19). Moreover, higher TIL grade predicted superior response to trastuzumab in $HER2^+$ patients (19). Infiltration by immune cells has been linked to a better clinical outcome also in non-small cell lung cancer (20, 21), ovarian (22), prostate (23) and colorectal (24, 25) carcinomas. These observations point out that infiltrating lymphocytes play an important, albeit not fully understood, role in cancer pathogenesis.

Importantly, it was shown that TILs are not constituted by a homogeneous subset of cells with solely anti-neoplastic properties. It is a heterogeneous group of multiple lymphocyte subsets with diverse and often opposing effects, which represents a population of $CD8^+$ cytotoxic lymphocytes, $CD4^+$ T helper lymphocytes, NK cells and $CD4^+$ regulatory T lymphocytes (Treg, $FoxP3^+$) (7). The most important subset of lymphocytes in the context of anti-tumor properties are cytotoxic $CD8^+$ lymphocytes, which have the ability to lyse neoplastic cells

directly or *via* humoral response. Upon activation, cytotoxic T lymphocytes (CTLs, $CD8^+$) express death activator designated Fas ligand (FasL) on their surface and the engagement of Fas/FasL pathway leads to mediated apoptosis of cancer cells (11, 12, 26). Interestingly, the opposite effect is induced by $CD4^+CD25^+$ T-regulatory cells ($Foxp3^+$), which are over-expressed in lymph nodes with metastatic melanoma. These cells, *via* contact-dependent mechanism, inhibit proliferation and cytokine production of the surrounding $CD4^+CD25^-$ and $CD8^+$ cells, which both have anti-tumoral properties (27). Moreover, recent data have demonstrated the implication of Tregs in the pathogenesis and progression of tumors (26).

To fully benefit from the possibility to target melanoma with infiltrating lymphocytes, we need to better understand the immunologicαλ nature of TILs (detailed immuno-phenotyping and correlation with long-term survival) and molecular mechanisms behind interactions between cancer and host immunity.

Conflicts of Interest

All Authors have no conflicts of interest.

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