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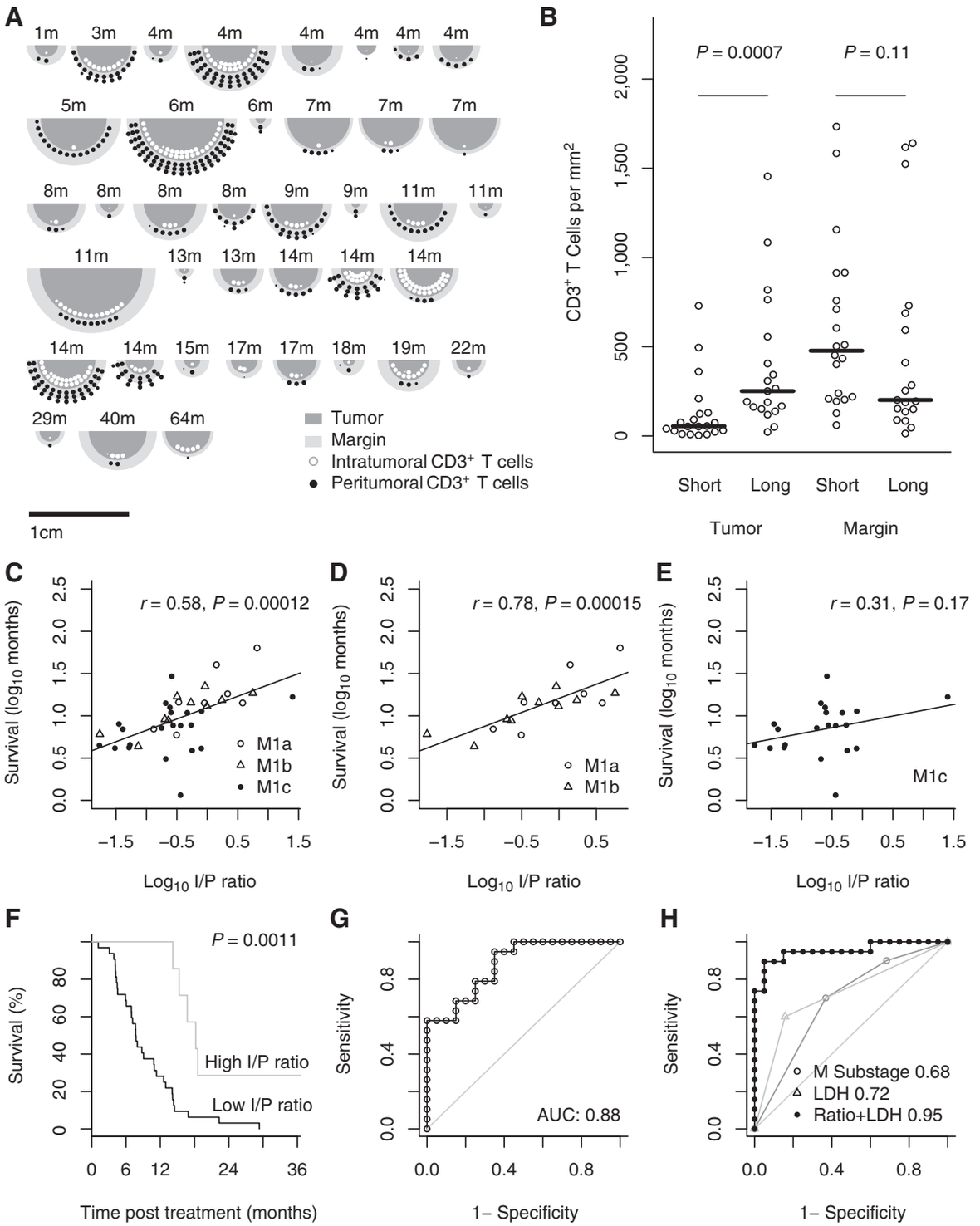

Figure 3.

Validation of the I/P ratio as a predictor of survival after DC vaccination. A, relative tumor size, size of peritumoral area, and $CD3^+$ count of all patients in the validation cohort. Overall survival is shown above each tumor in months. Each large bullet point represents 1,000 T cells within the tumor (white) or in the peritumoral area (black). B, peritumoral (margin) and intratumoral (tumor) $CD3^+$ T-cell densities ($CD3^+$ T cells/mm^2) shown separately for short and long survivors. C–E, correlation between I/P ratio and overall survival are shown on a log–log scale for all patients (C), patients with substage up to M1b (D), and patients with substage M1c (E). F, survival of patients with low (<1, $n = 33$) and high (>1, $n = 6$) I/P ratios ($P = 0.0011$, log-rank test). G, AUC analysis for discriminating short survivors (bottom 50% survival times) and long survivors (top 50%) using the I/P ratio (AUC of 0.88). H, comparison with two other relevant prognostic markers. A combined logistic regression model incorporating the ratio and LDH achieves an AUC of 0.95 (M substage alone: 0.68; LDH alone: 0.72), which is no longer improved by also taking the M substage into account.

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Table 2. Multivariate analysis

	Log OR (95% CI)	*P*
Intercept	0.82 (0.05–1.6)	0.044
I/P ratio (log_{10})	0.4 (0.19–0.61)	0.001
M stage at inclusion	0 (−0.18–0.19)	0.97
LDH	−0.37 (−0.64 to −0.1)	0.013
Gender	0 (−0.3–0.31)	0.98
Months to M stage	0 (0–0.01)	0.54
Breslow thickness	0 (−0.06–0.06)	0.96
Age at inclusion	0 (−0.01–0.01)	0.83

NOTE: Logistic regression model showing that the difference in ratio in the validation cohort is not explained by including M substage, LDH, and other possible confounders as covariates.

compared with LDH (AUC = 0.72) and M substage (AUC = 0.68). Combining the I/P ratio with LDH in a logistic regression model, we even obtained an AUC value of 0.95. In a larger multivariate analysis (Table 2), the I/P ratio was the strongest predictor (P = 0.001; log OR, 0.4; 95% CI, 0.19–0.61) for survival in the validation cohort. LDH level remained an independent, but weaker predictor (P = 0.013; log OR, −0.37; 95% CI, −0.64 to −0.1), whereas M substage at start of DC-based immunotherapy, gender, Breslow thickness, age, and time to M stage did not show significant prognostic value. These multivariate analyses provide compelling evidence that strong T-cell infiltration in the primary melanoma is an independent predictor of survival in metastatic melanoma patients receiving DC-based immunotherapy, despite the fact that several years typically pass between resection of the primary tumor and the onset of treatment.

T-cell infiltration is a potential predictive biomarker for DC vaccination

The I/P ratio in primary tumors would be tremendously useful as a predictive biomarker for (adjuvant) treatment selection due to its availability at an early stage of disease. Establishing a biomarker requires analysis of data from a randomized controlled trial, which is not available yet for DC vaccinations worldwide; so far, most DC studies are small stage I/II studies varying from 10 to 40 patients. Therefore, as an approximation, we compared our DC-vaccinated cohorts to a cohort of patients receiving standard treatment (chemotherapy) in the same timeframe, matched on known prognostic factors to minimize confounding. While this approach cannot provide definitive proof of biomarker status, it did allow us to probe our hypothesis that patients require strong intratumoral T-cell infiltration to benefit from immunotherapy.

We analyzed 19 primary melanomas of patients that had received chemotherapy (Table 1; Fig. 4A). Survival was evaluated with Kaplan–Meier estimates comparing patients with low (<1, n = 6) and high (>1, n = 13) I/P ratios (Fig. 4B). Although the survival curves suggest that strong T-cell infiltration may correspond to longer survival in chemotherapy-treated patients, the difference is not significant (P = 0.089). There was also no appreciable direct correlation between survival and I/P ratio (r = 0.12, P = 0.62; Fig. 4C), thus, the I/P ratio is not a strong predictor of survival in chemotherapy-treated patients. This is in line with our hypothesis that the effect of T-cell infiltration in the primary tumor needs to be augmented by immunotherapy to improve survival. To further support this hypothesis, we matched each chemotherapy patient to two DC vaccination patients based on LDH level and M substage (Fig. 4D). DC-vaccinated patients with low I/P ratios showed no significant difference in survival after the different treatments (Fig. 4E), indicating no survival benefit of DC-based immunotherapy when T-cell infiltration is weak. On the other hand, patients with high I/P ratios clearly survived longer after DC-based immunotherapy than patients with high I/P ratios treated with chemotherapy (Fig. 4F), and therefore seem to benefit from DC-based immunotherapy.

In summary, our matched analysis indicates that the I/P ratio could indeed be a predictive marker for DC-based immunotherapy: patients with high I/P ratios appear to benefit from receiving DC-based immunotherapy, whereas patients with low I/P ratios do not. It will be important to test this result in future randomized trials with DC vaccination and other immunotherapies.

Discussion

The rapidly evolving field of melanoma management and novel treatments mandates development of new biomarkers, especially given the high costs associated with current treatments and their substantial toxicity. While for targeted therapies, effective genetic tools are available to select patients who are most likely to benefit from treatment, that is, *BRAF* mutation status for vemurafenib, this is much less so for immunotherapy. Established factors that are known to correlate with survival after immunotherapy are mostly derived from metastases. When looking at immunotherapy in general, several studies have shown specific gene expression profiles, identified on pretreatment biopsies of melanoma metastases that correlate with outcome after immunotherapy (35). Besides gene profiling, multiple studies have focused on finding a predictive biomarker in the tumor microenvironment. For instance, the expression of FoxP3 and indoleamine 2,3-dioxygenase (IDO) in pretreatment biopsies of melanoma metastases have been linked to a positive association with clinical activity of anti-CTLA-4 antibody (36). Also, several studies investigated biomarkers for PD-1 pathway–blocking agents, in particular, the expression of the PD-1 ligand (PD-L1). Unfortunately, the many different PD-L1 clones and differences in assessment of the PD-L1 immunohistochemical staining complicate interpretation of the different studies (37). Nonetheless, in melanoma, there seems to be an association between PD-L1 expression on tumor cells and response to an anti-PD1-antibody (38). A recent study has focused both on the role of PD-L1 expression as well as $CD8^+$ T cells in melanoma metastases and has shown that the density of pre-existing $CD8^+$ T cells in both the tumoral area and the invasive tumor margin has a predictive value for the treatment outcome of patients receiving anti-PD-1 therapy (39). This study highlights the importance of pretreatment T-cell infiltration in the tumor area as it correlates with clinical outcome.

A disadvantage of most of these potential biomarkers is that tumor material from metastases can be difficult to access and only becomes available when patients reach systemic disease, and are therefore not suitable for decision-making at earlier stages of disease in which adjuvant immunotherapy might be beneficial. Furthermore, biomarkers observed in metastases might not be suitable for analysis of the primary tumor; for instance, the positive association between PD-L1 expression in metastases and melanoma-specific survival could not be confirmed in primary melanoma (40). Finally, markers like skin-infiltrating T cells isolated from DTH biopsies, although useful for monitoring

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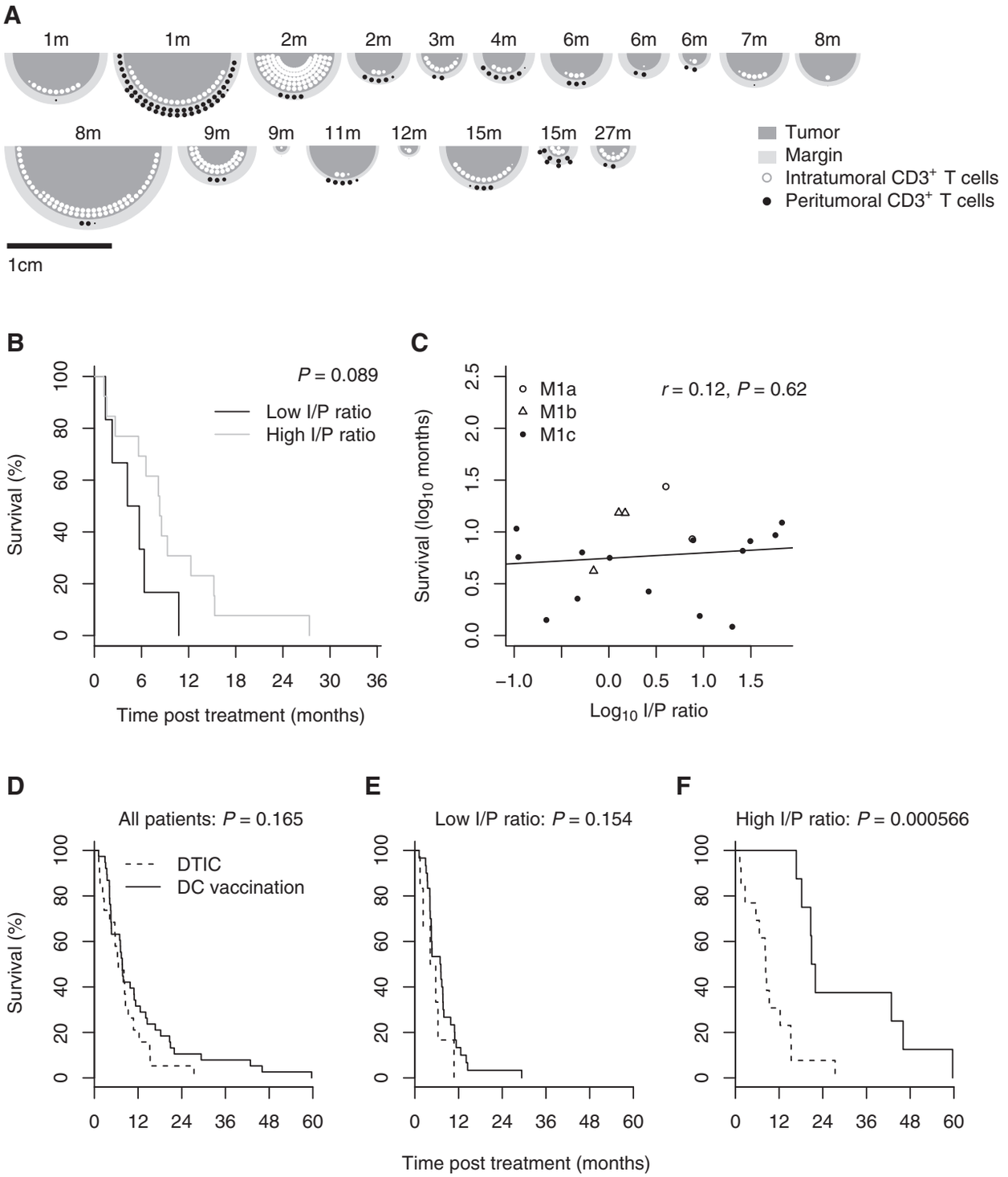

Figure 4.

Strong intratumoral T-cell infiltration is associated with a larger difference between DC vaccination and chemotherapy in a matched cohort. A, relative tumor size, size of peritumoral area, and $CD3^+$ count of all patients in the chemotherapy cohort. Overall survival is shown above each tumor in months. Each large bullet point represents 1,000 T cells within the tumor (white) or in the peritumoral area (black). B, survival of patients treated with chemotherapy with low (<1, $n = 6$) and high (>1, $n = 13$) I/P ratios ($P = 0.089$). C, correlation between I/P ratio and survival in months are shown on a log–log scale for all patients. D, analysis of a combined cohort in which each of the 19 chemotherapy patients was matched to two patients from the DC vaccination cohorts (giving $n = 57$) that were matched on LDH and M1c substage. E, longer survival upon immunotherapy is not apparent in patients with low I/P ratio (<1; $n = 36$). F, yet, there is a significant difference for patients with high I/P ratio (>1; $n = 21$).

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response to therapy, cannot be used for pretherapeutic decision-making.

In this study, we investigated whether the T-cell landscape within the primary tumor is relevant for survival of 77 patients with metastatic melanoma. Given partly contradicting results in the literature, our approach aimed to exploit a novel observer-independent methodology, and focused on patients who received immunotherapy as we expected the relevance of immune infiltration to be higher in this setting. Our results show that strong $CD3^+$ T-cell infiltration into the primary tumor is an excellent early predictor of longer survival in metastatic melanoma patients receiving DC-based immunotherapy. This was revealed in a discovery cohort and confirmed in a validation cohort, which showed a clear correlation between intratumoral $CD3^+$ T-cell infiltration and survival. Multivariate analyses showed that the I/P ratio improves upon and can be combined with LDH levels, measured at the time treatment decisions must be made. This led to the most accurate survival prediction in our validation cohort (AUC = 0.95), which also outperformed the conventional classification based on the TNM staging system.

T-cell recognition of tumor cells relies upon presentation of tumor-specific antigens on MHC molecules. Therefore, a potential mechanism explaining the relevance of T-cell infiltration might be that weak T-cell infiltration into the primary tumor reflects poor immunogenicity of the tumor and thus also of potential future metastases. Weak immunogenic tumors might also be more difficult to treat with immunotherapy. If so, then intratumoral T-cell infiltration should also be an appropriate marker for selecting patients for treatment with immunotherapy. Our analysis of the matched cohort provides support for this hypothesis. When data from randomized trials with DC vaccination become available, it will be possible to confirm this predictive biomarker status.

Consistently, with earlier studies, we found high intratumoral T-cell densities to be beneficial. However, in the cohorts studied here, a high T-cell density in the margin appears to be detrimental. In theory, a high marginal T-cell density could reflect an efficient homing rate of T cells from the circulation towards the tumor site. But it could also reflect a poor infiltration rate of T cells from the margin into the tumor. By measuring the I/P ratio, we focus on the infiltration rate, which predicts survival better than either of the individual densities. This might indicate that the infiltration rate is of particular importance in primary tumors, which may not be the case in metastases.

A major advantage of analyses of T-cell infiltration in primary tumors is that it can be assessed at initial diagnosis, which could select patients at high risk for metastatic disease and a high I/P ratio for adjuvant immunotherapy. This is indeed of interest, as we have recently shown that DC vaccination more frequently induced functional tumor-specific immune responses in stage III melanoma patients, compared with patients with metastatic disease, in which their presence correlated with clinical outcome (25, 41). Also, other studies have shown a benefit in terms of recurrence-free survival of treatment with the anti-CTLA-4 antibody in the adjuvant setting (42). Anti-CTLA-4 has recently been approved by the FDA for stage III melanoma patients; yet, it is still questionable whether anti-CTLA-4 is to be recommended as an adjuvant treatment because of its high toxicity, unknown late toxicities, and awaited data on overall survival. Identifying the right patients for adjuvant treatment (i.e., high risk patients who do not develop metastasis due to adjuvant treatment) would optimize outcome, lower toxicity, and reduce costs. However, as we have not analyzed T-cell landscapes in stage III melanoma patients who did not develop distant metastases, further research is needed to determine the value of T-cell infiltration at this stage of disease.

Often, the immune system is not capable of efficiently controlling tumor growth despite proper infiltration of immune cells in the tumor. This inefficiency may be caused by several mechanisms, including decreased function of tumor antigen-specific T cells by immunosuppressive strategies caused by tumor cells. Therefore, we believe it will be important to reveal the phenotype and function of the $CD3^+$ T cells. On the basis of previous studies examining the tumor microenvironment (8, 12, 43), we presume that the majority of the intratumoral cells are $CD8^+$ T cells, as cytotoxic T cells are considered to be the most important effector cells in the tumor microenvironment. However, given that T cells in the margin appear detrimental, the peritumoral T cells could have a distinct, perhaps regulatory, phenotype. Further research should focus on unraveling the phenotype of the $CD3^+$ T cells and the underlying mechanisms that give rise to the strong predictive value of the I/P ratio.

So far, most groups studying the correlation between TILs and patient survival classify the presence of TILs as absent, non-brisk and brisk (11, 15, 16). Some characterize TILs only as present or absent (44), while few use a more elaborate method that takes both the distribution and the density of TILs into account (12). Our method also takes distribution and density into account and does this in an automated and quantitative manner using the entire tumor slide. This way we are able to quantify all $CD3^+$ T cells and assess their exact location. Computing I/P ratio requires sufficient availability of both peritumoral and intratumoral tissue. In this study, we aimed for including peritumoral tissue within 500–700 μm around the tumor, which was possible in most cases. Thus, we believe that this method is practically applicable, provides highly accurate predictive information, and may lead to more reproducible results than manual qualitative assessment.

In summary, we have shown that T-cell infiltration into the tumor is a highly accurate predictor of survival in metastatic melanoma patients receiving DC vaccination: on average, patients with a high I/P ratio appear to benefit from DC-based immunotherapy, whereas patients with a low I/P ratio do not. The predictive power of the I/P ratio does not wane when taking into account established late-stage prognostic markers, which is remarkable given that DC vaccination was only administered at metastatic stage, years after resection of the primary tumor. This finding underscores the importance of the tumor microenvironment throughout the entire course of disease. From a clinical perspective, assessing the T-cell landscape at initial melanoma diagnosis is attractive for two reasons: primary tumors are more easily accessible than metastases, and the marker is available early on for diagnostic decision-making and, potentially, (adjuvant) treatment selection. As all immunotherapies are based on the same principle, this tool may be generally applicable for all types of immunotherapy. Further studies are warranted to confirm that strong intratumoral T-cell infiltration is indeed a predictive biomarker and to investigate the prognostic and predictive potentials of the T-cell landscape for other types of immunotherapy in melanoma. Finally, this approach may be useful for the investigation of other tumor types, especially for other immunogenic tumors.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Writing, review, and/or revision of the manuscript: A. Vasaturo, A. Halilovic, K.F. Bol, D.I. Verweij, W.A.M. Blokx, C.J.A. Punt, J.H.J.M. van Krieken, J. Textor, I.J.M. de Vries, C.G. Figdor

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Vasaturo, A. Halilovic, K.F. Bol, D.I. Verweij, P.J.T.A. Groenen

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T-cell Landscape in a Primary Melanoma Predicts the Survival of Patients with Metastatic Disease after Their Treatment with Dendritic Cell Vaccines

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