Immunotherapy is a promising strategy to treat melanoma, but the modest clinical responses so far call for improvement of therapeutic efficacy. To achieve this end, it is important to understand why some patients experience clinical benefit, while others do not. The efficacy may be compromised by immune-escape mechanisms in melanoma that inhibit the function of antitumor immunity, such as the presence of T-cell inhibitory factors or immunosuppressive cells within the tumor environment. It is conceivable that the starting condition of these features within the tumor of the patient has a great influence on the potency of the antitumor immune response and, consequently, clinical outcome.

Various markers and cell types are known to play an important role in regulating antitumor immune responses. Programmed death (ligand)-1 (PD-1/PD-L1; refs. 1, 2), indoleamine 2,3-dioxygenase (IDO), and galectin-1 and -3 are known to hinder T-cell function and/or promote T-cell apoptosis. Antigen-specific CD8+ T cells infiltrating melanoma reportedly express high levels of PD-1, causing functional impairment (3, 4). Although PD-L1 expression on tumors was found to correlate with unfavorable prognosis (5), its role in overall survival (OS) still remains controversial. Upregulation of IDO, an immunosuppressive enzyme involved in tryptophan catabolism, in metastatic melanoma can recruit regulatory T cells (Treg). Strong IDO expression was associated with a high number of FoxP3-expressing Tregs and shorter survival (6). Galectin-1 and -3 overexpression, frequently found in many human cancers (7, 8), may induce apoptosis of activated lymphocytes (9–12). Galectin-3 expression is correlated with apoptosis of tumor-associated lymphocytes in melanoma. Moreover, galectin-3 might distance the T-cell receptor from the CD8 molecule, causing impaired CTL activation and T-cell anergy (13). Immunosuppressive cells, including FoxP3+ Tregs (14–16), CD11b+CD33+ myeloid-derived suppressor cells (MDSC), and
tryptase-containing mast cells, may also interfere with T-cell effector function (17–19). Tolerogenic cytokines produced by melanoma cells, including interleukin (IL)-1, IL-6, IL-10, TNF-α, and TGF-β, further enhance the immunosuppressive environment in tumors (6, 20, 21). We previously found that impaired functional T-cell responses in HLA-A2+ patients correlated with significant loss of tumor HLA-A2, but not with the expression of melanocyte differentiation antigens (MART-1, gp100, and tyrosinase) or T-cell inhibitory factors PD-L1/PD-1 and FoxP3 in melanomas (22). Together, these findings indicate that the tumor and its environment greatly influence the potency of antitumor immune responses.

In this study, we investigated the influence of a large series of well-described immune (escape) markers (i.e., T-cell inhibitory factors, immunosuppressive cells, and tolerogenic cytokines) on clinical outcome [clinical response, progression-free survival (PFS), and OS] of patients with stage IV advanced melanoma receiving autologous granulocyte macrophage colony-stimulating factor (GM-CSF)–producing tumor cell vaccination. The results are informative for finding prospective biomarkers for the potential success of immunotherapy in individual patients with melanoma.

Materials and Methods

Patient material

Tumor biopsies of patients with stage IV metastatic melanoma, who were eligible to enter the study for treatment with autologous GM-CSF–transduced tumor cell vaccination (n = 43), were collected between 1994 and 1997 (M93CSF trial; ref. 23). Of these 43 patients, 16 patients were withdrawn from vaccination because of rapid disease progression during vaccine preparation. Biopsies were collected before immunotherapy and obtained after written informed patient consent using protocols approved by the medical ethical committee of the Netherlands Cancer Institute-Antoni van Leeuwenhoek Hospital according to the Declaration of Helsinki principles. All patients were staged according to the American Joint Committee on Cancer staging system. As reported previously (24), biopsies of (sub)cutaneous metastases or metastases in lymph nodes, lung, liver, or stomach were obtained by surgery and freshly frozen in liquid nitrogen or fixed in 4% buffered formalin.

Immunohistochemistry staining

Acetone-fixed 5-μm cryosections of tumors were incubated with 0.25% hydrogen peroxide (Merek) and 0.1% sodium-azide in TBS for 10 minutes at room temperature to block endogenous peroxidase. Subsequently, sections were preincubated with 10% normal goat serum (Dako) in TBS for 15 minutes. Sections of the tumors were stained with the following antibodies: mouse anti-gp100 (NK1-betel; MONOSAN), mouse anti-melan-A (clone A103; Dako), mouse anti-human CD4 (clone 346320; BD Biosciences), mouse anti-human CD8 (clone 346310; BD Biosciences), mouse anti-CD3 (Leu-4, clone SK7; BD Biosciences), mouse anti-HLA class-I (clone W6/32, Dako), rabbit anti-active caspase-3 (BD Biosciences), mouse anti-FoxP3 (Abcam), mouse anti-PD-1 (clone JI6; eBioscience), mouse anti-PD-L1 (clone M1H1; eBioscience), mouse anti-granzyme-B-PE (clone GB11; Sanquin), rabbit anti-CD11b (IM0530; Beckman Coulter), mouse anti-CD33-FITC (clone p67.6; BD Biosciences), and mouse anti-IL-10 (MAB217; R&D Systems). Bound antibodies, including fluorochrome-conjugated antibodies, were detected by either biotinylated polyclonal goat anti-mouse immunoglobulin (Ig) (Dako) or goat anti-rabbit Ig (Dako), followed by streptavidin–HRP (Dako).

In the case of TNF-α/β/on Willebrand factor (vWF) double staining, acetone-fixed frozen sections were incubated with a monoclonal antibody against human TNF-α (MONOSAN), followed by goat anti-mouse biotin (Dako) and streptavidin–HRP (PerkinElmer). Next, the sections were labeled with alkaline phosphatase–conjugated streptavidin (Dako) after using the tyramide signal amplification (TSA) system (PerkinElmer). Bound antibody complexes were visualized by the Alkaline Phosphatase Staining Kit (Vector Laboratories). After this staining, the sections were blocked with 10% normal mouse serum and incubated with vWF–HRP (Dako) to distinguish TNF-α–vascular endothelial cells from other TNF-α cells (e.g., tumor cells).

The following antibodies were used for paraffin-embedded tissue sections: mouse anti-CD4 (clone 4B12; Neomarkers), mouse anti-CD8 (clone C8/144B; Dako), mouse anti-tryptase (clone AA1; Dako), mouse anti-melan-A (clone A103; Dako), rabbit anti-human galectin-1 (kindly provided by Dr. V. Thijssen, VUMC, the Netherlands), mouse anti-human galectin-3 (BD Biosciences), mouse anti-IDO (clone 10.1; Millipore), mouse anti-TGF-β (TGFβ17; Abcam), goat anti-IL-1β (AF-201-NA), and goat anti-IL-6 (AF-206-NA; both from R&D Systems). Heat-induced epitope retrieval was performed in Tris/EDTA buffer (10 mmol/L/1 mmol/L; pH 9.0) for 10 minutes at 120°C using a pressure cooker. Bound antibodies were visualized using poly-HRP goat anti-mouse/rabbit IgG (BrightVision). For IDO, IL-1β, and IL-6, sections were labeled with streptavidin–HRP (Dako) after using the TSA system (PerkinElmer). Bound antibody was visualized with AEC (Vector Laboratories) and counterstained with hematoxylin (Klipath). Isotype-matched control antibodies were included in each assay and found to be negative. Cytokine expression in human cutaneous melanoma tissues has been reported previously (6, 20, 21).

Scoring of immunohistochemical staining

Scoring of the number of cells expressing the marker of interest was performed for each staining of serial sections of each tissue sample. Infiltration of immune-(suppressive) cells in the tumor tissue, as detected by antibodies against CD4, CD8, granzyme-B, PD-1, Foxp3, tryptase, CD11b, CD33 or IDO, was estimated as the percentage of infiltrating cells relative to the number of tumor cells, as detected by anti-melan A (MART-1) and/or anti-GP100. Expression of HLA class-I, melanocyte differentiation antigens, tolerogenic cytokines, galectin-1 and -3, PD-L1, and active caspase-3 by the tumor cells was estimated in the tumor area as the percentage of positive tumor cells relative to the total number of tumor cells. Scoring was performed by two observers independently (E.P.M. Tjin and G. Kreibers) using high-power field microscopy and comprised the analysis of the entire tumor area per section, excluding blood vessels or necrotic areas. Images (magnification, ×400)
were acquired using a Leica DMLB 100S microscope coupled to a Leica DC200 digital camera (Leica Microsystems) and a Qwin-based analysis system (Leica). Results were correlated statistically to the clinical outcome and OS.

**BRAF and NRAS mutation analysis**

The frequency of BRAF and NRAS hotspot mutations, in codon 600 and codons 12, 13, and 61 respectively, was assessed in all tumor samples. For this, DNA was extracted from 5-μm formalin-fixed, paraffin-embedded (FFPE) tumor sections according to the KAPA Express Extract protocol (Sopachem). PCR was performed on a LightCycler 480 (Roche Applied Science) in duplicates. Primers for the 129-bp NRAS exon 2 amplicon were forward 5'-TGTAAAACGACGGCCAGTCTCAGTACTGGTTTCCAAACAG-3' and reverse 5'-AAGTGGTGTCTGGATTACGGTA-3'. Primers for the 144-bp NRAS exon 3 amplicon were forward 5'-TGTAAAACGACGGCCAGTCTAGCAGTACTGGTTTCCAAACAG-3' and reverse 5'-TGGAATCTACAGCACAGAGA-3'. The universal M13 sequence is highlighted in bold. Each reaction mixture contained 1 μl of DNA, 500 nmol/L of each primer, 4 μl of LightScanner Master Mix (Bioké), additional 0.5 mmol/L of MgCl₂, and water to a final volume of 10 μl. PCR conditions were 95°C for 30 seconds, followed by 50 cycles of 5 seconds at 95°C, and 30 seconds annealing/extension at 64°C, and a final elongation for 1 minute at 72°C. Samples with a Ct value >40 were further analyzed. PCR product was treated with ExoSAP-IT For PCR Product Clean-up (USB/Affymetrix) according to the manufacturer's protocol. Direct sequencing reactions were performed in a reaction mixture of 1 μl BigDye Terminator v1.1 (Applied Biosystems), 750 nmol/L M13 primer 5'-TGTAAAACGACGGCCAGT-3', 3 μl of sequencing buffer, and 1 μl PCR product in a total volume of 10 μl. Sequencing was performed on the 3730 DNA Analyzer (Applied Biosystems).

**Statistical analysis**

Statistical analyses were conducted using SPSS 20.0 (IBM SPSS Inc.). For each marker, scored percentages of marker expression in the immunohistochemical stainings per patient were analyzed statistically for differences between vaccinated (nonprogressors vs. progressors) and nonvaccinated, using the one-way ANOVA test (Kruskal–Wallis) or the nonparametric Mann–Whitney test. Data are presented as median and interquartile ranges (IQR) of each marker analyzed. Correlations of immune-escape markers with OS of patients treated with autologous GM-CSF–producing tumor cell vaccine (median age, 48; range, 25–71 years). The feasibility, toxicity, and immunologic effects of the phase I/II vaccination have been reported previously (23). Sixteen of the 43 patients showed rapid disease progression during vaccine preparation and did not receive vaccinations, but were included in this study, whereas 18 patients had progressive disease (PD) during therapy, and 9 patients experienced stable disease or remained relapse free upon tumor resection [nonassessable disease (NAD)] upon vaccination, both manifesting prolonged PFS (Table 1). To date, 2 patients with NAD are still alive and disease free. The median OS of all 43 patients was 8.5 months (range, 1–214 months). Tumor tissues of these patients were analyzed for the infiltration of immune cells and the expression of immune-escape markers. Statistical analyses were performed by comparing responding patients with NAD or stable disease, resulting in prolonged PFS (nonprogresors, 5 male/4 female; median age, 46) with patients experiencing PD (progressors, 8 male/10 female; median age, 52) or included patients withdrawn from the vaccination protocol due to rapid disease progression (nonvaccinated, 7 male/9 female; median age, 45). The median OS for nonprogressors was 56 months (IQR, 22.75–142), whereas progressors had a median OS of 9.5 months (IQR, 5–17), and those nonvaccinated had a median OS of 3 months (IQR, 2–7). Because the presence of BRAF or NRAS mutation might have prognostic significance in melanoma (25–29), the mutation status of these patients was determined. BRAF p.V600E was found in 87% of the patients with melanoma, ranging from 71% in nonprogressors, 88% in nonresponders, to 93% in the nonvaccinated group. In 1 patient, the less-frequent BRAF p.V600K was found and in 1 patient a common NRAS mutation was found (p.Q61R; Table 1).

**Immune-escape mechanisms of melanoma and clinical outcome**

We examined the expression of an array of immune (escape) markers and the presence of (suppressive) cells in the tumor tissues of these patients. Our analyses included markers associated with T-cell inhibition and activation, PD-1/PD-L1, IDO, galectin-1 and -3, and granzyme-B; the loss of melanocyte differentiation antigens (MART-1 and gp100) or HLA class-I that are essential for the recognition of tumor cells by T cells; and the presence of tolerogenic cytokines IL-1, IL-6, IL-10, TGF-α, and TGF-β in tumor cells. Expression of active caspase-3 in melanoma cells was used to detect tumor cell apoptosis.

Differential expression of the various markers was found among melanoma (Figs. 1–3). Overall, the observed low expressions of FoxP3, tryptase, CD11b, CD33, and IDO suggest a minor role for the suppressive cells such as Tregs, mast cells, and MDSCs in these tumors. There were large variations in the infiltration of T cells in tumors, and in the expression of immune-escape markers (such as galectins, tolerogenic cytokines, and also the loss of melanocyte differentiation antigens) among patient samples (depicted by IQR). Significantly higher numbers of either CD4⁺ or CD8⁺ T cells (P < 0.030 and P < 0.019, respectively) were found in tumors of nonprogresors (median CD4, 30.0; median CD8, 6.3) as compared with tumors of progressors (median CD4, 5; median CD8, 0.5). In addition, significantly more granzyme-B⁺ T cells were...
### Table 1. Patient characteristics

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Abbreviations: CT, chemotherapy; DTIC, dacarbazine; HT, hyperthermia; MAGE, melanoma-associated antigen; NA, not available; PD, progressive disease; RT, radiotherapy; SD, stable disease; WT, wild-type.
present in the tumors of nonprogressors \( (P < 0.003) \), suggesting that there were activated CTLs and/or natural killer cells in the tumors (Fig. 3 and Table 2). Progressors and nonvaccinated patients were comparable in their low expression of T-cell (activation) markers in the tumors. Nonprogressors and progressors of the vaccinated group (i.e., median, 17.5) both had higher IL-10 expression in the tumor than in nonvaccinated patients (median, 0.5; \( P < 0.002 \)). These results suggest that the

Figure 1. Immunohistochemistry of T-cell inhibitory factors and suppressive cells in melanoma tissues. (Very) low to moderate–high numbers of T cells, including CD4\(^+\), CD8\(^+\), PD-1\(^+\) T cells, FoxP3\(^+\) regulatory T-cells, and cytotoxic marker granzyme-B (left), and the presence of suppressive cells such as PD-L1\(^+\) cells, tryptase\(^+\) mast cells, CD11b\(^+\) MDSCs, IDO\(^+\) cells (right) in melanoma tissues. The percentages of these infiltrating cells, designated as (very) low, ranged between 0.5% and 40%, whereas moderate to high ranged between 40% and 100%. Because of the overall low expression of PD-1, FoxP3, and tryptase in the tumor, both images shown represent (very) low. Original magnification, \( \times 400 \).

Figure 2. Immunohistochemistry of immune-escape markers expressed by melanoma cells. (Very) low to moderate–high expression of melanocyte differentiation antigens (MART-1 and GP100), apoptosis (active caspase-3), tumor-associated galectin-1 and -3 in tumor cells (left) or tolerogenic cytokines produced by tumor cells (right) in melanoma tissues. The percentages of expression of these markers, designated as (very) low, ranged between 0.5% and 40%, whereas moderate–high ranged between 40% and 100%. Original magnification, \( \times 400 \).
Immune-Escape Mechanisms in Melanoma Patients

Figure 3. Immune/tumor (escape) markers in relation to clinical outcome in patients with advanced melanoma upon tumor cell vaccination. Box plots show percentages of median expression of indicated markers in the tumor in relation to clinical outcome by comparing between the three groups: nonprogressors, progressors (both vaccinated), and nonvaccinated. The significance between the three groups is calculated by the one-way ANOVA test. Only the significant P values (P < 0.05 or P < 0.01) from the ANOVA test are depicted in the box plots. The P values between two groups, as calculated by the nonparametric Mann–Whitney test, are shown in Table 2.

Discussion

In this retrospective study of patients with stage IV metastatic melanoma, we investigated a large panel of well-described immune (escape) markers in melanoma for their association with clinical outcome and OS upon subsequent (immuno)therapy. We have compared various T cell–related markers and immune-escape markers of melanoma, including preexisting level of IL-10 within the tumor did not influence the outcome of the GM-CSF–transduced tumor cell vaccination. Age and gender did not differ significantly between nonprogressors, progressors, and the nonvaccinated (P > 0.25 and P > 0.832, respectively). Therefore, the differences found between these groups are not influenced by these population characteristics. Taken together, our results show that T-cell infiltration and activation in the tumor tissue are more informative for clinical outcome than immune escape following autologous tumor cell vaccination.

Relation between immune-escape mechanisms in melanoma and OS

Correlations between immune (escape) markers and OS of vaccinated patients (i.e., nonprogressors and progressors together) were analyzed further by Spearman correlation tests (Fig. 4). Significant positive correlations were found for CD4, CD8, granzyme-B (P < 0.05), and OS. No significant correlation was found between OS and age and gender (P > 0.433 and P > 0.155, respectively), indicating that the results found are not influenced by these parameters. In addition, no prognostic significance of BRAF mutation status in our cohort of patients with stage IV melanoma was found (P > 0.634; Fig. 4).

Because the statistical tests (one-way ANOVA, Mann–Whitney, and Spearman tests) revealed a strong relationship between CD4, CD8, and granzyme-B with OS, these markers could be a clinically informative determinant in tumor tissue before starting immunotherapy for the clinical outcome of patients receiving an autologous tumor cell vaccine.
suppressive cells, T-cell inhibitory factors, and tolerogenic cytokines in one comprehensive analysis.

Our data show that the presence of CD4 T cells, CD8 T cells, and granzyme-B in the tumor tissue is associated significantly with a favorable clinical outcome, manifested in prolonged PFS and/or OS in patients receiving autologous tumor cell vaccination. These data indicate that assessment of these markers in the tumor tissue before immunotherapy, at least for autologous tumor cell vaccination, is informative for the OS of patients. The presence of suppressive cells such as Tregs, mast cells, and CD11b+/CD33+ MDSC, T-cell inhibitory factors IDO, galectins, PD-L1, and the loss of HLA class-I or melanocyte differentiation antigens was not associated with OS of patients in our study.

Successful eradication of tumor cells by T cells depends on the number of antigen-specific T cells within the tumor (TIL) and their ability to recognize tumor cells. Tumor cells can avoid T-cell–mediated attack by downregulating the expression of melanocyte antigen and/or HLA molecules (30–32) or by manifesting other immune-escape mechanisms. Previously, a positive correlation was found between the presence of intratumoral T cells and preserved HLA class-I expression in patients with stage IV melanoma that had failed at least one palliative chemotherapy regimen (32). We have reported that the loss of HLA-A2 expression in melanoma inversely correlated with the functional activation of melanoma-reactive T-cell responses in patients with advanced melanoma (22), indicating that the presence of HLA on tumor cells determines the T-cell effector function and consequently the antitumor immune response. In this clinical study, this relationship between the tumoral HLA-A2 expression and the clinical outcome was not assessable because of the low number of HLA-A2+ patients. Importantly, we show in the current study that increased numbers of intratumoral (cytotoxic and/or activated) T cells before immunotherapy positively correlate with the clinical outcome (i.e., PFS and OS) of patients with stage IV melanoma. Although our study is small in size, our results are in accordance with previous studies showing that the presence of tumor-infiltrating T cells is associated with a favorable outcome for these patients (33–37). These studies focused on (cutaneous) tumor or sentinel lymph nodes of patients with varying stages of melanoma. We hereby extend

| Nonprogressors vs. progressors vs. nonvaccinateda | 0.0260c | 0.0163c | 0.0027d | 0.0067d |
| Nonprogressors vs. progressorsb | 0.0303c | 0.0185c | 0.0034d | 0.0978 |
| Progressors vs. nonvaccinatedb | 0.5721 | 0.5128 | 0.8924 | 0.0095d |
| Nonprogressors vs. nonvaccinatedb | 0.0096d | 0.0097d | 0.0028d | 0.0050d |

NOTE: The table shows P values of the markers CD4, CD8, granzyme-B, and IL-10 in relation to clinical outcome, by comparing three groups (using one-way ANOVA test) or between two groups (using the nonparametric Mann–Whitney test). Bold P values are statistically significant.

aP value calculated by one-way ANOVA.
bP value calculated by the nonparametric Mann–Whitney test.
cP < 0.05.
dP < 0.01.

Figure 4. Correlation between immune (escape) markers and OS in patients with advanced melanoma treated with autologous tumor cell vaccine. The Spearman correlation test was performed to calculate the correlation coefficient $R$ between the marker and OS. Markers with more than 1% expression were subjected to this test. Only the markers significant at $P < 0.05$ or $P < 0.01$ are depicted in the box plots. ‘*, $P < 0.05$; ‘**, $P < 0.01$.  

<table>
<thead>
<tr>
<th>Mutation (BRAF/NRAS)</th>
<th>CD4</th>
<th>CD8</th>
<th>Granzyme-B</th>
<th>IL-10</th>
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<tr>
<td>CD4</td>
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<td>CD8</td>
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<td>Granzyme-B</td>
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<td>Active caspase-3</td>
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<td>TGF-β</td>
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these data by analyzing metastatic tumor tissues of a specific patient population, i.e., patients with stage IV melanoma before they receive immunotherapy. Together, published data and our data strengthen the importance of TILs for the prognosis of clinical outcome upon immunotherapy. Whether the prognostic value of T-cell infiltration in tumor tissue differs between various types of (immuno)therapy needs further investigation.

In our study, T-cell inhibitory factors and suppressive cells in the tumor tissue did not correlate with OS, suggesting that these immune-escape mechanisms do not have significant influence on the patients’ therapeutic outcome. However, the limited number of patients in this study does not allow the drawing of definite conclusions. A possible relationship between these parameters and OS may be more subtle and will be detectable in a larger cohort size. Many T-cell inhibitory factors and suppressive cells such as IDO, PD-L1, and MDSCs can inhibit T-cell activation both at the tumor site and in the tumor-draining lymph nodes. It is, therefore, possible that these immune-escape mechanisms might relate more to clinical outcome or OS when measured in the tumor-draining lymph nodes or other organs, than in the tumor tissue. This further indicates the importance of the compartment-specific analysis of biomarker expression for the potential association with clinical parameters.

In conclusion, our data indicate a more prominent role for T-cell infiltration and activation in the tumor tissue for clinical outcome than immune-escape mechanisms. Analysis of these tumor tissue characteristics before immunotherapy may, therefore, provide physicians with a tool to optimally select patients who will have increased chances of a favorable clinical outcome from the immunotherapy.

Disclosure of Potential Conflicts of Interest

C.J.M. Melief is employed as CSO of ISA Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biosstatistics, computational analysis): E.P.M. Tjin, E.H. Rosenberg, J. Sanders, P.M. Nederlof
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