T-Cell Activation Marker Expression on Tumor-Infiltrating Lymphocytes As Prognostic Factor in Cutaneous Malignant Melanoma

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ABSTRACT
The central role of T cells in antitumor immunity is well established. However, tumor progression, often seen in the presence of substantial lymphocytic infiltration, suggests that these T cells are not capable of mounting an effective immune response to control tumor growth. Evidence has accumulated that T lymphocytes infiltrating human neoplasms are functionally defective, incompletely activated, or anergic. Therefore, when characterizing the immune competent cells within lymphoid infiltrates of tumors, it is important to assess their activation state. We investigated the expression of two T-cell activation markers, interleukin 2 receptor α (CD25) and OX40 (CD134), by immunohistochemistry in primary cutaneous melanoma samples of 76 patients and analyzed it in relation to tumor stage and tumor progression (>5 years follow-up), as well as to patients’ survival. We found that the degree of infiltration by CD25+ and intratumoral OX40+ lymphocytes showed a tendency to decrease in thicker melanomas. The frequency of samples with high numbers of peritumoral CD25+ and OX40+ cells was significantly lower (P = 0.0009 and P = 0.0087, respectively) in melanomas developing distant visceral metastases, compared with nonmetastatic or lymph node metastatic tumors. For both activation markers studied, high peritumoral densities were associated with longer survival by univariate analysis (P = 0.0028 and P = 0.0255 for CD25 and OX40, respectively), whereas peritumoral OX40+ lymphocyte infiltration had an impact on survival also in multivariate analysis (P = 0.035). The results suggest that the presence of lymphocytes expressing the T-cell activation markers CD25 or OX40 shows correlation with tumor progression as well as with patients’ survival in cutaneous malignant melanoma.

INTRODUCTION
Human solid tumors are often infiltrated by lymphocytes [tumor-infiltrating lymphocytes (TILs)], mostly T cells. CTLs capable of specific lysis of autologous tumor cells and tumor-specific CD8+ T cells could be isolated from lymphocytes infiltrating human neoplasms, and an increasing number of tumor-associated antigens have been discovered that can stimulate CD4+ or CD8+ TIL after presentation by MHC class I or II, inducing tumor-specific cytokine production or lysis (1, 2). Moreover, selective expansion of tumor-specific CD8+ T cells at the tumor site, demonstrated in several tumor types, is probably indicative of an ongoing immune response (3, 4). However, as tumor progression is often seen in the presence of substantial lymphocytic infiltration, this immune response is apparently incapable of controlling tumor growth. There are several potential mechanisms that might explain the lack of effectiveness of antitumor immune mechanisms, including (among others) the suboptimal activation of TIL due to the loss or down-regulation of tumor-associated antigens or MHC class I molecules, insufficient presentation of antigenic peptides, the lack of costimulation, or the production of immune suppressive factors by cells in the tumor microenvironment (reviewed in Ref. 5). Evidence has accumulated that T lymphocytes infiltrating human neoplasms are functionally defective, incompletely activated, or anergic. T cells from freshly isolated TIL often have depressed proliferation capacity, antitumor cytotoxicity, or cytokine production (6, 7). Tumor cells have been shown to induce clonal anergy in antitumor T cells in vitro (8), as well as in vivo (9). In several tumor types, abnormalities in the T-cell receptor-associated signal transduction pathway have been found in tumor-infiltrating and peripheral blood T lymphocytes in cancer patients (reviewed in Ref. 10).

Although lymphoid infiltration has been considered to be a manifestation of host immune response against cancer, the pathophysiological importance of TIL has remained controversial. The presence of TIL has been correlated to tumor size, stage, and patients’ survival in a variety of human cancers, including colorectal, prostate, and stomach carcinomas (11–13). No such correlation was found in other tumor types, as in esophageal carcinoma (14), and an association of T-cell infiltration with shorter survival was reported in renal cell carcinoma (15). In several cases, overall degree of lymphoid infiltration was not related to survival, and an impact on prognosis could be found only if a distinction was made with respect to lymphocyte types, localization, tumor characteristics, or patient subgroups (16–18). In cutaneous melanoma, the published results are
contradictory; although some studies reported that a prominent lymphocytic infiltrate was an independent parameter associated with good prognosis, especially when lymphocytes infiltrating the tumor in vertical growth phase were taken into consideration (19–21), in others, partly based on similar methodology, no significant correlation was found, or lymphocyte infiltration was not independent predictor of survival (22–24).

The detection of TIL in itself might not be a sensitive marker of the immune response; therefore, the analysis of their activation state is equally important. There are few studies where functional activity or the expression of activation markers of infiltrating cells was taken into consideration. In renal cell carcinoma, the proliferative activity of intratumoral CD8+ cells proved to be independent prognostic factor (15). Poor survival was demonstrated in oral squamous cell cancer patients with stage III and IV tumors and in gastric carcinoma patients where TIL exhibited absent or low expression of the T-cell receptor-associated ζ chain (25, 26). Among known T-cell activation markers, HLA-DR and CD69 did not seem to correlate with the actual activation state of TILs being expressed on a high proportion of cells (generally higher than in normal peripheral blood lymphocytes; Refs. 7, 27, 28) probably because the expression of these markers persists for a protracted period after activation. In contrast, CD25 (interleukin 2 receptor), expressed on a relatively small proportion of TIL in most tumor types (6, 7, 27, 28), is considered as a result of recent antigen stimulation and at the same time has functional significance, playing a crucial role in the regulation of T-cell proliferation. It has been used for predicting or monitoring the effects of immunotherapeutical protocols on T-cell activation in melanoma, head and neck carcinoma, and colorectal carcinoma patients (29–31).

Another less frequently used but extremely specific marker of T-cell activation is OX40 (CD134). It is a transmembrane glycoprotein belonging to the tumor necrosis factor superfamily and expressed on recently activated T lymphocytes, primarily of the CD4+ subset (reviewed in Ref. 32). The expression of its ligand, OX40L (gp34), is less restricted, being found on a number of cell types, including B cells, dendritic cells, macrophages, and endothelial cells (32, 33). The engagement of OX40 by its ligand causes costimulation of the T cells and prevents their activation-induced cell death, enhancing long-term survival (32, 34, 35). Moreover, the OX40-OX40L interaction has been shown to be involved in the adhesion of activated CD4+ T lymphocytes to vascular endothelium (36). In murine models of autoimmune diseases, OX40+ T cells were confined to the inflammatory compartment, representing the T cells recognizing the local autoantigen (32). Similarly, OX40+ T lymphocytes have been detected in a series of autoimmune diseases in humans, where they are limited to the sites of active inflammation (37, 38). Recently, it was demonstrated that OX40 is also present on a portion of T lymphocytes in TIL and draining lymph nodes of mouse tumors as well as human melanomas, head and neck, breast, and colorectal carcinomas, where these cells are believed to represent tumor-specific T cells (39–42).

In this retrospective study, we investigated the level of expression of CD25 and OX40 by immunohistochemistry in primary tumor samples obtained from 76 patients with cutaneous melanoma and evaluated their relationship with tumor stage and the development of subsequent visceral metastases. We also attempted to correlate the extent of CD25+ and OX40+ T cell infiltration with the 5-year survival of the patients to assess their prognostic importance.

**PATIENTS AND METHODS**

**Patient Characteristics.** Archival tissue samples were obtained from 76 patients with primary cutaneous melanoma who underwent surgery between 1980 and 2000 at the Institute of Dermato-Venerology, Semmelweis University and at the National Institute of Oncology (Budapest, Hungary). Patients were selected to obtain a study group involving a higher number of medium thick or thick (>1.0 mm) melanoma samples than their normal ratio, which have a more uncertain prognosis than thin tumors. None of the patients received any anticancer treatment before surgery. With regard to pathology, slides of all cases were re-reviewed for the purpose of the study. Clinical and pathological characteristics are summarized in Table 1. The tumors were grouped into four thickness categories based on the current American Joint Committee on Cancer staging system (Ref. 43; ≤1.0, 1.01–2.0, 2.01–4.0, >4.0 mm) and into three categories according to disease progression during the follow-up period (nonmetastatic, lymph node metastatic, and visceral metastatic). Thirty-four patients had no metastases developed during the follow-up period, whereas 11 patients had metastases confined to regional lymph nodes, which were excised. Thirty-one patients had developed distant visceral metastases in addition to lymph node involvement. All surviving patients had follow-up data for at least 5 years. The 5-year

<table>
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<td>11/11 (100)</td>
<td>2/31 (6)</td>
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</table>

a Only regional lymph node metastases during the follow-up period (5 years).

b SSM, superficial spreading melanoma; NM, nodular melanoma; ALM, acral lentiginous melanoma; LMM, lentigo maligna melanoma.
survival of patients in both the nonmetastatic and the lymph node metastatic groups was 100%, whereas only 2 patients developing distant visceral metastases survived for >5 years (62 and 72 months); no patients died of melanoma-unrelated causes. The majority (61 of 62, 98%) of tumors thicker than 1.0 mm and 4 of 14 (29%) of thin melanomas entered vertical growth phase (defined according to criteria based on Clemente et al. (Ref. 44: Histopathologic Diagnosis of Melanoma, WHO Melanoma Programme Publications, no. 5). Ulceration was defined as absence of an intact epidermis overlying a major portion of melanomas (43). Tumors with clinical regression and/or histological signs of late regression (45) were not included in the study.

### Immunohistochemical Detection of T-Cell Activation Markers in Melanoma Samples

Three-μm sections cut from formalin-fixed, paraffin-embedded cutaneous melanoma samples were used in the studies. Immunohistochemistry was performed on deparaffinized serial sections after microwave antigen retrieval. Endogenous peroxidase activity was blocked with incubation of the slides in 3% H2O2 in methanol, and nonspecific binding sites were blocked with 20% goat serum. Monoclonal anti-CD25 (diluted 1:100; Novoceastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom) and anti-CD134 (diluted 1:40; PharMingen, San Diego, CA) were used as primary antibodies and mouse IgG1 (Sigma, St. Louis, MO) for negative control. Biotinylated antimouse/antirabbit immunoglobulin was used as secondary reagent, followed by streptavidin-peroxidase treatment (LSAB2 System, HRP: Dako, Glostrup, Denmark). Antibody binding was visualized with 3-amino-9-ethylcarbazole (Vector Laboratories, Inc., Burlingame, CA), then the slides were counterstained with hematoxylin.

**Double Immunohistochemical Labeling.** Double staining for activation markers CD25 or CD134 and lymphocyte subset markers CD3, CD4, CD8, or CD20 was performed in a subset of cases. Sections were treated as for single staining and incubated with the first primary antibody (monoclonal anti-CD25 or anti-CD134), followed by biotinylated antimouse/antirabbit immunoglobulin and streptavidin-peroxidase treatment (LSAB2 System, HRP: Dako). The peroxidase reaction was detected using Vector SG (gray; Vector Laboratories, Inc.). Then the second primary antibody was applied (polyclonal anti-CD3, monoclonal anti-CD8, and monoclonal anti-CD20cy, all from Dako diluted 1:100 or monoclonal anti-CD4, Novocas-

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**Fig. 1** A, OX40+ lymphoid cells in the periphery of thick (Breslow 5.6 mm) melanoma; B, OX40+ lymphoid cells in thin (Breslow 1.0 mm) melanoma. Although the majority of the cells is in the intratumoral stroma (S), OX40+ cells are also seen in contact with tumor cells (→). C, CD25+ lymphoid cells in a thin (Breslow 0.4 mm) melanoma. Note the positive cells both intratumorally (M) and in the peritumoral lymphoid infiltrate (P); D, ulcerated surface area of a medium thick (Breslow 2.8 mm) melanoma, labeled for OX40. Note the high density of positive lymphoid cells underneath the necrotic superficial zone (N). Pictures were taken using ×40 (A–C) or ×20 (D) objective.
tra Laboratories Ltd., diluted 1:20), followed by biotinylated antimouse/antirabbit immunoglobulin and streptavidin-alkaline phosphatase treatment (LSAB2 System, Alkaline Phosphatase; Dako). Sections were developed using fuchsin (fuchsia color; Vector Laboratories, Inc.); no counterstaining was applied.

**Evaluation of the Immune Reactions.** Slides were examined using a graticule of 10 × 10 squares, calibrated as 0.25 mm² at ×200 magnification. All counting was performed by the same investigator (A. L.), with the supervision of another pathologist (J. T.), both blinded to the clinical information. Because the distribution of stained cells in the tumors was heterogeneous, the entire tumor area was analyzed in every case, and density of positive cells/mm² is given. The number of CD25⁺ and OX40⁺ lymphocytes was registered separately in intratumoral (lymphocytes infiltrating melanoma cell nests) and peritumoral areas (lymphocytes distributed in the infiltrate along the margin and the base of melanomas). In the case of ulcerated tumors, lymphocytes infiltrating the areas of ulceration were not taken in account. The proportion of patients with significant densities of the different cell types was also calculated, using cutoff values set up separately for each cell type, based on the mean of the given variable in the whole patient group, with minor adjustment for better discriminating power in the case of peritumoral CD25⁺ cells.

**Statistical Analysis.** Statistical comparisons between cell densities in different tumor groups was made using the Mann-Whitney U test and Kruskal-Wallis test, whereas the standard χ² test was used for comparing the proportions of samples with high cell densities. The correlation between CD25⁺ and OX40⁺ cell densities was evaluated by using the Pearson test. The univariate analysis of survival was performed by the Kaplan-Meier method, and the statistical analysis was carried out by the log-rank (Mantel-Cox) test. In multivariate analysis, independent prognostic factors were determined by the Cox proportional hazards model. All statistics were calculated using the BMDP Statistical Software Pack.

**RESULTS**

**Patient and Tumor Characteristics.** Seventy-six patients with primary cutaneous melanoma were included in the study (Table 1). The patients, followed for a minimum of 5 years, were grouped in three categories according to disease progression: nonmetastatic; lymph node metastatic; and visceral metastatic. There was no significant difference between these groups in the distribution according to sex, localization, or the histological type of the tumor (Table 1). However, compared with the other two categories, a lower proportion in the non-

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**Fig. 2** Double staining of lymphoid cell populations for CD25 (developed by Vector SG, gray signal), and CD3 (A), CD20 (B), CD4 (C), or CD8 (D) markers (developed by fuchsin, red signal). A, in a CD3⁺ T-cell population, a few CD25⁺ cells can be identified. B, no double-positive (CD20⁺CD25⁺) cells are seen. C, expression of CD25 on CD4⁺ lymphocytes (black arrows). D, colocalization of CD25 and CD8 (black arrows). Cells solely positive for CD25 can also be found (white arrows). Pictures were taken using ×40 objective.
metastatic tumor group was found to be ulcerated because of their overrepresentation in the lowest thickness category (≤1.0 mm; Table 1). All patients with nonmetastatic or lymph node metastatic tumors survived for at least 5 years, whereas only 2 of 31 patients (6%) with visceral metastases lived >60 months (median survival: 32 months; range: 6–72 months).

**Immunohistochemical Detection of the Infiltration by CD25+ and OX40+ Cells.** CD25 and OX40 expression was detected predominantly on small lymphocytes inside melanoma cell nests and in the stroma surrounding tumor deposits (Fig. 1). Other cell types with morphology of histiocytes, monocytes, dendritic cells, or plasma cells were not labeled. Occasional larger lymphoid blast cells expressing these two markers were observed as well. Melanoma cells in the close vicinity of CD25+ and OX40+ lymphocytes did not show morphological signs of degeneration. Double stainings for the activation markers and lymphocyte subset markers (CD3, CD20, CD4, and CD8) demonstrated the expression of CD25 or OX40 in CD3+ (T-cell) areas of melanoma infiltrates, with the absence of expression on CD20+ B cells (Fig. 2, A and B). Expression of the activation markers on CD4+ and, less frequently, on CD8+ T cells has been observed (Fig. 2, C and D).

The density of CD25+ and OX40+ lymphocytes was determined in the primary tumors, distinguishing intratumoral (lymphocytes infiltrating the melanoma cell nests) and peritumoral infiltrate (lymphocytes surrounding the tumor deposits). In the majority of cases, peritumoral density of mononuclear cells positive for the two markers exceeded their intralesional density, CD25+ cells being more numerous in both locations (CD25+ intratumoral, 12.4 ± 15.4 cells/mm²; peritumoral, 88.2 ± 66.4 cells/mm²; OX40+ intratumoral, 4.9 ± 6.3; and peritumoral, 19.8 ± 21.0 cells/mm²). Positive cells for either CD25 or OX40 were found only occasionally in the uninvolved skin or dermal areas. A considerably high (up to 200 cells/mm²) density of mononuclear cells expressing the studied activation markers were observed in ulcerated areas in 14 of 38 (37%) of cases with ulceration (Fig. 1D); this was considered to result from reactive inflammatory infiltrates secondary to ulceration, and such areas were excluded from the evaluation. The density of CD25+ and OX40+ lymphocytes in the melanoma samples correlated with each other (P < 0.001). With the exception of peritumoral OX40+ cells, the intensity of infiltration showed a trend of decreasing in thicker melanomas, which was statistically not significant (Fig. 3).

**Correlation of Infiltration by Activated T Cells with the Metastatic Potential of the Tumors.** The intensity of infiltration by CD25+ and OX40+ lymphocytes was studied in tumors that either did not metastasize or gave regional lymph node metastases during the follow-up period (5 years) and compared with tumors developing visceral metastases (Fig. 4). Nonmetastatic and lymph node metastatic cases were evaluated together because there was no significant difference in the intensity of infiltration of these tumors by either of the cell types studied (data not shown). Moreover, the relative malignancy of the disease, reflected by survival data, was identical in patients belonging to these two groups (100% 5-year survival). Intratumoral infiltration by CD25+ mononuclear cells did not show correlation with the metastatic behavior of the tumors. Intratumoral density of OX40+ lymphocytes, on the other hand, was more pronounced in nonmetastatic or lymph node metastatic tumors, compared with visceral metastatic ones (P = 0.0552, borderline significance). CD25+ lymphocytes showed a decreased peritumoral density in visceral metastatic tumors (P = 0.0079). Similar tendency was observed in the case of OX40+ lymphocytes.
cells, but the difference did not reach statistical significance ($P = 0.1070$; Fig. 4).

**Correlation of the Ratio of Patients with Significant Density of Activated T Cells with Patient and Tumor Characteristics.** For both activation markers studied, cutoff values (based on the mean number of the appropriate cell population in the whole patient group; shown in Fig. 4) were introduced, and the proportion of patients with “significant cell density,” defined as higher than the cutoff value, was calculated. The distribution of patients with significant (intratumoral or peritumoral) density of cells expressing T-cell activation markers was analyzed according to patient and tumor characteristics (Table 2). There was no significant difference in the ratio of patients with high cell densities when the tumors were distinguished according to localization, tissue type, ulceration, or the gender of the patient. Similarly to the absolute numbers of labeled cells/mm$^2$ (Fig. 3), the proportion of patients with significant CD25$^+$ or intratumoral OX40$^+$ lymphocyte frequencies exhibited a tendency of decreasing in melanomas thicker than 2.0 mm (Table 2; not significant).

**Correlation of the Ratio of Patients with Significant Density of Activated T Cells with the Metastatic Potential.** Using the cutoff values described above, we evaluated the relationship between the proportion of patients with marked density of lymphocytes expressing CD25 or OX40 and the metastatic behavior of the tumor. There was no difference between the nonmetastatic or lymph node metastatic tumors compared with visceral metastatic ones in the ratio of patients with marked intratumoral CD25$^+$ or OX40$^+$ cell infiltration. However, highly significant difference was found between the two patient groups when peritumoral densities of both cell populations were compared (Table 3). The difference remained significant even if tumors of $\leq 1.0$ mm thickness were excluded (this group, consisting exclusively of nonmetastatic tumors, was characterized by the highest level of infiltration by lymphocytes expressing either CD25 or OX40).

**Correlation of the Ratio of Patients with Significant Density of Activated T Cells with Survival.** The intratumoral density of CD25$^+$ or OX40$^+$ cells did not show correlation with the survival of the patients (Fig. 5). However, high peritumoral infiltration by either CD25$^+$ or OX40$^+$ lymphocytes provided significant survival advantage ($P = 0.0028$ and $P = 0.0255$, respectively). In the case of CD25, the difference remained significant when only tumors thicker than $1.0$ mm were included ($P = 0.0032$), with a borderline significance in the case of OX40 ($P = 0.0775$). The percentage of patients with $>5$ years survival was higher in the case of tumors characterized by significant numbers of peritumoral CD25$^+$ or OX40$^+$ lymphocytes ($78$ versus $46$ and $79$ versus $52\%$, respectively). Multivariate analysis (Cox’s proportional hazards model) considering the density of CD25$^+$ and OX40$^+$ lymphocytes, taken together with other prognostic factors (tumor thickness, localization, histological type, ulceration, and patient sex), identified the thickness of the tumors (Breslow index) and peritumoral OX40$^+$ cells as significant independent prognostic markers ($P = 0.001$ and $P = 0.035$, respectively).

**DISCUSSION**

Pathological and clinical factors predicting outcome of malignant melanoma have been extensively studied in multivariate analyses, including significant numbers of patients. In most studies, Breslow thickness has proved to be the most powerful predictor of survival in localized melanomas, followed with ulceration according to the largest, recent prognostic factor analysis comprising 17,600 patients (43). However, unexpected behavior of a certain proportion of melanomas can be observed, which cannot be explained on the basis of these or other prog-
nostic factors (e.g., invasion level, anatomical site, mitotic rate, or patient’s age or sex). Overall error rates using tumor thickness alone or a six-variable model were reported to be 24.2 and 15.9%, respectively (20), but the accuracy of prediction is lower in patients with thick melanomas. Thus, new markers that prove to be of prognostic value could be useful to better predict patient outcome.

In the present study, we analyzed the expression of the T-cell activation markers CD25 (interleukin 2R/IL2RA) and CD134 (OX40) in lymphoid cells infiltrating human malignant melanomas. We demonstrated, for the first time, that the intensity of infiltration by lymphocytes expressing these molecules has a significant impact on patients’ survival. Patients with high peritumoral density of CD25+ or OX40+ lymphocytes had superior survival when compared with patients with low numbers of these cells. Importantly, in the case of CD25, this difference in survival remained significant when only melanomas thicker than 1.0 mm were included in the study, representing a group characterized with heterogeneous metastatic potential and prognosis, unlike melanomas ≤ 1.0 mm that were all nonmetastatic with 100% survival in this study (Table 1). In multivariate analysis evaluating the factors significant in univariate analysis, taken together with other established prognostic indicators in human melanoma, peritumoral OX40+ lymphocyte infiltration,

<table>
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<th>CD25</th>
<th>OX40</th>
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<td>Visceral metastatic</td>
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</tbody>
</table>

Data are expressed as number (%) of patients with significant cell density (defined as values exceeding population average).

n.s., not significant.

ALM (2) and LMM (1) cases are not shown.

Table 3  Correlation of the density of activated T cells with metastasis formation

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Data are expressed as number (%) of patients with significant cell density (defined as values exceeding population average).

n.s., not significant.
beside Breslow index, was identified as significant independent predictor of survival. The effect of peritumoral CD25⁺ cell density was not significant in this case, probably because of a weak association ($r = -0.1749$, $P < 0.15$) between the expression of this activation marker and the thickness of the tumor.

In accordance with their effect on survival, higher CD25⁺ and OX40⁺ cell densities were found in melanomas that did not develop visceral metastases during the follow-up period (5 years). At the same time, a (statistically not significant) inverse correlation was observed between the degree of infiltration by the studied cells and the thickness of the tumors, with the exception of peritumoral OX40⁺ lymphocytes. A similar association was found in a previous study for CD25 (46), however, without referring to the localization of the infiltrate (intratumoral or peritumoral) or to the metastatic potential of the tumors, although it is mentioned that melanoma metastases contained decreased numbers of interleukin 2R-positive cells.

Although several studies used CD25 as a marker of T-cell activation for the evaluation of the immunological effects of immunotherapeutical modalities or in search for factors predicting the effectiveness of such treatment schedules (29–31), to our knowledge, it has not been studied as a potential prognostic marker in melanoma or other tumor types. Still, less data are available on the presence and importance of OX40 expression in lymphocytes infiltrating human tumors. OX40-positive cells had been detected by flow cytometry in primary tumors and draining lymph nodes in melanoma and head and neck carcinoma patients and by immunohistochemistry in breast carcinoma patients (39, 40). Recently, a pioneer study using semi-quantitative evaluation found that increased expression of the OX40 molecule in TIL correlated with survival in colorectal cancer (42). Collectively, our results on melanoma and the findings on colorectal carcinoma patients suggest that the density of OX40-positive lymphocytes might prove to have relevance as a prognostic factor in human neoplasms of different origin.

In the present study, peritumoral density of lymphocytes expressing OX40 or CD25 was associated with survival of melanoma patients while intratumoral infiltration was not. The reason for this discrepancy could be that the intratumoral density was much lower for both markers than their peritumoral density, and apart from a few outstanding values, patient-to-patient variation was low. Thus, these variables did not differentiate well between samples. Also, the lack of an effect of intratumoral infiltration by CD25⁺ and OX40⁺ lymphocytes on patient outcome might indicate that the majority of these cells are CD4⁺ lymphocytes, with a helper rather than effector function, which would not require a direct contact with tumor cells. In accordance with this, the most intense infiltration by CD25⁺ and OX40⁺ lymphocytes was seen within peritumoral infiltrates, generally not in close contact with melanoma cells, whereas intratumoral-positive cells were scattered in most cases. The lack of direct cytotoxicity by CD25⁺ or OX40⁺ cells seems to be supported by our finding of the absence of significant numbers of melanoma cells with morphologically detectable signs of degeneration or cell death in the vicinity of lymphocytes expressing these markers.

With regard to the lymphocyte subsets expressing the two
activation markers, OX40 had been previously detected exclusively on T cells, with a preferential expression on CD4+ lymphocytes in both murine models and humans (32, 37, 47), although, to a lesser extent, CD8+ T cells expressing the OX40 molecule could be identified after stimulation of human or mouse lymphocytes or in TIL of certain mouse tumors (34, 41, 47). CD25, on the other hand, can be expressed by both T-cell subsets, although in resting or activated PBL, as well as in TIL in the case of several tumor types, a higher number of CD4+ than CD8+ cells was found to express interleukin 2 receptor (28, 48, 49). In harmony with these data, in human melanoma samples, both CD25 and OX40 expression was found to be restricted to T cells, and colocalization with CD4 and on some cells with CD8 has been observed.

An intriguing observation in our study was the increased frequency of infiltrating lymphocytes expressing CD25 and OX40 in ulcerated areas in several melanoma samples. The high expression of activation markers was probably due to the effect of bacterial and cytokine milieu in the areas of tissue damage. This is in accordance with the finding that lipopolysaccharide up-regulated OX40 expression on antigen-activated mouse T cells, probably through the induction of inflammatory cytokines (50). According to the application of the “danger theory” to tumor immunology, the CD25- and OX40-expressing lymphocytes in ulcerated areas of melanomas could represent T cells recognizing tumor antigens, fully activated by antigen, costimulated by antigen-presenting cells in ulcerated areas by chemoattractants. In any case, the demonstrated expression of activation markers was probably due to the effect of bacterial and cytokine milieu in the areas of tissue damage.

In conclusion, we have shown that the increased density of CD25+ and OX40+ lymphocytes in the peritumoral infiltrate of the primary tumor was associated with a lower occurrence of distant metastases and longer survival in patients with cutaneous melanoma. Besides its prognostic importance, evaluation of the expression of these activation markers on TILs could prove useful in defining subgroups of melanoma patients that would most probably benefit from immunotherapeutical protocols, as well as in monitoring the response to such treatment modalities.

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T-Cell Activation Marker Expression on Tumor-Infiltrating Lymphocytes As Prognostic Factor in Cutaneous Malignant Melanoma

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