Utility of tumour-infiltrating CD25+FOXP3+ regulatory T cell evaluation in predicting local recurrence in vertical growth phase cutaneous melanoma

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Abstract. Tumour-infiltrating lymphocytes (TILs) represent the local immune response to cancer, however, their correlation with tumour behaviour is not unanimously considered in the literature. Most studies have not characterized TILs, that are known to comprise distinct subsets, bearing different roles in the complex tumour microenvironment. Characterization of patient lymphocytes has been mainly performed in peripheral blood, that is not always representative of the local immune status. Only few investigations have been performed at the tissue level in cancer, including melanoma. TILs encompass different populations of effector and regulatory T cells (Tregs), and the relevance of the latter in tumour progression is widely accepted. The transcription factor gene product FOXP3 is considered the most reliable marker of Tregs. However, it has not been extensively evaluated in primary cutaneous melanoma. We analyzed 66 vertical growth phase primary cutaneous melanomas, aiming at finding differences in TIL subsets between two groups of cases, that behaved differently in terms of local recurrence. In our study, the percentage of Tregs, as characterized by CD25 and FOXP3 expression, both among tumour cells, inside tumour parenchyma and at its periphery, and among TILs, at the tumour-stroma boundary, was significantly higher in cases that recurred than in those that did not ($p=0.00065$; $p=0.00014$; $p<0.00001$, respectively). TIL characterization by immunohistochemistry in melanoma diagnostic reports, could add further information. The analysis of a larger series of patients and correlation with other clinical parameters, such as distant metastases and/or patient survival, are mandatory for validating its use as a prognostic indicator.

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Introduction

Tumour-infiltrating lymphocytes (TILs) are considered a prognostic factor in human cutaneous melanoma, thus they should be evaluated in diagnostic reports (1,2). However, some studies failed to support their prognostic role in cancer, and their pathophysiological importance remains controversial (2-6). In the tumour microenvironment, TILs encompass various subsets of lymphocytes with different and sometimes opposite functions. Their immunophenotypic characterization is still incomplete. Furthermore, additional factors including cytokines, as well as mechanisms of tumour cell immune evasion can influence TIL effects, resulting in a complex humoral and cell-mediated immune response (7-9).

The majority of TILs are CD3+ T cells: they include CD4+ and CD8+ subsets, the latter comprising cytotoxic lymphocytes, which are important effectors against melanoma cells; their numbers usually correlate with a more favourable prognosis (1,2). Cytotoxic CD8+ lymphocytes include CD3+ and CD3- (natural killer) cells, and both are characterized by the expression of granzyme b, a serine protease, and TIA-1, a membrane-associated protein, which can induce apoptotic cell death, therefore resulting in tumour cell killing and melanoma regression (9,10). The expression of CD25 (the α chain of the IL-2 receptor) allowed for grouping CD4+ cells in two broad subsets, T helper and T regulatory cells, and showed quite different characteristics (11). The CD8+ T cell activity against tumour and patient survival is enhanced by CD4+ CD25- (T helper) cells, and downregulated by CD4+ CD25+ (T regulatory, Treg) cells, that mediate active suppression, another mechanism that sustain unresponsiveness towards self-antigens, besides deletion or functional inactivation of self-reactive lymphocytes (11,12). However, CD25 was found to be expressed also by activated effector T cells (13). This partly explains the contrasting results of many previous studies, that did not look at more specific markers of Tregs (13,14).

Recently, a population of CD4+ lymphocytes with strong regulatory activity on effector T cells was isolated in the thymus and peripheral blood. Additionally to the high levels of CD25, they specifically express the forkhead-winged helix transcription factor gene product Foxp3 (CD4+CD25high+

FOXP3+ Treg cells) (14). FOXP3 is required for the generation and activity of the Tregs, and, is considered their most reliable marker, being specifically expressed by CD4+CD25high+ Treg cells (13,14). Natural CD4+CD25high+ Foxp3+ Tregs are able to prevent reactivity to both self- and nonself-antigens (14). A population of CD4+CD25+FOXP3+ Tregs (called 'adaptive Tregs'), which inhibits the CD4+CD25- effector T cells, was demonstrated to develop outside the thymus and peripheral blood (15,16). Adaptive Tregs are induced by different stimuli, including the presence of IL-10 and TGF-ß; alike natural Tregs, peripheral Tregs are characterized by cytokine dependence (15,16).

Recently, Tregs were identified in normal skin (17), where they may regulate the local immune response. Tregs have been demonstrated in experimental and naturally-occurring cancerous and non-cancerous diseases, in the lesional tissue, where they may be important in inducing peripheral tolerance and inhibiting effector T cells (13,15,18). In human cancer, they have usually been correlated to an unfavourable course of the disease, although there is a limited number of studies at the tissue level that used FOXP3 as their specific marker (13). A higher frequency of CD4+CD25+ Treg cells in both peripheral blood and tumours was reported in patients with melanoma (19,20).

Overexpression and increased activity of CD25+FOXP3+ Tregs, as well as a decreased function of CD8+ T cells, have been observed at the tissue level in melanoma metastases, and correlated with tumour progression (20,21). CD4+CD25+ Foxp3+ Tregs have not yet been extensively investigated at the tissue level, in primary cutaneous melanoma.

In this retrospective study, we analyzed CD4+/CD25+ FOXP3+ Tregs by immunohistochemistry in primary tumour samples obtained from 66 patients with cutaneous melanoma in the vertical growth phase and evaluated their correlation with tumour relapse.

Materials and methods

Cases. Archival tissue samples were obtained from the files of Pathological Anatomy - Department of Human Pathology and Oncology of the Siena University.

We examined 66 cases of vertical growth phase primary cutaneous melanomas, as defined by current histological criteria (22), from patients who underwent surgery between 1990 and 2000. Patients with a 5-year minimum follow-up were selected who did not receive any immunotherapy or chemotherapy before surgery. Melanoma stage was established according to the American Joint Committee on Cancer (Table I) (23).

All cases were revised by two pathologists (C.M., P.L.) and diagnosis of melanoma was confirmed in all cases, according to the current histological criteria (22). In all cases, melanomas were removed, with excisional margins 1 cm away from the tumour. Cases were grouped into two categories: group A, comprising of 35 cases that neither recurred nor metastasized, and group B, comprising of 31 cases that recurred locally, in the dermis and subcutis within the scar area. Clinical and pathological characteristics are summarized in Table I. The two groups were similar for main histological prognostic factors (i.e. melanoma thickness and level, mitotic rate,

ulceration, regression, vascular/perineural invasion). Group A included 31 level IV, and 4 level V melanomas (median thickness: 2.2 mm; range: 1.3-4.2 mm). Group B included 29 level IV and 2 level V melanomas (median thickness: 2.1 mm; range: 1.2-4.4 mm).

Written informed consent was obtained before each patient was included in the study, that was approved by local ethics committees, and performed in accordance with the principles of the World Medical Association Declaration of Helsinki.

Immunohistochemical procedures. Tumour-infiltrating lymphocytes (TILs) were characterized in 4 *μ*-thick serial sections cut from formalin-fixed, paraffin-embedded, tissue specimens of the most representative tumour areas. Immuno-histochemistry of deparaffinated sections was carried out for CD3, CD4, CD20, CD8, granzyme b, CD25 and FOXP3. CD3 (CD3 polyclonal antibody, Bio-Optica, Milan, Italy, dilution 1:1000) and CD4 (CD4 polyclonal antibody, 4ß12, Menarini, Florence, Italy, dilution 1:50) antibodies were applied to sections for 60 min at room temperature, after antigen retrieval in Wcap buffer (pH 6.0, 98˚C, for 40 min), and after using the Ultravision detection system anti-polyvalent HRP (LabVision, Bio-Optica). Diaminobenzidine (Dako, Milan, Italy) served as chromogen.

Microwave pre-treatment and the Ultravision LP detection system AP polymer (Lab Vision) were used for CD20 (CD20 monoclonal antibody, clone L26, Neomarkers, BioOptica, dilution 1:150), CD8 (CD8 monoclonal antibody, CD8-144B clone, Dako, dilution 1:50) and granzyme b (granzyme b monoclonal antibody, GZBO1 clone, Bio-Optica, dilution 1:100); after incubation with the antibodies for 60 min at room temperature, staining was developed by using Fucsin (Dako) as chromogen. Anti-human CD25 (CD25 polyclonal antibody, Bio-Optica, dilution 1:50; trypsin pre-treatment) and FOXP3 (FOXP3 monoclonal antibody, Abcam, DBA, Milan, Italy; dilution 1:50, microwave pre-treatment) were used for double immunohistochemistry. Briefly, after antigen unmasking in Wcap, the Ultravision LP detection system AP polymer was used.

For nuclear staining, FOXP3 was applied for 60 min at room temperature, and Fucsin (Dako) was used as chromogen. For cytoplasmic staining, sections were put in 3% H_2O_2 for 10 min. The ultravision detection system anti-polyvalent HRP was used; sections were then incubated with CD25, for 60 min at room temperature, and DAB was used as chromogen. Double immunohistochemistry for FOXP3 and CD4 was also performed, by following all the steps above as described for the single stains. For each case, a negative control was obtained by replacing the specific antibody with non-immune serum immunoglobulins at the same concentration of the primary antibody. All sections were counterstained with Harris hematoxylin, dehydrated in alcohol, cleared in xylene, and coverslipped. The slides were independently evaluated by two observers (C.M., P.L.).

Evaluation of the tumour infiltrating lymphocytes (TILs). CD3+ and CD4+ percentage was calculated on the total number of lymphocytes. CD8+ granzyme b+ tumour-infiltrating cyto-toxic lymphocytes, and CD4+/CD25+FOXP3+ Treg cells were

Table I. Patient and tumour characteristics.

	All patients	Group A	Group B
No. of patients	66	35	31
M age (R)	65 (48-77)	66 (50-77)	63 (48-72)
Sex			
Female	36	20	16
Male	30	16	14
Localization			
Head	29	16	13
Extremities	22	10	12
Trunk	15	9	6
Type[a]			
ALM	3	1	2
LMM	29	16	13
NM	3	1	2
SSM	31	16	15
Level IV	60	31	29
Level V	6	4	2
Thickness-M(R)	2.2 (1.2-4.4)	2.2 (1.3-4.2)	2.1 (1.2-4.4)
Mitotic rate	5 (0-10)	5 (0-10)	4 (0-9)
Ulceration			
Present	21	13	11
Absent	45	22	20
Regression (f,m,mr)	27 (13:f; 13:m; 1:mr)	12 (5:f; 6:m; 1:mr)	15 (8:f; 7:m)
Brisk LI	7	4	3[b]
VI, PnI	VI(60); PnI(7)	VI(31); PnI (3)	VI(29) PnI(4)
Metastases	5	0	5 (SnLN)
Stage[c]			
I B pT2a N0 M0	31	18	13
II A pT2b N0M0	24	13	11
II B pT4a N0 M0	6	4	2
III A pT2a pT3a N1a	5	0	5
Recurrence[d]	31	0	31
5-year survival (%)	66 (100%)	35 (100%)	29 (94%)[e]

M(R), Median (range). [a]ALM, acral lentiginous melanoma; LMM, lentigo maligna melanoma; NM, nodular melanoma; SSM, superficial spreading melanoma; Brisk LI, brisk lymphocytic infiltrate; LNM, lymph node metastatic; Regression: (f,m, mr) (focal, moderate, marked), see ref. 2. VI, vascular invasion; PnI, perineural invasion. [b]All of the 3 patients developed visceral metastases during the last two years of follow-up. SnLN, sentinel lymph node. [c]Stage according to the current American Joint Committee on Cancer staging system, see ref. 22. [d]All melanomas recurred within two years; in 25 out of 31 cases recurrence occurred during the first year after surgical excision. [e]Two patients developed visceral metastases and died of disease (40 and 57 months after surgery, respectively).

evaluated in immunostained sections. Immunostained TILs were counted in serial sections and in at least 10 randomly-chosen high power fields (x40 objective and x10 eyepiece; 0.16 mm² per field), both at the tumour parenchyma, at its periphery (tumour-host interface), and at the tumour-stroma boundary. Ulcerated areas were avoided. When a radial growth phase was present, it was excluded from the counts, and only the vertical growth phase was evaluated. Numbers of labelled TILs both per 100 tumour cells (inside tumour parenchyma and at tumour periphery), and per 100 lymphocytes (at tumour-stroma boundary) were assessed. Quantification of the absolute numbers of cytotoxic lymphocytes was performed both on granzyme b- and CD8-stained sections. Quantification of absolute numbers of Tregs was performed both in FOXP3-CD25 and in FOXP3-CD4 double immunostains. As for quantification among TILs, for cytotoxic lymphocyte
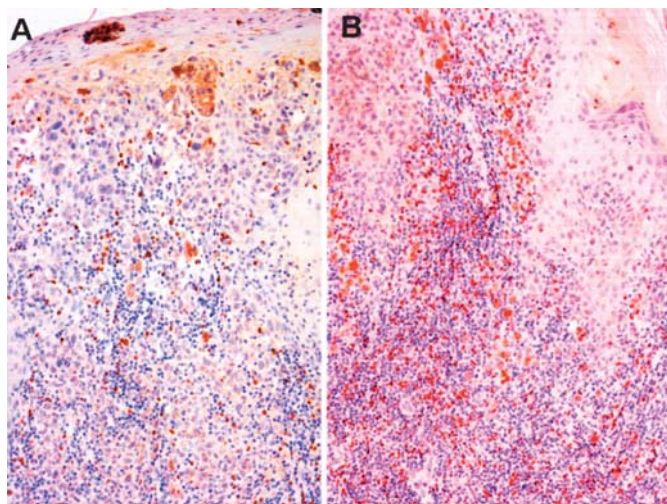
Figure 1. Tregs are less numerous in a case from group A (A), than another from group B (B) melanomas, both showing brisk TIL. The latter melanoma recurred and metastasized to the brain. Nuclei of Tregs are stained in red. CD25-FOXP3 double immunostaining; original magnification of x100.

Figure 3. A case from group B showing Tregs inside melanoma parenchyma (A) and sparse infiltrating lymphocytes at tumor periphery, among which Tregs are detectable (B). A case from group A showing few Tregs inside tumor parenchyma (C; C, inset). CD25-FOXP3 double immunostaining; original magnification: A and C, x200; B and C, inset, x400.
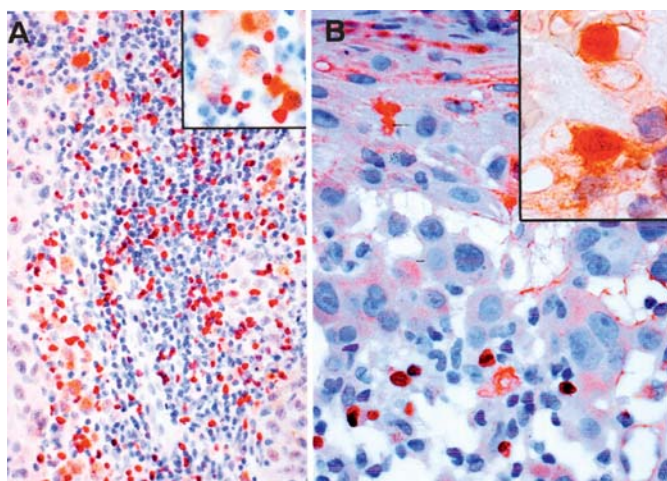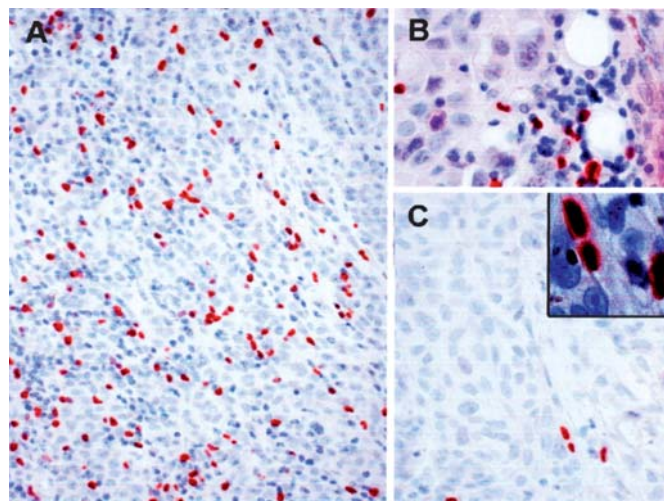

Figure 2. The same case of group B as depicted in Fig. 1B at higher magnification: admixed with tumor cells and lymphocytes (A), many macrophages are recognizable, some of which very close to Tregs (A, inset). A case of group A showing CD25+FOXP3+ cells intermingled with lympho-cytes and neoplastic cells (B); detail of CD25+FOXP3+ cells (B, inset). CD25-FOXP3 double immunostaining; original magnification: A, x200; A, inset, x400, B, x400; B, inset, x1000.

assessment, the percentage of CD8+ and granzyme b+ lymphocytes was calculated on the total number of TILs; for Tregs, the percentage of CD25+FOXP3+ Tregs was assessed among CD4+ lymphocytes.

Reproducibility of all features was assessed by 2 independent observers (C.M., P.L.) in 6 cases (3 group A and 3 group B), by repeating counting procedures 3 times. The coefficient of intra- and interobserver correlation was always >0.9.

Statistics. Mean percentages + SD of immunostained TIL were calculated and compared in each group and between the two groups of patients by the non-parametric Kruskal-Wallis test. The correlation among variables in each group was studied by means of Pearson's correlation coefficient. The significance level was set at p<0.05.

Results

Histological examination revealed that TILs were sparse-to-moderate in 32 out of 66 cases. A heavy-to-moderate lymphocytic infiltrate was demonstrable in 34 out of 66 cases, in 27 cases (12, group A; and 15, group B) it was associated with focal/moderate features of intermediate regression (abundant pigment-laden macrophages, fibrosis, vessel proliferation), according to Barnhill *et al* (22). In 7 cases (4, group A; and 3, group B), there were brisk TILs, as defined by Clemente *et al* (1). In all cases, most TILs were at the tumour-stroma boundary. In the tumour parenchyma, they were more numerous at tumour periphery.

Immunohistochemical evaluation. In both groups, >90% of TILs expressed CD3, and most (90-99% among CD3+ TIL) were CD4+, whereas CD20 cells constituted a minority of cells (1-5%). With both CD4-FOXP3 and CD25-FOXP3 double staining, Treg nuclei were stained in red; their cytoplasm was brownish. Some representative cases are depicted in Figs. 1-3. Almost all FOXP3+ cells were CD4+ T cells, and the majority of the FOXP3+ cells were CD25+. In both groups, CD4+/CD25+FOXP3+ cells were more numerous at tumour periphery, and admixed with lymphocytes, at the tumour-stroma boundary; in some cases they were also detectable in the epidermis. In most cases, when present inside the melanoma parenchyma, CD4+/CD25+FOXP3+ cells were not associated with other lymphocytes; in many group B cases they were very close to tumour infiltrating macrophages.

Table II. Mean percentage and SD values of CD25+FOXP3+ and granzyme b+ TILs as calculated on tumour cells in the melanoma parenchyma (%/TCc), at its periphery (%/TCp), and at tumour-stroma boundary, respectively on CD4+ (CD4+L), and total lymphocytes (TL) in groups A (a) and B (b).[a]

a, Group A

	CD25+FOXP3+ TILs			Granzyme b+ TILs		
	%/TCc	%/TCp	%/CD4+L	%/TCc	%/TCp	%/TL
M	0.015 (0.0-0.05)	0.042 (0.0-0.2)	0.844 (0.0-6)	3.56 e-3 (0.0-0.08)	0.016 (0.0-0.08)	4.2 (1-10)
SD	0.018	0.039	1.44	0.02	0.03	2.5

b, Group B

	CD25+FOXP3+ TILs			Granzyme b+ TILs		
	%/TCc	%/TCp	%/CD4+L	%/TCc	%/TCp	%/TL
M	0.752 (0.0-4)	1.66 (0.0-10)	13.78 (5-25)	0.013 (0.0-0.1)	0.108 (0.0-2)	4.02 (0.05-9)
SD	1.22	2.38	5.46	0.024	0.373	2.4952

	%/TCc	%/TCp	%/CD4+TL
CD25+FOXP3+ TILs (Group A vs. Group B), p=	0.00065	0.00014	<0.00001

	%/TCc	%/TCp	%/TL
Granzyme b+ TILs (Group A vs. B), p=	NS	NS	NS

[a]Range of values are reported in brackets. p-values are also reported. M, Mean; SD, standard deviation; NS, not significant.

In most cases, CD8+, granzyme b+ TILs, in both groups, were less numerous than CD4+/CD25+FOXP3+ cells; they were less frequently detectable in the melanoma parenchyma. The two groups did not show statistically significant differences in CD3+ and CD4+ percentage of lymphocytes. In each case, there were no statistically significant differences either between the absolute number of CD8+ and granzyme b+ TILs (p>0.05), or between the absolute number of CD25+FOXP3+ and CD4+FOXP3+ TILs (p>0.05). Therefore, the percentage of granzyme b+ lymphocytes on total TILs, and the percentage of CD25+FOXP3+ on total CD4+ TILs were used for cytotoxic and Treg cell percentage assessment, respectively.

Cytotoxic and Treg cell percentage did not significantly differ when comparing various histogenetic types in each group. There were no statistically significant differences between group A and B in cytotoxic lymphocyte percentage. The percentage of CD25+FOXP3+ Tregs was, instead, significantly higher in group B than in group A, both when calculated in melanoma cells in the tumour parenchyma (p=0.00065), at its periphery (p=0.00015), and, at tumour-stroma boundary, among CD4+ TILs (p<0.00001). There were

no significant correlations among variables in each group. Mean percentage values with standard deviation (SD) and significant p-values are reported in Table II.

Discussion

The immune system plays an important role not only in inhibiting cancer development but also in promoting its growth, based on a process that has been called 'cancer immunoediting' (18). Melanoma is immunogenic enough to induce host responses, and cell-mediated immunity plays a determining role, as documented by the occurrence of local, spontaneous complete regression, although tumour progression is the rule (2). Tumour-infiltrating lymphocytes (TILs) are involved in the control of tumour development; they have been related to tumour size, stage, and patient survival in melanoma, as well as in other human cancers, although the results of these studies were contradictory (1,2,4-6). In diagnostic reports, the intensity of TILs is evaluated and categorized as brisk, non-brisk, and absent (1). Brisk intratumoural T-cell infiltrates have been correlated with a low rate of tumour recurrence and increased patient

survival (1,2). However, melanoma progression is often seen in the presence of brisk TIL, as we also observed in three patients of group B.

Besides numbers and localization at the tumour site, immunophenotypic differences among TIL subsets, as well as impaired lymphoid cell differentiation and functions are relevant in local immune response (5,24). The detection of TILs in itself, is not always informative on the local immune response status and, therefore, might not be a reliable prognostic marker. There is a growing agreement on the necessity of characterizing TILs, as well as studying their functionality, for prognostic and therapeutic purposes (4,5,25,26). TIL functionality may be investigated through methods that are still indaginous and hampered by practical problems, whereas their characterization is today facilitated by the existence of commercial antibodies. In our study, TIL immunophenotyping was informative and allowed for distinguishing cases that recurred (group B) from cases that did not (group A), after a 5-year follow-up.

In agreement with Hussein *et al* (4), we found that the majority of TILs were $CD3^+$ (including $CD4^+$ and $CD8^+$ cells), with a negligible $CD20^+$ component. In our experience, most TILs were $CD3^+CD4^+$, with granzyme b^+ cytotoxic cells that constituted 1-10% and 1-9% of TILs in group A and in group B, respectively. This means that cytotoxic TILs were not determinant in contrasting tumour relapse in our cases. Groups A and B did not even differ in the percentages of $CD4^+$ cells, that ranged from 90 to 99% of total $CD3^+$ TILs in both groups. What significantly distinguished the two groups of patients was the percentage of Tregs, both among tumour cells and lymphocytes, as assessed after double immuno-staining with CD25 and FOXP3 antibodies. Lymphocytes bearing cytotoxic properties comprise two effector cell populations: $CD3^+CD8^+$ cytotoxic lymphocytes that become activated following recognition of tumour-specific antigens and require a proper MHC-1 complex, and $CD3^-CD8^+$ natural killer cells, that induce tumour cell death in absence of MHC-1 (7,27). We observed low percentage of cytotoxic TILs. This is in line with other investigations, that demonstrated a decrease of cytotoxic TILs in advanced cancer (28).

Among TILs, the subset of $CD4^+CD25^+FOXP3^+$ Tregs mediate immune suppression through a cell-cell contact mechanism and inhibits the effects of cytotoxic TILs (14,16). The transcription factor FOXP3 specifically identifies a subset of $CD4^+CD25^+$ Tregs, that represents a small fraction (5-10%) of the overall $CD4^+$ T cell population and is essential for downregulating immune responses to both endogenous (self) and exogenous antigens (14). Originally identified in the peripheral blood and thymus, Treg cells have also skin homing properties (14,17), that implies their role in the local control of disease, and stimulates interest in their possible therapeutic ramifications (25). Abnormalities in number and functions of peripheral Tregs are recognized in some human autoimmune and inflammatory diseases (15).

In cancer, $CD4^+CD25^+$ Tregs have been shown to be increased in the peripheral blood and in tumour-draining lymph nodes of patients, however, discordant results have been reported on their prognostic role (13). Treg numbers have been associated with an unfavourable prognosis in ovarian cancer (13,29), and with high tumour grade in glial neoplasms

(30), whereas they were not found of prognostic value in anal squamous cell carcinoma (31), and, vice versa, were associated with improved survival rates in patients suffering from Hodgkin lymphoma (32). However, many studies on Tregs did not use specific markers, such as FOXP3, and few studies were performed at the tissue level, in which immune status may differ from the general one (21). Adaptive Tregs develop from both natural Tregs and $CD25^-$ T cells under continuous antigen stimulation and in a cytokine (i.e. TGF-ß, IL-2, IL-10, IL-4, IFN-γ)-dependent manner (16,18). Natural Tregs control autoimmune responses, whereas adaptive Tregs are involved in immune response control not only to self-antigens, but also to a wide variety of nonself-antigens (15).

Melanoma environment is a continuous source of antigens as well as cytokines, therefore ideal to the development and maintenance of adaptive Tregs. $CD4^+CD25^+FOXP3^+$ TILs may be involved in thwarting the T-cell response against the tumour, and might therefore have a relevant role in local immune tolerance, allowing for uncontrolled melanoma growth and progression. We did not observe significant differences in cytotoxic TIL percentage between the two groups of patients. This is not surprising since Tregs are thought to affect functionality of effector cells, that is therefore impaired, despite the presence of the latter in tumour microenvironment (7). Investigation on cross-talk between Tregs and other cell types in the tumour habitat, could also be informative. Recently, mast cells have been demonstrated to be essential in mediating peripheral tolerance induced by $CD4^+CD25^+FOXP3^+$ Tregs (33).

In our study, especially in group B patients, in many cases we observed $CD4^+/CD25^+FOXP3^+$ Treg cells very close to tumour-associated macrophages, that are a well-known source of cytokines, and usually correlated to a poor prognosis in advanced melanoma (27). Macrophages could also be involved in Treg-tumour homeostasis, however this observation needs further investigation. Although differences in $CD25^+FOXP3^+$ Treg percentages between the two groups of our patients were significant, some overlapping results were registered, as observable by the range of reported values (Table II). It is known that the functionality of $CD25^+FOXP3^+$ cells might depend on various factors, such as environmental agents and their proliferation status (35). Furthermore, the complexity of players in the immune response should be taken into consideration, and that there are also other non-$FOXP3^+$ subsets of induced T cells, that can play a role in immune tolerance against melanoma antigens (26).

It has also been assumed that FOXP3 expression in humans, unlike mice, may not be specific for cells with a regulatory phenotype and may be only a consequence of their activation status (34). The double $CD25^-FOXP3$ stain is, therefore, mandatory for identifying cells with regulatory properties. Waiting for a more complete TIL characterization with commercial antibodies, and for less indaginous tests aiming at investigating $CD25^+FOXP3^+$ Treg functionality, we think that, the immunohistochemical evaluation of $CD25^+FOXP3^+$ cells is a practical and reliable approach in tissue biopsies. In humans, FOXP3 protein is detected as a doublet by immunoblot analysis: it has recently been demonstrated that both full-length FOXP3 and the splice variant forms of the protein are functional repressors of $CD4^+$ T cell activation

(35,36). This further supports the use of the commercially available antibodies for diagnostic purposes.

The relevance of Treg evaluation as a spy of local immune response status is also sustained by the evidence that they would exert a 'dominant' form of immune tolerance on many different cell types, such as natural killer and CD3$^+$CD8$^+$ effector cells (18). As we observed in our study, the evaluation of CD4$^+$/CD25$^+$FOXP3$^+$ Tregs in tumour sections could be helpful in foreseeing melanoma behaviour: CD4$^+$/CD25$^+$ FOXP3$^+$ Tregs might represent a key marker for an immuno-suppressive microenvironment contributing to tumour immune escape.

In our experience, CD25$^+$FOXP3$^+$ Tregs were associated with melanoma recurrence, also in three cases that showed brisk TILs. Although limited to a phase of tumour growth, our conclusions are consistent with those of an increasing number of studies, that support the relevance of CD25$^+$FOXP3$^+$ Tregs, and of an increased CD25$^+$FOXP3$^+$ Treg/T effector ratio as a positive indicator of tumour aggressiveness and/or reduced patient survival (18,20,28,29).

It is conceivable to add TIL characterization in diagnostic reports of melanoma, by using a panel of antibodies including CD8, granzyme b, CD4, CD25 and FOXP3. Prospective studies on larger series comprising tumours in various growth phases, as well as metastases, and correlation with patient survival, are warranted to validate CD4$^+$CD25$^+$ FOXP3$^+$ Treg evaluation at the tissue level as a prognostic indicator in cutaneous melanoma.

Acknowledgements

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