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Review

Focus on TILs: Prognostic significance of tumor infiltrating lymphocytes in human melanoma

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Tumors contain variable numbers of lymphocytes, referred to as tumor infiltrating lymphocytes (TILs). In melanoma, the intensity of this lymphocytic infiltrate is believed to correlate with outcome, though there is some debate about the applicability of this finding for all melanomas. Much research has gone into classifying TILs with respect to antigen receptor structure and the antigen to which melanoma-specific T cells react. However, these studies for the most part did not immunophenotype TILs, and recent data has revealed that the composition of tumoral lymphocytes is not homogenous, but rather represents varying contributions from many lymphocytic subsets. Furthermore, the function of TILs is often compromised as a result of the accumulation of immunoregulatory cells and various tumor escape mechanisms. These recent insights stress the need to collect more data on the composition and function of TIL infiltrates before definitive conclusions about the prognostic significance of TILs can be drawn. Advances in immunology have also facilitated the development of immunotherapeutic strategies, examples of which will be discussed with a special emphasis on blocking antibodies against CTLA-4, which are prototypical immunotherapeutic agents. This flurry of novel "biological" therapies will undoubtedly complicate our already incomplete understanding of TIL immunobiology as each of these agents has the potential to uniquely distort the series of immunological events which normally occur in untreated melanoma. Therefore, considerable research is needed to better elucidate the function and prognostic significance of TILs in both untreated melanoma and tumors treated with "biological" therapy.

Keywords: human, melanoma, tumor-infiltrating lymphocytes, prognosis, biological therapy, CTLA-4

Evolution of the TIL concept

More than 100 years ago, malignant tumors were first noted to contain variable numbers of lymphocytes (1), which have come to be known as tumor infiltrating lymphocytes (TILs). Initially, these TILs were thought to reflect the origin of cancer at sites of chronic inflammation (1), and later it was debated whether TILs provided a favorable environment for cancer growth or were evidence of the host's attempt to eliminate cancer (2). A relationship was first identified between the extent of immune cell infiltration and prognosis in 1949 in cases of breast cancer (3). In 1969, Clark *et al.* (4) first described the lymphocytic infiltration of primary cutaneous melanoma, a finding which Day *et al.* (5) and Tuthill *et al.* (6) later found to be of prognostic significance. Patients with a moderate-to-marked lymphocytic infiltrate within their primary melanoma had a significantly better prognosis and a 3-times higher 5-year survival rate than patients with a sparse or absent lymphocytic infiltrate (5). Elder *et al.* (7) differentiated the lymphocytic infiltrate into brisk, non-brisk, or absent, according to its intensity, and demonstrated that TILs were of prognostic significance only in vertical growth phase (VGP) melanoma. In contrast, the extent of lymphocytic infiltration had no prognostic influence in radial growth phase (RGP) melanomas, regardless of whether the melanoma was *in situ* or invasive (7), findings which were verified by Clemente *et al.* (8). The 5- and 10-year survival rates were 77% and 55% in melanomas with brisk VGP infiltrates; 53% and 45% with non-brisk VGP infiltrates; and 37% and 27% without VGP infiltrates (8). Also, the number of TILs in the primary tumor has been found to be inversely correlated with the probability for lymph node metastases (8). Patients with brisk TIL infiltrates in their primary tumors showed a 3.9% probability of a positive sentinel lymph node (SLN), compared to a 26.2% probability in patients with TILs absent from their primary melanoma (9). Furthermore, of those patients with regional lymph node metastases, patients with more marked lymphocytic responses in their metastatic melanoma showed a significantly higher 30-month disease-free survival rate (81.3% for patients with a brisk TIL infiltrate; 46.8% for patients with a non-brisk infiltrate; and, 29.3% for patients with TILs absent from their lymph node metastases) (5, 10). However, other studies could not convincingly demonstrate that brisk TIL infiltrates were associated with improved survival in melanoma patients (11-13). These discrepant results may in part be explained by differences in patient populations investigated, with particular reference to the thickness of patients' melanomas (9). The study by Clemente *et al.* (8) found the impact of TILs most pronounced in patients with high-risk lesions, thicker than 1.7 mm but less than 6 mm in Breslow depth (9). This suggested that the briskness of the TIL infiltrate was prognostic for T2-T4 (TMN system) (14) primary cutaneous melanoma (PCM), though the prognostic significance of TILs was lost in very thick lesions (advanced T4). In contrast, Barnhill *et al.* (11) did not find any survival advantage to be associated with brisk TIL

infiltrates; however, patients with both RGP and VGP were included in this study (11), even though other studies did not demonstrate a prognostic significance of TILs in RGP PCM (7, 8). Furthermore, only 25.6% of patients in Barnhill's study had lesions thicker than 1.7 mm (11) while 82% and 71% of patients had lesions thicker than 1.7 mm in the studies by Clemente (8) and Tuthill (6). Taylor *et al.* (9) did not find an impact of TILs on survival (44% of patients had lesions thicker than 2 mm); however, they did show that TILs are an independent predictor of SLN positivity, which by itself is the most important independent predictor of recurrence and survival in malignant melanoma patients (15). Nevertheless, due to technical limitations at the time, the vast majority of these studies did not immunophenotype TILs and, therefore, did not examine the difference in composition or function of tumoral lymphocytes.

TIL targets and their mechanism of recognition

The antigens which T cells recognize are comprised of peptides and a population of polymorphic cell-surface proteins called major histocompatibility complex (MHC) Ags, which associate with peptides via their peptide binding groove (16). CD4 "helper" T cells (T_H) recognize peptides of at least 13 amino acids in length presented by MHC class II (HLA DR, DP, DQ) Ag, whereas CD8 "cytotoxic" T lymphocytes (CTLs) recognize 8-10 amino acid peptides presented by MHC class I (HLA A, B, C) Ags (16). MHC class I Ags are essentially expressed on all nucleated cells (16) and hence have a broad distribution, including tumor cells; however, as will be discussed later, these may be lost during tumor progression (17). In contrast, MHC class II Ag expression is normally limited to "professional" Ag-presenting cells (APC); however, these Ags may be expressed aberrantly on tumor cells as a result of peritumoral inflammation (18) or as a direct result of neoplastic transformation (19). In fact, a significant percentage of melanomas express cell surface MHC class II molecules (20), and treatment with interferon-gamma (IFN-γ) can induce class II expression on the majority of melanomas (21). MHC Ag expression in malignant melanoma has also been shown to have prognostic relevance as both expression of HLA-DR in melanoma lesions and a decreased expression of HLA-A, -B, -C Ags in loco-regional metastases are associated with an unfavorable prognosis (19). Although all nucleated cells normally express MHC class I molecules and have the capability to upregulate MHC class II molecules in an inflammatory milieu, professional APCs (macrophages, dendritic cells and B cells) are best equipped for the priming of a T cell response given their constitutional expression of MHC II Ags and their ability to express numerous T cell co-stimulatory molecules (16). Thus numerous studies are targeting the function of professional APCs, particularly the dendritic cell (DC) subset of these cells, to bolster anti-tumor immunity.

The majority of melanoma peptide Ags have been identified by: screening cDNA expression libraries against melanoma-reactive T cells (22); mass spectrometry following their elution from purified HLA molecules (23); or, prediction from the genomic sequence based upon the need for specific anchor residues (16) to fit into respective "pockets" of the HLA peptide binding groove (23). Recently, the study of tumor-specific T cells has been facilitated by the development of multimer technology, which allows one to track and enumerate Ag-specific T cells by flow cytometry (16, 24). Through the use of these techniques, various classes of tumor Ags have been observed in human melanoma, which include autologous tumor-specific (specific point mutations such as in the β-catenin gene), tissue-specific (e.g., MART-1/Melan-A from here on referred to as MART-1), and common cancer-specific (e.g., MAGE family) Ags (22). However, how the immune system differentially responds to these various Ag types has not yet been fully explored.

TILs derived from melanomas may lyse MHC-matched allogeneic tumors (25), suggesting that some tumor-associated Ags (TAAs) are commonly expressed by tumors from different patients (22). Given the high frequency of this phenomenon (22), it would be unusual for such cross-reactivity to involve sporadic mutations which result in novel peptide Ags (autologous tumor-specific Ags). In keeping with this hypothesis, many melanoma-specific Ags recognized to date have been non-mutated peptides derived from proteins involved in melanin synthesis (22, 26). These so-called melanosomal proteins or melanocytic differentiation proteins (tissue-specific tumor Ags) include MART-1, gp100, tyrosinase, TRP-1, and TRP-2 (22, 26). Peptides derived from these proteins have been recognized in the context of the HLA-A1, -A2, and -A3, HLA molecules that are expressed in 26%, 49%, and 25% of the Caucasian population respectively, with the HLA-A2-binding MART-1 peptides 27-35 and 26-35 being the most frequently detected peptide Ags recognized in melanoma patients (22). Melanosomal protein-derived peptide Ags have relatively low HLA binding affinities (26), due to the absence of optimal amino acid anchor residues, suggesting that they may be expressed at a low density on the melanocyte surface (22), although a subgroup of these Ags are strongly immunogenic as the therapeutic capacity of TILs often correlates with anti-gp100 and anti-TRP-2 specificity when used in adoptive immunotherapy (27). Evidence that effective anti-melanoma immunity can be directed against these melanosomal Ags includes tumor regression in some melanoma patients immunized with MART-1, gp100, or tyrosinase peptides (28, 29), as well as the different biological behavior of same-patient metastases correlating with the expression level of melanosomal Ags (22). Furthermore, a significant correlation has been observed between vitiligo development and tumor regression in patients receiving immunotherapy (22), suggesting that the T cells mediating melanoma regression also recognize Ags expressed by non-neoplastic melanocytes. Interestingly, large numbers of melanosomal-specific T cells are present not only in the blood of melanoma patients but also in healthy persons, with the frequency of MART-1/A2 tetramer positive cells being approximately 10^{-3} of the phenotypically naïve CD8+ T cells in the peripheral blood of healthy HLA-A2 positive donors (30). The high frequency of these cells is unusual and, to date, MART-1 is the only known tumor Ag for which Ag-specific T cells can be detected in the blood, without any prior *in vitro* stimulation (30). Why these cells are maintained at such a high concentration remains enigmatic; however, it has been suggested that this high frequency may be explained by the phenomenon of epitope mimicry (31). Nevertheless, melanosomal-specific cells in healthy individuals exhibit a naïve immunophenotype (CD8+ CCR7+ perforin-) (32), perhaps reflecting a low affinity interaction with low-density melanosomal peptides on the cell surface. In the vast majority of melanoma patients, however, a detectable accumulation of MART-1-specific T cells, possessing an Ag-experienced memory/effector immunophenotype (CD8+ CCR7- perforin+) (32), occurs in metastatic tumors (30). The explanation of how the same Ag can have such differential effects is unclear, but may reflect more efficient Ag presentation by mature professional

APCs or the lowering of the T cell activation threshold by a rich cytokine milieu within the tumor environment.

While the melanocyte differentiation Ags are expressed constitutively by melanocytic cells, some melanoma tumor Ags are expressed as a result of malignant transformation (33), such as certain "common cancer-specific" Ags of which the MAGE family of genes is one of the best described (22). MAGE proteins are members of the cancer-testis (CT) family of TAAs, which also includes the BAGE, GAGE, and PRAME proteins (23). The biological function of these CT Ags is not yet known; however, these proteins are broadly expressed by tumors of diverse histologies (23) - for example, *MAGE-6* is expressed in more than 70% of metastatic melanomas and more than 50% of carcinomas of the lung, esophagus, bladder, and head and neck (34). CT Ags are concentrated upon the X chromosome (though multiple non-X chromosome CT Ags have also been described) (35), and their expression appears to be related to hypomethylation (22). Some studies have shown that CT Ag expression is more frequently seen in more advanced melanomas (36, 37); nevertheless, preliminary studies have shown that immunization of melanoma patients with epitopes derived from MAGE proteins may result in significant tumor regression (38). Surprisingly, in these vaccinated patients with evidence of a therapeutic response, no sign of systemic immunization could be observed in the peripheral blood (38). This apparent discrepancy between the therapeutic effectiveness of MAGE-specific T cells and the ability to detect these cells *in vivo* may reflect a difference in the immune response to CT Ags relative to the melanocytic differentiation Ags. For example, some studies have shown that immunogenic peptides derived from MAGE proteins are presented by HLA class II molecules, such as the presentation of MAGE-6 peptides by HLA-DR4 (expressed by 15-20% of the North American population) (39). These observations highlight how our knowledge of tumor-specific lymphocytes is biased by early studies which focused on CD8+ cytotoxic T cells circulating in the peripheral blood or present within tumor. However, other lymphocyte subsets which may be equally important in the defense against melanoma, such as helper T (T_H) cells, may follow alternate trafficking patterns and may be more represented in other compartments, such as the draining lymph nodes.

Another subset of "common cancer-specific" Ags is a newly described family of molecules called "stress ligands" (40). These proteins, which are best classified as "common-cancer Ags", include the non-classical MHC Ib molecules MICA/B that are expressed by a variety of cancer cell types (41). These proteins are different from the aforementioned MHC molecules in that they represent non-peptide presenting ligands, a subset of which are recognized by the NKG2D "stress ligand receptor", which is an activating/co-stimulating molecule on the surface of T cells and natural killer (NK) cells (41, 42). A subset of innate-like T cells (Vδ1 γδ T cells, see below), which are present in high numbers in various epithelial compartments (43) and which constitutively express NKG2D (44), possess a T cell receptor (TCR) that also directly recognizes MICA/B (45). These intraepithelial T cells, through the coordinate binding of the γδ TCR and NKG2D to the same stress ligand, are capable of the immediate rejection of transformed cells and thus are believed to be prototypical sentinel lymphocytes (46).

Another potential target for immunotherapy, but for which the least amount of data is available, are mutated protein Ags (autologous tumor-specific Ags), some of which are associated with tumorigenesis (22). Mutated peptides appear to be potent rejection Ags in murine tumor models (22) and some data indicates that mutated peptide Ags (22), as well as T cells directed against such Ags (47), can be found in melanoma patients with a more favorable prognosis. One of the reasons for this potency is that such Ags are "non-self" and thus T cells with high affinities for such Ags, which are capable of generating a successful immune response, are not deleted in the thymus during normal T cell ontogeny, a process which helps to ensure self-tolerance (16). Targeting these mutated Ags with immunotherapy may be a strategic approach (48), given that the altered proteins may also confer a growth advantage to the tumor that precludes the development of Ag-loss variants (17, 49) - though the loss of MHC molecules by melanoma cells may immunologically produce a functional loss of these Ags. Mutated proteins that have been isolated from human melanoma include ras, β-catenin, melanoma ubiquitous mutated 1 (MUM-1), and CDK4 (50) and, although these may serve as excellent targets for future immunotherapy, such an approach necessitates an extensive genomic understanding of each individual tumor since these Ags will not be shared between different HLA-matched melanoma patients.

TCR repertoire of melanoma TILs

Many studies have examined whether a restricted usage of TCR variable (V) genes is employed by T cells to recognize melanoma tumor Ags (25, 51); however, the results have been contradictory, perhaps reflecting the complexity of tumor immunity. In some early studies, limited TCR V gene segment usage by melanoma TILs was found (25). For example, in two representative studies, only 3 Vα gene families (Vα13, Vα15, and Vα16) were predominantly expressed by TILs from 24 melanomas examined (51), whereas TILs of uveal melanoma demonstrated a preferential expression of Vα7 genes in 7 of 8 melanoma samples (52). Using a polymerase chain reaction (PCR) approach, Strohal *et al.* (51) demonstrated that while lymphocytes from normal skin samples showed a heterogeneous expression of TCR Vα chains, the TILs present in or around the tumor had a restricted Vα chain repertoire, expressing only Vα13, Vα15 or Vα16. Further studies implicated a spatial organization of this TCR repertoire restriction (30, 53). For example, Clemente *et al.* (53) demonstrated that TILs in VGP melanoma and lymph node metastases of the same patients exhibited the same restricted repertoire of TCR Vβ chains, whereas lymphocytes present in extra-VGP areas showed no β chain restrictions. Furthermore, it was shown that TCR repertoires in the peripheral blood of melanoma patients were not restricted (30), perhaps reflecting the ability of only high affinity clones to enter and expand within the tumor microenvironment. Interestingly, even within the same individual, different T cell clones can predominate at different sites of disease, perhaps reflecting diverging subclones of melanoma exhibiting unique patterns of Ag loss that stimulates unique infiltrates of melanoma-specific T cells (54). Despite these earlier studies, it is now widely accepted that the repertoire of T cells against certain melanoma Ags (MART-1 for example) is diverse and mostly non-overlapping among different individuals (25, 30). Tetramer-based studies have revealed a highly diversified repertoire utilizing most Vβ chain families (30), with only some clones showing partial conservation of TCR structure (25, 30). Therefore, understanding how different subsets of T cells contribute to an effective anti-tumor response and assaying the functional characteristics of these cells is beginning to overshadow the significance of TCR repertoire analysis of TILs.

Immunophenotyping and subtyping TILs

An explosion of immunological data has resulted in a greater characterization of T cell subsets, which has led to an effort to immunophenotype T cells within the tumor microenvironment (9, 10, 55-61). Studies on IL-2-cultured TILs demonstrated that the T cell composition within the tumor microenvironment varies in individual patients, ranging from an infiltrate with 90% CD4+ T cells to an infiltrate with 90% CD8+ T cells (59-61), with highly specific cytolytic activity and patient outcomes correlating with the presence of tumor-specific, CD8+ T cells (9, 10, 55). Most research has focused on CD8+ cells, which are known to infiltrate tumors and, by virtue of their recognition of tumor-specific peptides presented by classical HLA class I molecules (16), are suspected to have a role in mediating the cytotoxic destruction of transformed cells (62, 63). However, recent studies that tested the function of CD8+ TILs, such as the ability of tetramer-positive cells to express IFN-γ after *in vitro* stimulation (64), demonstrated that many melanomas are populated by inactivated/anergic cells (65, 66). This observation stresses the importance of some form of functional assessment to best prognosticate TIL significance. Furthermore, the significance of other immunocyte populations in melanoma is uncertain despite a heterogeneous mixture of inflammatory cells often present within the tumor microenvironment (47, 57, 58, 67, 68). For example, although the role of CD4+ TILs in melanoma is not yet understood, several studies suggest that they may have an important role. For example, melanoma-specific CD4+ TILs have been shown to possess the ability to directly lyse tumor cells (58, 69) and eliminate melanoma in animal models (56, 70). Moreover, work by Rosenberg's group has demonstrated that the co-transfer of CD4+ and CD8+ T cells is more beneficial than the transfer of CD8+ T cells alone (71), and a recent report has demonstrated that the adoptive transfer of *in vitro* expanded autologous CD4+ T cell clones with specificity for the melanoma-associated Ag NY-ESO-1 may induce durable responses in some patients with metastatic melanoma (72). Additional work has revealed that CD4+ TILs in thinner, regressing lesions secrete a pattern of cytokines typical of T_H1 CD4+ cells (IFN-γ, lymphotoxin, IL-15, IL-2) whereas thicker, non-regressing lesions contain a greater number of TGF-β- and IL-10-liberating CD4+ cells (73-75), likely belonging to the T_H2 or a regulatory T cell lineage. Given that T_H1 cells promote strong cell-mediated immune responses while T_H2 cells promote allergic responses and/or secrete immunosuppressive factors (16, 76), the proportions of these different cell subsets will likely influence the tumor microenvironment.

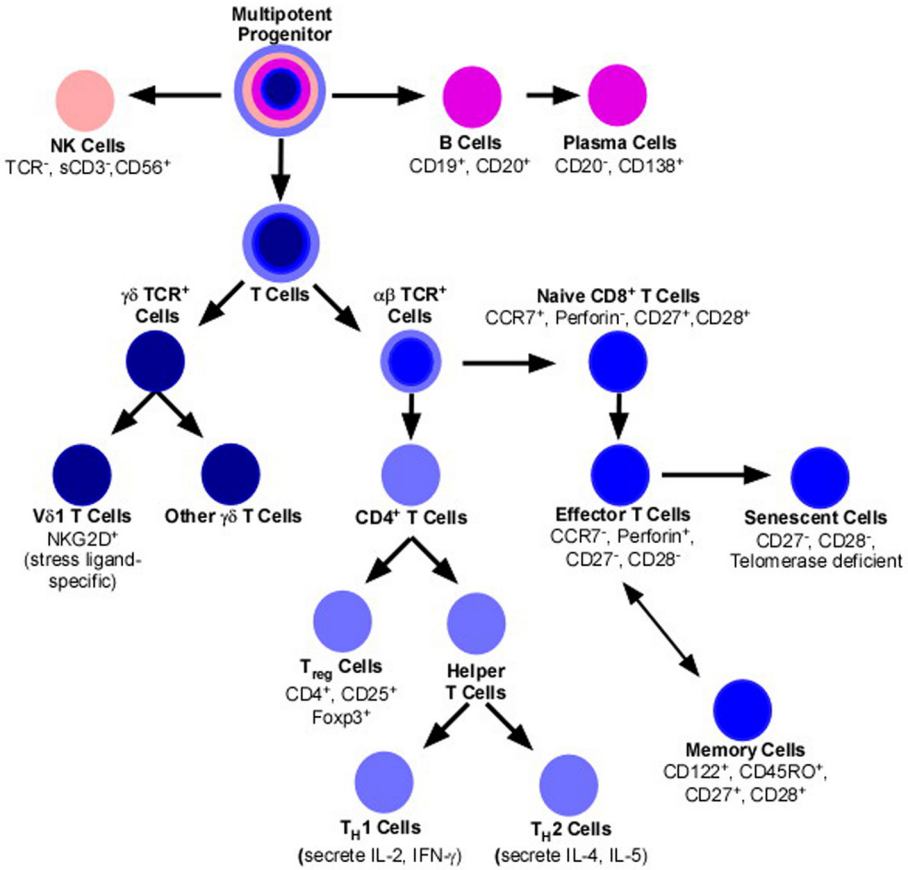
As alluded to above, another subset of CD4+ T cells variably present within the tumor environment (68, 77-80) are the CD4+ CD25+ regulatory T cells (Tregs). Tregs express a CD4+/CD25+high/Foxp3+ immunophenotype and represent 5-10% of human CD4+ T cells (81). A deficiency of Tregs, either occurring naturally (82, 83) or induced experimentally (84, 85), is associated with massive T cell lymphoproliferation and multi-organ autoimmunity (82-85), illustrating how a subset of these cells are important for mediating self-tolerance (natural Tregs) (86, 87). Another subset of Tregs appear to modulate the response of immunocytes to non-self Ags (induced Tregs) (86, 87), thereby limiting the immune response to foreign Ags. Interestingly, Tregs are significantly increased in patients with epithelial malignancies (twice the number of Tregs relative to healthy volunteers) (88) and, in experimental models, depletion of Tregs evokes effective anti-tumor immunity (84, 85). Treg TILs have also been shown to be more represented in advanced human melanoma lesions, with more Treg TILs in metastatic lesions (68) and in deep VGP lesions relative to shallow VGP and RGP lesions (89). Thus the accumulation of these cells may be associated with disease progression (89, 90), a hypothesis which is supported by the finding that a higher percentage of Treg TILs is associated with a significantly higher risk of melanoma recurrence (91). It is also tempting to speculate that the increased frequency of Tregs in advanced malignant lesions (68, 78, 79) may, in part, explain the anergy of tumor-specific CD8+ T cells observed in such lesions (65, 66). Studies are ongoing to investigate whether a correlation exists between melanoma survival and the frequency of Treg TILs, similar to what has been described for ovarian cancer patients (92).

Melanoma-specific B cells have also been demonstrated in limited studies (58, 93), and high levels of B cell TILs have been correlated with a favorable prognosis in certain types of cancer (93, 94). Interestingly, immunization of mice with TRP-1 protein resulted in the induction of auto-Abs and the protection against growth of TRP-1-expressing melanoma cells (95). Furthermore, human studies on the immune response to NY-ESO-1-expressing melanomas have demonstrated that CD8+ T cell responses to this Ag do not occur in patients who do not develop NY-ESO-1-specific Abs and that the titer of NY-ESO-1-specific Abs falls with the successful therapy of melanomas (96). These data suggest that melanoma-specific Abs may have a role in opsonizing tumor cells for phagocytosis and optimal Ag presentation, and that measuring a humoral immune response to vaccination can identify patients who will likely respond to therapy. However, the significance of humoral immunity in human melanoma has yet to be clarified since studies to date have shown that Ig deposits and B cells are only infrequently present within the tumor microenvironment (97).

NK cells are another lymphocyte population whose role in the melanoma immune response has not yet been closely examined. NK cells use an assortment of germline-encoded receptors, including inhibitory receptors for MHC molecules (16), that enable them to recognize cells that have aberrantly upregulated or downregulated cell surface markers as a result of cellular transformation (16, 98). Cells which have lost expression of self-MHCs are essentially marked for NK cell-mediated destruction (missing-self model) (98, 99). Similarly, activating receptors expressed by NK cells, such as NKG2D, recognize transformation-associated stress ligands, which function as another trigger for the NK cell-mediated destruction of tumor cells (98, 100). The linkage of NK receptor signaling to the release of cytotoxic granules is the basis for the immunosurveillance function of these cells (98). Although NK cells are either absent or present only infrequently within the melanoma microenvironment (97), one study demonstrated that the presence of NK TILs was seen only in responding melanoma patients but not in those with progressive disease (101).

Yet another subset of melanoma TILs which has not received much recent attention in the literature is the γδ family of T cells. While most melanoma TILs express the αβ heterodimeric TCR (102), a subset of TILs express a γδ TCR (67). γδ TCRs are assembled in a similar fashion as αβ TCRs (16); however, the γδ receptor complex is characterized by different Ag recognition properties (46). MICA/B-specific Vδ1 γδ T cells have been demonstrated within the melanoma microenvironment (67) and several studies have demonstrated a relationship between the stage of melanocytic lesions and the frequency of these cells (54, 103-106). For example, a greater number of Vδ1 γδ T cells were found to be present in dysplastic melanocytic nevi relative

Figure 1



Stylized outline of major lymphocyte subsets.

to invasive and metastatic melanoma (106). This observation supports an important immunosurveillance function of these cells in early lesions (46), which is in keeping with the upregulated expression of cellular stress ligands during the transformation of dysplastic nevi to invasive melanoma (107). The lesser role of γδ TILs in more advanced melanocytic disease likely reflects the tendency of invasive melanomas to downregulate the surface expression of these stress ligands (108), and it is tempting to speculate that enhancing stress ligand expression by dysplastic melanocytes or melanoma cells may prove to be an effective treatment strategy in future clinical trials.

Clearly a number of different lymphocyte subsets contribute to the immunological response to melanoma. A greater understanding of the Ag specificity and immunobiology of the different lymphocyte subsets is clearly needed to better predict the prognostic significance of TILs and to more effectively modulate their immunosurveillance of melanoma. A basic subdivision of lymphoid cells is illustrated in Figure 1, and a list of markers for comprehensive TIL immunophenotyping is proposed in Table 1. Given this greater capability to immunophenotype TILs, future studies may show that the composition is as important as the "briskness" of the lymphocytic infiltrate.

Immunomodulating strategies to augment anti-melanoma immunity

Recently, certain forms of immunotherapy have been shown to be of potential therapeutic use in some patients with metastatic melanoma (109, 110). Unfortunately, with the implementation of such therapies, it is likely that our limited understanding of the prognostic significance of TILs will be further disadvantaged by therapy-specific immune distortions that change the usual pattern of TIL composition and infiltration. For this reason, it will be critical for those who interpret histopathological material to gain an understanding of the underlying immunobiology of such therapies.

Attempts to augment melanoma-specific immunity have involved various techniques. Vaccination of patients with melanoma Ags, or augmentation of natural Ag presentation *in vivo* through the use of various adjuvants to expand tumor-specific lymphocytes (22, 50, 111), has been a major focus in melanoma research. Steps taken to enhance natural Ag presentation have included the administration of a number of cytokines. IL-2, perhaps the most important of lymphocyte growth factors (16), has been investigated for more than 20 years in the therapy of melanoma (59, 109, 112), and likely works by either promoting the expansion of Ag-specific T cells or by enhancing their cytotoxicity (113, 114). Initial studies of IL-2 therapy for melanoma demonstrated that the administration of this agent resulted in substantial increases in tumoral lymphocytes (115). Subsequent prospective clinical trials demonstrated some utility of IL-2 therapy for malignant

Table 1
Proposed markers for immunophenotyping TILs.

Marker	Specificity
Foxp3	Together with CD3, CD4, and CD27 can identify Tregs.
CD27	Together with CD3, CD4, and Foxp3 can identify Tregs. Together with CD3, CD8, CD28, and markers such as KLRG1 and CD57 can identify differentiation states of CD8+ T cells.
CD3 and CD5	Identifies T cell subsets. Negative CD5 staining and CD3 surface staining can be used as an indicator of the NK lineage.[(a)]
CD8	An indicator of the cytotoxic lineage of T cells.[(b)]
CD4	An indicator of the "helper" or T regulatory lineage of T cells.[(c)]
CD28	Together with CD3, CD8, CD27, and markers such as KLRG1 and CD57 can identify differentiation states of CD8+ T cells.
CD57 and KLRG1	Together with CD3, CD8, CD28, and CD27 can identify differentiation states of CD8+ T cells. Staining with these markers in the absence of CD5, surface CD3, and TCR staining indicates an NK lineage.
Vδ1 γδ TCR	Defines a subset of γδ T cells with a conserved TCR that is capable of directly binding to MICA/B stress ligands.
Pan-γδ TCR	A marker of γδ T cells.[(d)]
Pan-αβ TCR	A marker of αβ T cells.[(d)]
CD20	A fairly specific marker of the B cell lineage.[(e)] Can be used together with markers such as CD19 (flow cytometry only), PAX5, and surface Ig (BCR) to identify B cells.
CD16 and CD56	Can be used together with CD2, CD7, and NK receptors such as KLRG1 to identify NK cells.[(f)] NK cells do not express CD5, surface CD3 (sCD3), or surface TCR.
CD25 (IL-2Rα)	Moderate expression is a marker of T cell activation. Bright CD25 expression with other markers (Foxp3, CD27, CD4) defines Tregs.
CD69 (Leu 23)	An activation marker on T cells. Found on T cells recently activated through the TCR (212).
CD122 (IL-2Rβ)	A marker of T cells with previous Ag exposure. Also constitutively expressed by NK cells.
CD79a	A marker of all B cell differentiation stages. Positive staining is found on normal bone marrow B cell precursors (hematogones) through to terminally differentiated plasma cells.
CD138	Together with CD38 and CD79a is a marker of plasma cells. CD138 positive cells are terminally differentiated and do not stain for PAX5 and CD20, and may be CD45 negative.
CD45RO/CD45RA	Isotypes of leukocyte common Ag. Similar to CD122, CD45RO can be used to demonstrate previous Ag exposure.
Perforin/Granzyme	Cytotoxic granule-associated molecules. Present in effector T cells (predominantly CD8+).
NKG2D	Stress ligand receptor/co-stimulatory molecule expressed constitutively by some cells with immunosurveillance function (45).

[(a)] NK lymphocytes do not express surface CD3; however, they frequently express cytoplasmic CD3 chains. Therefore, although CD3 is a relatively specific marker of the T cell lineage, only surface expression of CD3 should be considered indicative of the T cell lineage and should be confirmed by surface expression of a T cell receptor when indicated.
[(b)] Dim levels of CD8 are expressed by NK cells ($sCD3^-$, $CD5^-$).
[(c)] Dim levels of CD4 can be seen on immature monocytes ($CD3^-$, $CD68^+$) and Langerhans cells ($CD3^-$, $CD1a^+$, $S100^+$, $CD68^+$).
[(d)] Surface expression of the αβ or γδ T cell receptor is mutually exclusive.
[(e)] A small population of normal T lymphocytes expresses a low level of CD20 (213).
[(f)] Activated/memory T cells can upregulate markers once thought to be specific for the NK lineage, such as CD16.

melanoma, with a 16.3% response rate, including a 6% complete response rate, observed in one trial (109). In this study, median duration of response was 6.5 months, with 60% of complete responders remaining progression-free at 5 years (109). Of 24 patients who experienced a complete regression in this trial, only five have experienced a recurrence and 19 remain in clinical remission for 46 to 137 months or more (109). These numbers, albeit small, are the first evidence for potential cure in metastatic melanoma and have led to the U.S. Food and Drug Administration's approval of IL-2 therapy in such patients (109). IL-15, a cytokine whose receptor consists of IL-2's β and γ chain (CD122 and CD132, respectively) as well as the specificity-determining IL-15α chain, is also considered a promising agent in immunotherapy (116) as it possesses a similar effect on T cell proliferation without IL-2's effect on inducing T cell apoptosis (117). Immunotherapy with interferon-alpha (IFN-α) has also shown reproducible activity in metastatic melanoma, with 15% to 20% response rates reported in some series (118), and with clinical responders having significantly denser TIL infiltrates (119). The mechanism of IFN-α immunotherapy is not fully understood; however, it is proposed to work by both augmenting the immune response and by exerting a direct effect on melanoma cells, which may involve activation of STAT proteins (120). IL-12 has also been used as an enhancer of the immune response (121-123). IL-12 acts by inducing T_H1 differentiation and, by inducing cytokine secretion, promotes the proliferation and the cytolytic activity of NK and T cells (16, 122). IL-12 has also been shown to reverse Ag-specific T cell anergy (124) and can boost the frequency of circulating tumor-specific lymphocytes (123). In trials using IL-12, these aforementioned effects were associated with brisk melanoma TIL infiltrates and encouraging treatment effects (121-123). Another cytokine found to be a useful adjuvant to tumor vaccination is granulocyte macrophage-colony stimulating factor (GM-CSF) (16). GM-CSF treatment in melanoma results in the accumulation of large numbers of professional APCs (125), an observation leading to the integration of GM-CSF in a vaccination protocol which employs irradiated autologous tumor cells that have been genetically engineered to produce

large amounts of this factor (125-128). In a phase I clinical trial employing this approach, 10 out of 16 patients with stage IV melanoma developed dense lymphocytic infiltrates with extensive necrosis of metastatic lesions with one complete response, one partial response, and one mixed response (128).

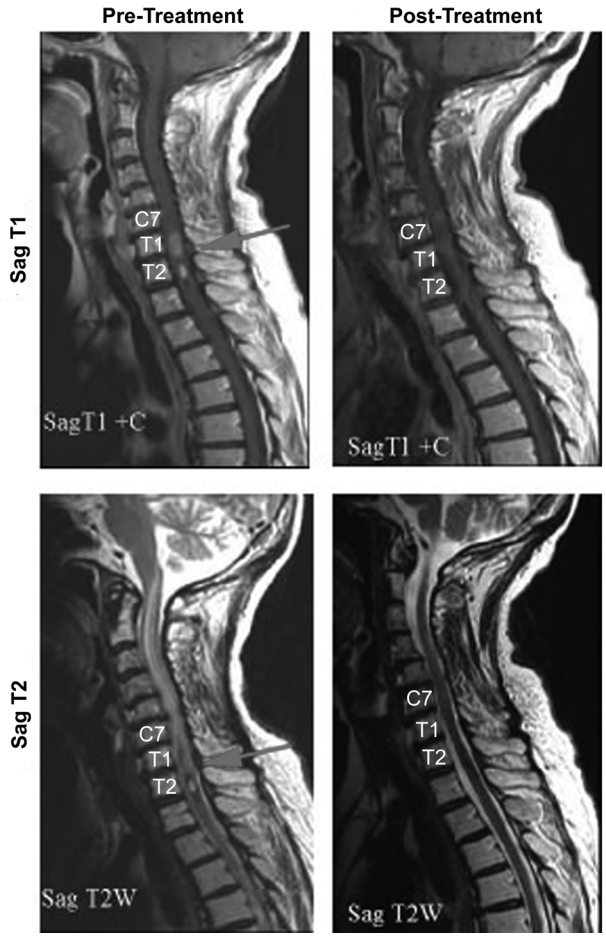
Various other immunomodulating strategies have been attempted for the treatment of melanoma, including vaccination with tumor cells, tumor lysate or tumor-specific peptides, especially those derived from melanosomal Ags, with or without DCs (50, 111, 129-134). Tumor regression and tumoral lymphocytic infiltration has been observed in some melanoma patients immunized with melanosomal peptide Ags (22, 132, 135, 136) and, although some of the results have shown promise in the treatment of melanoma (50, 132, 135, 136), the great variability in the protocols used in these studies has led to a perplexing collection of data (131-138). This variability, in part, reflects different maturation states of DCs used in immunotherapy trials (133), since immature DCs are weak immunogens and can be tolerogenic, even resulting in the induction of Ag-specific Tregs (133). The collection and *in vitro* expansion of TILs followed by their adoptive transfer has also been used to augment anti-melanoma immunity (139, 140). This strategy is especially suitable for immunocompromised patients who may not optimally respond to a tumor vaccination approach. The administration of radio-labeled, melanoma-specific lymphocytes (as assessed with MART-1 tetramers), followed by imaging studies with a gamma camera, confirmed that transferred cells indeed localize to sites of metastatic tumor (141), which is consistent with the brisk TIL infiltrates following adoptive transfer of *in vitro* expanded, TAA-specific T cells (71, 142). While successful tumor eradication in murine models has been achieved by TIL transfer (143), the translation of this technique into clinical practice has been cumbersome, though it is considered by many to be the most promising immunotherapeutical strategy to date (144-146). In addition to these aforementioned approaches, novel strategies for the treatment of melanoma are being developed at a rapid rate and include immunization with recombinant viruses or plasmids encoding tumoral Ags (22) and the administration of a host of monoclonal Abs (mAbs) targeting critical regulators of immune function, such as a triggering mAb against 4-1BB (CD137) (147) and a blocking mAb against the cytotoxic T cell lymphocyte Ag-4 (CTLA-4, CD152) (111). Anti-CTLA-4 mAbs are a prototypical example of how our growing understanding of immunobiology is being translated into potentially useful immune modulating agents and thus these and related therapies will be discussed in detail.

Examples of novel biological agents in melanoma immunotherapy with a focus on CTLA-4 blockade

CTLA-4 is critically important for the contraction of immune responses (148), which is necessary to ensure that other T cell clones are not dangerously diluted by unopposed clonal expansions (148). CTLA-4 is not expressed on Ag-naïve T cells, but is upregulated upon the surface of T lymphocytes approximately 3 days following Ag-specific T cell activation (149, 150). CTLA-4 is a high affinity receptor for the B7.1 and B7.2 ligands (151) that are expressed on mature APCs during an immune response (16, 152) and which are critical for delivering the classically-described "co-stimulatory" signal or "signal 2" to a naïve T cell (16). This co-stimulation is necessary for the optimal activation and proliferation of responding Ag-specific T cells (16, 148), and is transduced through CD28 (16) and related cell surface molecules upon initial Ag encounter (148). CTLA-4 is believed to antagonize T cell activation/expansion by at least two possible mechanisms, the first of which involves CTLA-4's 100-to-2000-fold greater affinity for B7.1/B7.2 relative to that of CD28 (148, 151), which effectively eliminates co-stimulatory signaling by the sequestration of B7.1/B7.2 away from CD28 (151, 153). The second possible mechanism involves the recruitment of an inhibitory phosphatase (SHP-2) to the immunological synapse by the SH2-binding domain of CTLA-4 (151), leading to dephosphorylation of critical tyrosine residues and the subsequent extinguishment of downstream TCR signaling pathways (154, 155). The synergistic effect of these two processes is to halt further expansion of Ag-specific T cells and enhance the attrition of the expanded clonal population, probably by depriving T cells of survival signals which are obtained through low-level TCR signaling (156). Thus the blockade of CTLA-4 function with a mAb was proposed for use as an adjuvant to increase the frequency of tumor-reactive T cells by prolonging the clonal expansion phase following tumor vaccination or during natural tumor Ag presentation (157). Various animal models have validated the effectiveness of CTLA-4 blockade at increasing the clone size of Ag-specific T cells when used in association with tumor vaccination (158-160). Effective CTLA-4 blockade was also found to be associated with better tumor control and prolonged survival in these model systems (158, 161-163). Based on these experimental findings, two human anti-CTLA-4 IgG1 mAbs have been developed (Ipilimumab® and Ticilimumab®) that are in advanced clinical trials for the treatment of a variety of malignancies (164-169). Preliminary data from these early trials of anti-CTLA-4 mAbs alone or in association with tumor vaccination have been encouraging (164-170), resulting in better tumor control in some recipients even within immunologically privileged sites such as the brain (Figure 2) (170), which is somewhat surprising given the role of the blood-brain barrier in preventing the influx of therapeutic mAbs into the brain parenchyma (171). One study of melanoma patients treated with anti-CTLA-4 mAb therapy alone showed an overall response rate of 21%, with two complete and one partial remission in 14 treated patients (165), while another study of melanoma and ovarian cancer patients demonstrated extensive tumor necrosis in 5 out of 9 patients following CTLA-4 blockade (164).

Studies of CTLA-4 blockade have also yielded information on how different TIL subsets interact within the tumor environment. For example, while marked TIL infiltrates were observed in many anti-CTLA-4 mAb studies (164, 172), in one study of advanced melanoma patients where TILs were more comprehensively immunophenotyped, the data suggested that Tregs limit the cytotoxic response to melanoma TAAs (172). In this study (172), an inverse relationship existed between the frequency of Treg TILs in metastatic melanoma and both the extent of necrosis and the frequency of cytotoxic T cells in such lesions (Figure 3). The reduction of Treg TILs noted in some of the patients in this study may have been attributable to a mAb-mediated depletion, given the constitutive expression of CTLA-4 by Tregs (173). Such Treg depletion may account for the autoimmune-like adverse effects that occur in many patients treated with CTLA-4 blockade (165, 166, 174), which are strikingly similar to the phenotypic changes in FoxP3-mutated Scurfy mice that are naturally-deficient of Tregs (175-177). Interestingly, other tumor vaccination strategies have been shown to be inexplicably accompanied by increases in Treg TILs, which may have adversely affected the clinical response in

Figure 2



CTLA-4 blockade results in significant tumor regression in some patients. MRI images of the cervicothoracic spine from a patient with metastatic malignant melanoma which reveal enhancing intraspinal metastases with extensive cord edema prior to treatment with CTLA-4 blockade. Post-therapy images demonstrate complete resolution of the metastases and the accompanying edema. [Adapted from Hodi *et al.* (170)]

these studies (178). Based upon these observations, it has been proposed that melanoma patients may benefit from Treg depletion, either as monotherapy or in association with tumor vaccination and, therefore, a variety of strategies are being investigated for this purpose.

Treg-depleting strategies currently being tested include agents which bind to the interleukin-2 (IL-2) receptor alpha chain (IL-2Rα, CD25) which, similar to CTLA-4, is not found on Ag-naïve T cells (16) but is expressed constitutively by Tregs (16, 179). These CD25-targeted therapies include anti-CD25 mAbs (180) and recombinant cytotoxic proteins composed of portions of bacterial toxins conjugated to either human IL-2 (181-183) or an antibody against CD25 (184, 185), that after internalization by CD25-expressing cells leads to cell death (181-183). Although CD25-directed therapies have shown some success in the depletion of Treg TILs (184), scant data exist on how these therapies immunomodulate other TIL subsets. Furthermore, these strategies to abrogate Treg function have generated mixed results in clinical trials (181-185), and it is unclear why these therapies have been less efficacious than anticipated. One possible explanation may be the unwanted depletion of tumor-specific T cells, since upregulation of CD25 is one of the earliest events in T cell activation (16, 186), and therefore, the tumor-specific population of cells one hopes to expand may in fact be depleted by CD25-directed agents.

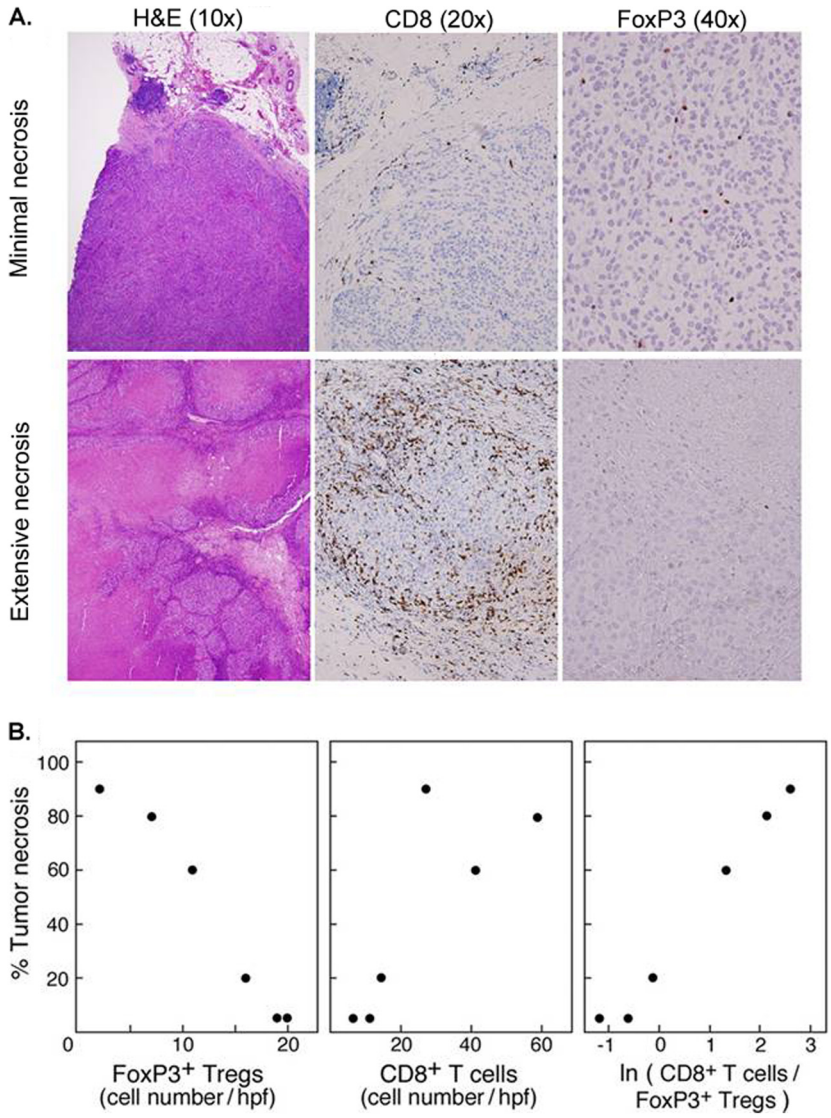
An alternative strategy to abrogate the function of Tregs *in vivo* involves interrupting the migration of these regulatory cells into the tumor microenvironment by utilizing mAbs directed against chemokines and their receptors. The majority of Tregs express high levels of chemokine receptors CCR4 (receptor for CCL22) (187, 188) and CCR6 (receptor for CCL20) (189). Antagonizing these chemotactic networks with mAbs against chemokines or their receptors has proven to be effective in experimental models; for example, a mAb against CCL22 reduced Treg migration to ovarian tumors (190). Accordingly, it is anticipated that an on-going clinical trial using a mAb against CCR4 for the treatment of hematological malignancies (191, 192) will be expanded to include patients with various other malignancies, including malignant melanoma.

The effect of melanoma immunotherapy on TILs

The frequent failure of melanoma immunity is highlighted by data from both human (193) and murine (194) tumor models which show that TILs are sometimes composed of quiescent and/or functionally anergic effector/memory T cells (58). For example, some TIL cell lines do not lyse but rather release GM-CSF in response to autologous tumor (195), and while normal donor lymphocytes were able to secrete IFN-γ in response to MAGE-6-derived peptides, cells from melanoma patients were unresponsive to this stimulation (39). Therefore merely demonstrating the presence of TILs may not be an entirely accurate method of predicting patient outcomes. Myriad tumor escape mechanisms, described in detail elsewhere (17) and briefly summarized in Table 2, likely work together to affect this immune compromise. Mechanisms particularly important to recognize include: loss of tumor Ags, which occurs in 5-20% of patients with metastatic melanoma in the form of selective loss of tumor Ags or the concordant loss of multiple melanosomal proteins (196); altered expression of classical and non-classical MHC molecules (17); and, the ability of tumors to liberate chemotactic factors for Tregs (197).

The frequency of these tumor escape tactics appears to increase in metastatic melanoma when compared to primary melanoma (180, 198), perhaps reflecting clonal evolution of the tumor and the selection of multiple escape mechanisms. However, while T cell incompetence is likely attributable to many of the processes listed in Table 2, it may also reflect problems invoked by current immunotherapy protocols. Although most melanoma patients treated with TIL adoptive transfer show a brisk T cell rich infiltrate (71, 142), the frequency of TAA-specific TILs does not necessarily correlate with a strong *in vivo* anti-tumor response (140). Similarly, vaccination protocols utilizing melanoma TAAs are frequently associated with unsatisfactory clinical responses (133). In experimental studies, one finding that correlated very strongly with positive therapeutic responses was the *in vivo* persistence of tumor-specific T cells (71, 139). This persistence has been defined in cell transfer techniques as the ability of at least one clonotype to remain in the peripheral blood one month after transfer at 5% or greater of the total CD8+ T cell population (199). While persistent and non-persistent TILs shared a

Figure 3



The ratio of tumor infiltrating CD8+ T cells to Foxp3+ Treg TILs following anti-CTLA-4 treatment is tightly correlated with the extent of tumor necrosis. (A) Representative photomicrographs demonstrating CD8+ and Foxp3+ Treg TILs in melanoma metastasis exhibiting minimal (top) and extensive (bottom) necrosis. (B) Graphical demonstration of the relationship between FoxP3+ Treg TILs, CD8+ TILs, and tumor necrosis. [Adapted from Hodi *et al.* (172)]

remarkable degree of similarity in the expression of activation markers (CD69, CD25, and CD40L) and homing molecules (CCR7, CXCR4), it was shown that a greater number of CD27-expressing TILs is associated with greater persistence and better outcomes (200, 201). CD27 is stably downregulated in late effector stage T cells (200), which are terminally differentiated and have significantly shorter telomeres and extremely poor telomerase activity attributable to defective Akt phosphorylation (202). Telomere shortening has been shown to be induced by prolonged *in vitro* culture, which is consistent with the finding that human T cells become senescent after 20 to 30 population doublings *in vitro* (199). Therefore, cells cultured from a small number of harvested TILs may be functionally compromised and unable to persist or perform their immunosurveillance function after adoptive transfer (203). Immunophenotyping TILs with the panel of markers listed in Table 1 can help to estimate the frequency of senescent T cells, especially if fresh material for flow cytometry is available where multiple markers can be evaluated simultaneously on each cell (16).

Another aspect of earlier clinical trials which may have adversely affected TIL function was the selection of Ags used for *in vivo* or *in vitro* expansion of tumor-specific T cells. Although a peptide may have anchor residues necessary to bind to a particular MHC molecule (16), such a peptide may not result in the generation of useful tumor-specific T cells. For example, among a panel of 10 different MART-1 peptides containing the HLA-A2 binding motif, only one was able to induce CTL lines with specific recognition of melanoma cells (204). This inability of certain peptides to induce functional tumor-specific T cells may reflect: a low affinity of interaction between relevant T cell receptors and the Ag, which at physiological levels of Ag

Table 2
Selected tumor escape mechanisms that mitigate the anti-tumor immune response.

Mechanism	Proposed Basis of Tumor Escape Mechanism
FasL expression	Tumor-derived Fas-ligand (FasL, CD178) binds TIL Fas (CD95) to induce apoptosis (58, 214).
RCAS1 expression	Tumor-derived RCAS1, expressed on a minority of melanoma cells, acts as a ligand for a putative activation-associated lymphocyte receptor that inhibits growth and induces apoptosis of TIL. RCAS1 expression may also skew T cells towards T_H2 differentiation through secretion of IL-10/TGF-β (58, 215-217).
Tumor expression of B7-H1	Engagement of the lymphocytic programmed death 1 molecule (PD1) by tumor-associated B7-H1 results in exhaustion/apoptosis of tumor-specific TILs (17, 58, 218).
High tumor cell burden	Chronic exposure to tumor Ags, particularly without co-stimulatory signaling, leads to exhaustion/deletion of T cells (58), in part, due to defective T cell and NK cell signaling by reduced expression/loss of CD3-ζ chains (219) and associated signaling molecules (Zap-70, $p56^{lck}$) (219, 220). Exhaustion may also be manifested by dysfunctional IL-2 signaling caused by reduced Jak3 expression (198). Chronic exposure to tumor antigen can also lead to activation-induced cell death (AICD) (58).
Release of ROS	Macrophages and melanoma cells are both sources of reactive oxygen species (ROS) (221). Although melanoma is relatively resistant to ROS because of an extensive antioxidant network (221), ROS may contribute to the loss of the aforementioned signaling molecules in TILs (i.e., CD3-ζ) (58).
Tumor expression of CCL22/CCL17	Tumor-derived CCL22 and CCL17 chemoattract Treg TILs that downregulate anti-tumor immunity by cell-to-cell or cytokine-mediated suppression (58, 222).
Tumor expression of IDO	Tumor expression of indolamine 2,3-dioxygenase (IDO), an enzyme which catalyzes tryptophan degradation, results in an impairment in the accumulation of tumor-specific T cells (223). IDO seems to block proliferation of T cells, which are extremely sensitive to tryptophan shortage (223).
Generation of epitope loss variants	Because some melanoma Ags are not important for tumor cell survival (melanosomal proteins), loss of tumor associated antigens (TAAs) occurs in 5-20% of patients with metastatic melanoma (17). These clones can be selected for by immunotherapy protocols targeting a single TAA.
Tumor HLA Ag loss	Loss of HLA class I Ags, can occur selectively or more generally due to mutations/deletion of β2-microglobulin genes (17), as β2-microglobulin stabilizes HLA class I molecules (16). This loss abrogates tumor recognition by Ag-specific T cells (17, 224).
Tumor expression of certain HLA class Ib molecules	The HLA class Ib molecules, HLA-E and HLA-G, protect from NK cell destruction by masking the loss of HLA class I molecules (see "missing self" hypothesis of NK cell-mediated lysis) (58, 99, 225). HLA-E presents a peptide derived from the membrane localization signal of HLA-G, maintaining expression of "self" without presenting immunogenic peptides (225). This also serves to inhibit effector/memory T cell function, as activated T cells often upregulate inhibitory NK receptors (58).
Tumor expression of MMPs	Tumor-derived matrix metalloproteinases (MMPs) may cleave the high affinity IL-2 receptor (CD25, IL-2Rα), abrogating the activation and subsequent proliferation of tumor-specific T cells (33, 58).
Impairment of peptide processing	Downregulated expression of transporter-associated with antigen processing (TAP) and immunoproteosome components, such as LMP-2 and LMP-7, sequester or abrogate the production of immunogenic peptides (17, 226).
Tumor expression of CEACAM1	Carcinoembryonic Ag cell adhesion molecule 1 (CEACAM1) is broadly expressed by immunocytes and by melanoma, especially those melanomas associated with a bad prognosis (227). CEACAM1 homophilic interactions inhibit TIL effector functions (227).
Mitigation of proinflammatory signals	The constitutive activity of the signal transducer and activator of transcription 3 (Stat3) molecule in some melanoma cell lines (17, 228) inhibits the production of proinflammatory cytokines (TNF-α, INF-β) and induces tolerogenic factors (IL-10), which inhibits the functional maturation of dendritic cells, and thereby may lead to the tolerization of TILs (17).

expression will fail to result in the recognition and/or efficient lysis of tumor cells; or, a failure to generate such peptides *in vivo* by the cellular machinery responsible for generating peptides for Ag presentation (proteasome) (16, 205), thereby rendering tumor cells invisible to a population of highly efficient cytotoxic cells (205, 206). With respect to this latter explanation, a similar "invisibility" may occur when Ag-specific T cells are generated from stimulation with altered peptide ligands, such as the modified high-affinity HLA-A2 binding gp100 peptide ($gp100_{209\text{-}2M}$). This peptide was generated from the substitution of a threonine residue with a methionine at the anchor residue P2, which results in a 10-fold higher HLA-A2 binding affinity that more efficiently produces high frequencies of tetramer-binding CD8+ cells compared to the native epitope (207). In one study where altered peptide ligands produced Ag-specific T cells with a spectrum of functional avidities, the cells were incapable of lysing HLA-A2-expressing melanoma cells, even at 100:1 effector-to-target-cell ratios, illustrating this potential pitfall (208). The failure to generate CTLs with some natural high-affinity peptides, such as to some MART-1 peptides (204), may also be explained by the induction of T cell tolerance toward these high-affinity self-peptides, reflecting the normal immunobiology of T cell Ag recognition (16). The finding that only studies showing T cell responsiveness to physiological levels of Ag demonstrated any clinical response (64, 209, 210) exemplifies these aforementioned complications of immunotherapy. One promising avenue of research, which aims to circumvent such problems invoked by normal T cell immunobiology, involves genetically-engineered T cells that express high affinity TCR for naturally-processed peptide Ags expressed at physiological concentrations (211); nevertheless, this approach may be associated with considerable autoimmune adverse effects.

These findings emphasize the potential necessity of moving beyond enumerating TILs with routine hematoxylin and eosin (H&E) staining and simple immunohistochemistry panels, towards the use of advanced immunophenotyping and possibly functional assessments of TILs. An emphasis on cellular senescence and reactivity towards physiological levels of cognate Ags may help to best prognosticate their significance.

Concluding remarks

Since the prognostic significance of TILs was proposed, much has been learned about the immunobiology of lymphocytes and the small molecules that govern the behavior of these cells. The sometimes contradictory results of earlier studies likely reflect the great immunophenotypic and functional heterogeneity of melanoma TILs. By applying today's greater immunobiological insight to the re-evaluation of previous data and the design of future studies, the significance of TILs in melanoma will surely be elucidated allowing for definitive recommendations for the routine management of melanoma to be developed.

Abbreviations

CT, cancer-testis; TAA, tumor-associated antigen; VGP, vertical growth phase

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