Identifying and Overcoming Immune Resistance Mechanisms in the Melanoma Tumor Microenvironment

Thomas F. Gajewski

Abstract The continually growing list of defined tumor antigens is broadening the potential applicability of tumor antigen-targeted cancer therapies. Although cancer vaccines and adoptive T-cell transfer have been shown to increase the frequency of circulating tumor antigen-specific T cells, these approaches cause clinical responses in a few patients. In melanoma, approximately one third of metastatic lesions contain activated T cells, including those specific for tumor antigens, arguing that the priming phase has occurred already in such individuals even without vaccination. These observations indicate that tumor resistance to immune destruction may dominate in many instances, arguing for a thorough analysis of the melanoma tumor microenvironment in individual patients. Recent work has suggested that T-cell anergy, the influence of CD4+CD25+ regulatory T cells, the expression of inhibitory ligands, such as PD-L1, and the activity of nutrient-catabolizing enzymes, such as indoleamine 2,3-dioxygenase, may be involved. Preclinical murine models have shown that interfering with each of these processes can translate into T-cell–mediated tumor control. Importantly, each of these targets is amenable to clinical manipulation. Clinical translation of these approaches to counter negative regulation of antitumor immunity should receive high priority.

The identification of specific antigens expressed by tumor cells and the realization that CD8+ T cells from patients can recognize these antigens and kill cancer cells in vitro have fueled numerous clinical studies aiming to gain a greater understanding of antitumor immunity in patients. Much effort has been focused on active immunization strategies that aim to promote expansion and differentiation of tumor antigen-specific T cells in vivo. Although several vaccine clinical trials have achieved this goal (1–3), particularly in melanoma, detection of elevated numbers of antitumor T cells as measured in the blood only rarely is associated with tumor regression. The alternative approach of adoptive transfer of many in vitro expanded tumor antigen-specific T cells can result in the presence of even greater numbers of activated T cells that are known to have cytolytic activity and to produce proinflammatory cytokines (4), but still clinical responses are infrequent.

More recent studies in patients with metastatic melanoma have revealed the presence of spontaneously generated tumor antigen-specific CD8+ T cells even without exogenous immunization (see example for Melan-A-reactive cells in Fig. 1) and even the presence of such cells within the tumor microenvironment. An example of a metastatic melanoma lesion showing a large accumulation of lymphocytes is depicted in Fig. 2. In fact, expanded tumor-infiltrating lymphocytes (TILs) have been explored as an adoptive immunotherapy in their own right (5). The existence of antigen-experienced T cells within growing tumors suggests that tumor resistance at the effector phase of an antitumor immune response may be dominant in many instances. When analyzed directly ex vivo, TILs often show defective antigen-specific proliferation, cytolytic activity, and cytokine production (6, 7). Recent observations have correlated poor cytolytic activity with lack of perforin expression (8, 9). Thus, it is likely that factors in the tumor microenvironment lead to dysfunction of activated T cells, resulting in tumor escape from immune-mediated destruction. This concept has led to a renewed search for immune-inhibitory mechanisms in the tumor microenvironment, with the hope of identifying processes or factors amenable to therapeutic manipulation.

Melanoma Microenvironment Analysis

One approach that has been applied toward the search for clinically relevant inhibitory factors in the melanoma tumor microenvironment is gene expression profiling. Marincola et al. were the first to explore patterns of transcripts that might be associated with a favorable clinical outcome in melanoma patients treated in various vaccine clinical trials. Using cDNA arrays applied to retrospective fine-needle aspirate samples, they identified a set of transcripts present in pretreatment lesions that seemed to be associated with a subsequent complete response to therapy. These included multiple immunologically relevant genes, including EBI3, TIA1, IRF2,
Purified CD8+ T cells were stimulated with Melan-A peptide-pulsed T2 cells and an ELISPOT assay was performed to measure cytokine secretion. The number of spots per 10^5 CD8+ T cells per well was determined. Several patients showed strong responses, while others showed no effect.

### T-Cell Anergy

Analysis of TILs has revealed antigen-specific hyporesponsiveness on analysis directly ex vivo, consistent with a form of T-cell anergy (6). The fact that anergy is a reversible state raises hope that cytokolytic activity and cytokine-producing capability of these TILs can be restored. Using a mouse preclinical model, Sotomayor et al. (12) showed anergy of CD4+ tumor antigen-specific T cells that correlated with lack of tumor rejection in vivo. We recently developed a CD8+ T-cell receptor transgenic model in which anergy of tumor antigen-reactive T cells also occurs with tumor progression. Based on the observation that anergic T cells can regain function on culture in cytokines that engage the common γ-cytokine receptor chain (13), we investigated whether homeostatic proliferation in lymphopenic recipients (which is dependent on interleukin-7 and interleukin-15) would restore the function of CD8+ T cells anergized in vivo. Indeed, anergic T-cell receptor transgenic T cells recovered function and rejected tumors in RAG2−/− recipients. These results indicate that one strategy for maintaining the function of antitumor T cells that is amenable to clinical translation is adoptive transfer into lymphopenic hosts, an approach that is in fact already being evaluated using TIL adoptive transfer at the National Cancer Institute (5).

### CD4+CD25+ Regulatory T Cells

Human melanoma metastases also have been shown to contain FoxP3-expressing CD4+CD25+ regulatory T cells (14). These cells can suppress the activation of tumor antigen-specific effector T cells in vitro (15). We have confirmed variable expression of FoxP3 in melanoma metastases by real-time reverse transcription-PCR and have commonly observed CD4+CD25+ T cells among TILs. Using a mouse preclinical model, we have investigated whether elimination of CD25+ T cells from bulk T cells in an adoptive transfer system could lead to improved control of B16 melanoma tumors in vivo. In fact, transfer of CD25-depleted splenic T cells could transiently suppress tumor growth, whereas total T cells had no effect, and this tumor control was achieved without any additional therapeutic intervention. However, tumors eventually did grow out, indicating that regulatory T-cell depletion alone is unlikely to be sufficient for complete tumor elimination. Importantly, two strategies for clinical depletion of regulatory T cells are possible to consider. The first is the interleukin-2/diphtheria toxin fusion protein Ontak, which was developed for treatment of CD25+ T-cell lymphomas (16). Interestingly, preliminary evidence has indicated that Ontak can transiently deplete regulatory T cells from the circulation in patients. The second is the use of anti-CD25 monoclonal antibody-coupled magnetic beads, which can deplete regulatory T cells from T-cell products to be used for adoptive transfer protocols. Clinical trials that use these strategies are under development.

### Inhibitory Ligands: The Example of PD-L1

Several membrane-associated factors expressed by tumor cells have been identified that have been shown to inhibit T-cell function. Arguably, the most important of these is PD-L1/B7-H1, which engages the inhibitory receptor on activated T cells called PD-1 (17). In contrast to the ligands for the alternative inhibitory receptor CTLA-4, PD-L1 can be expressed directly on tumor cells. Chen et al. showed the presence of PD-L1 protein by immunohistochemical analysis in a wide range of cancer types, including melanoma (18). We have confirmed frequent expression of PD-L1 expression in metastatic melanoma by real-time reverse transcription-PCR, flow cytometry, and immunohistochemical analysis. To study the function of PD-L1 in a preclinical setting, we analyzed a panel of mouse tumor cell lines and observed that most tumor cells up-regulate PD-L1 expression on IFN-γ treatment, including B16 melanoma (19). Using T-cell receptor transgenic/CD1−/−-deficient T cells, absence of PD-1 was associated with markedly improved tumor rejection in vivo.
under conditions in which even CTLA-4—deficient T cells did not reject (18). In addition, administration of a polyclonal antibody against PD-L1 has been shown to promote tumor rejection in other models (20). Together, these results support the development of reagents to block PD-1/PD-L1 interactions for clinical investigation.

**Metabolic Dysregulation: The Example of Indoleamine 2,3-Dioxygenase**

Our own gene expression profiling experiments have revealed that transcripts that encode metabolic factors that limit T-cell function also are commonly expressed in metastatic melanoma lesions. The molecule furthest along in exploration is indoleamine 2,3-dioxygenase (IDO). IDO is inducibly expressed by IFN-γ and has been observed to be expressed in melanoma tumors (20). A role for IDO has been established in suppressing T-cell responses through tryptophan depletion and via the generation of proapoptotic metabolites (22, 23). Interestingly, the effects of tryptophan depletion have been shown to be mediated in part via a stress-activated kinase called GCN2 (24). The importance of IDO in immunologic tolerance in vivo is illustrated by its involvement at the maternal-fetal interface (25). Uyttenhove et al. (20) have used a mouse preclinical model to show that IDO expression by tumors can be immunosuppressive and that inhibition of IDO activity with 1-methyltryptophan can improve T-cell–mediated tumor control in vivo. The availability of a straightforward inhibitor of IDO activity makes it possible to consider clinical investigation of this strategy.

Of note, controversy exists regarding whether 1-methyl-L-tryptophan or 1-methyl-D-tryptophan has superior inhibitory effects on IDO activity.

**Future Directions**

Now that several attractive candidate negative regulators have been identified that could limit the efficacy of antitumor T cells within the melanoma tumor microenvironment, prospective clinical studies should be pursued to determine if any of these are predictive of clinical outcome in response to immune-based therapies. Gene expression profiling of metastatic tumors before and after therapy also should be done to use an unbiased approach toward identifying factors that predict clinical response. At the same time, sufficient evidence has been acquired from preclinical models that reversal of T-cell anergy, depletion of regulatory T cells, blockade of PD-1/PD-L1 interactions, and antagonism of IDO can promote tumor rejection to warrant pursuit of similar strategies in patients with advanced melanoma. A summary of the proposed interventions toward uncoupling negative regulation of antitumor effector T cells is given in Table 1.

**Open Discussion**

**Dr. Atkins:** Are the elements that you’ve found in metastatic tumors to be potentially important to the tumor escape from the host of prognostic significance? Have they been looked at in lymph nodes? If you have antibodies, can they be looked at in paraffin tissue from primary tumors? We’ve not talked about tumor-infiltrating lymphocytes (TILs) in primaries, but is there some way to use this as a prognostic marker for early-stage disease?

**Dr. Gajewski:** That has not been done in melanoma, to my knowledge. I don’t know if TIL is the right word; maybe it should be tumor-associated lymphocytes, because we are unsure if they are really infiltrating the melanoma. It should be possible to see if there are regulatory cells among those tumor-associated lymphocytes and determine if that has predictive value.

**Dr. Elder:** TILs as judged by simple hematoxylin-eosin sections have been strongly associated with prognosis of melanoma since 1989, when a 12-fold difference in survival was demonstrated for patients with what were called brisk TILs compared with absent TILs. These were literally
show that autoimmunity and clinical response might be differences or somatic differences between tumors? There are population, is that heterogeneity due to germ-line genetic therapeutic interventions, if there is heterogeneity among the therapy approaches.

How much is controlled purely by host genetic factors. This information may help us select patients for immuno-therapy approaches.

There appear to be two patient populations. The first is a population of patients who develop autoimmunity and who don’t develop regulatory T cells and their immunosuppressive effects in their tumor. The second population has these various immunosuppressive factors that influence their T-cell function, so that they will never respond to immune stimulation. We need to sort out how much of this distinction is related to the tumor itself and how much is controlled purely by host genetic factors. This type of information may help us select patients for immunotherapy approaches.

Dr. Gajewski: Similar to the general response to immunotherapeutic interventions, if there is heterogeneity among the population, is that heterogeneity due to germ-line genetic differences or somatic differences between tumors? There are some data, as you know with the anti-CTLA-4 antibody, that show that autoimmunity and clinical response might be associated with a certain CTLA-4 gene polymorphism. But the rest of the heterogeneity would have to be host-related heterogeneity in the tumor cells, the tumor stroma, or the immune repertoire.

Dr. Keilholz: Regarding the minimal tumor load, looking at which of those factors is relevant in the minimal disease setting is something that could be done in your animal models.

Dr. Gajewski: It’s obviously a doable experiment. I’m sure we could find some model that would give us a result that says indoleamine 2,3-dioxygenase (IDO) is the most important factor or perhaps another model where programmed death ligand 1 (PD-L1) is the most important factor. I want to know the relevance and value of interfering with those pathways in patients at this point. We need to know the heterogeneity in patients because of the poor reliability of transplantable tumor models in mice to predict these more complicated tumor-host interaction phenomena in the clinic in human patients.

Dr. Slingluff: If we’re talking about moving toward being able to monitor this in a patient-specific way and designing therapy in that setting, what do you think is the right technology to do that with? Gene chip analysis on everybody or fine-needle aspiration biopsies for certain genes?

Dr. Gajewski: If I had to choose a single test, I would do a gene array. Flow cytometry can be used to look at tetramer-reactive cells, and you can focus your attention on the tumor antigen-specific population. We’re doing gene expression profiling on cells from large resected metastases to compare that to well-defined transcriptional profiles of T-cell differentiation states.

Dr. Ross: How comfortable are people in general that performing small biopsies or sampling represents the milieu of the tumor? How do you know you’re not getting a gene array of lymphocytes as opposed to tumor?

Dr. Gajewski: We’ve done core biopsies or excisional biopsies. When we’ve looked at serial biopsy specimens of the same tumor, they look similar. They cluster together on the nonsupervised hierarchical clustering. We have to keep in mind that normal nonmalignant cells might also be present in the biopsy specimen. We make an effort to dissect away grossly what is not tumor, but certainly there are a few normal cells from that particular target tissue where the metastasis came from that are also contributing to the transcriptional profile in the biopsy. I feel fairly strongly that in situ stains, either immunohistochemistry or in situ hybridization, are important to see what cell subsets are expressing the genes that seem to be differentially represented.

Dr. Hwu: It appears that there is exciting information about inhibitory pathways that could potentially lead to clinical interventions. What are your thoughts about future reagents that can be used in the clinic?

Dr. Gajewski: We have to develop new reagents against some of these targets. Even for IDO, 1-methyltryptophan is not a great inhibitor, and there are new drugs that have been reported on recently that are more potent inhibitors of IDO than 1-methyltryptophan. We have to continue to work toward developing agents to block IDO. For the interaction between PD-L1 and programmed death 1 (PD-1), my bias is toward neutralizing anti—PD-1 antibodies because of the possibility of another receptor for PD-L1 and PD-L2 that could have a positive costimulatory function like CD28 and

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<th>Inhibitory process</th>
<th>Proposed interventions</th>
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<tr>
<td>T-cell anergy</td>
<td>Homeostatic proliferation, Exogenous interleukin-7 and/or interleukin-15, Intratumoral B7, Small molecules to block anergy-associated factors</td>
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<tr>
<td>Regulatory T cells</td>
<td>Ontak, CD25 depletion of T cells for transfer, Small molecules to inhibit FoxP3 function</td>
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<td>PD-L1/PD-1 interactions</td>
<td>Anti – PD-1 or anti – PD-L1 monoclonal antibodies, Small molecules to inhibit PD-1 signals</td>
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<tr>
<td>IDO</td>
<td>1-Methyltryptophan, Small molecules to inhibit GCN2</td>
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Table 1. Candidate negative regulatory processes that suppress antitumor T-cell effector function and potential interventions that should be developed to reverse them in vivo.
CTLA-4. Then, we have reagents available to promote homeostatic proliferation and to deplete at least CD25+ cells in cancer patients. The attractiveness of denileukin difitox as opposed to anti-CD25 monoclonal antibody is the short half-life. The other attractive research and development aspect is small-molecule inhibitors against some of these other pathways: the kinase downstream from tryptophan catabolism and the signaling pathway downstream from PD-1.

References
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