Molecular profiling to identify relevant immune resistance mechanisms in the tumor microenvironment

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Summary
The molecular identification of tumor antigens initially catalyzed substantial enthusiasm for the development of tumor antigen-based vaccines for the treatment of cancer. However, numerous vaccine approaches in melanoma and other cancers have yielded a low rate of clinical response, despite frequent induction of specific T cells as detected in the peripheral blood. This observation has prompted several investigators to begin interrogating the tumor microenvironment for biologic correlates to tumor response versus resistance. Evidence is beginning to emerge suggesting that distinct subsets of tumors may exist that reflect distinct categories of immune escape. Lack of chemokine-mediated trafficking, poor innate immune cell activation, and the presence of specific immune suppressive mechanisms can be found to characterize subsets of tumors. A non-inflamed tumor phenotype may predict for resistance to cancer vaccines, suggesting a possible predictive biomarker and patient enrichment strategy. But in addition, characterization of these subsets may pave the way for catering therapeutic interventions toward the biologic features of the tumor in individual patients.

Introduction: Molecular profiling of human melanoma metastases

The observation that clinical responses to tumor antigen-based vaccines have generally not correlated well with biologic changes as measured in peripheral blood lymphocytes has led to focused interrogation of tumor sites for possible predictive factors based on features of the tumor microenvironment. One strategy for capturing a large amount of information in a single assay has been the use of gene expression profiling of the total cellular composition of tumors from pre-treatment biopsy material. This was first explored in our own group through a pilot study in 19 patients vaccinated with multiple melanoma antigen peptides and the cytokine IL-12. Supervised hierarchical clustering comparing clinical responders versus non-responders identified a small set of genes, some upregulated and some downregulated, in tumors from patients with a favorable clinical outcome [1]. A second melanoma vaccine clinical trial was similarly analyzed in collaboration with Gerold Schuler's group from Erlangen. In that dendritic cell-based immunization against class I and class II epitopes, T cell responses were induced in the majority of patients, but no correlation between clinical response and T cell parameters as measured in the blood was observed. In contrast, analysis of gene expression profiling of pre-treatment tumor biopsies identified a set of immune transcripts that clearly was associated with a favorable clinical outcome [2]. A third study

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was performed by GSK-Bio in the context of a MAGE-3 protein-based vaccine in melanoma and non-small cell lung cancer. In that case as well, gene expression profiling of pre-treatment tumor biopsies identified a cluster of transcripts that predicted outcome to treatment [3]. Together, these studies support the notion that molecular profiling of the tumor microenvironment is a candidate predictive biomarker for clinical response to these relatively potent melanoma vaccines [4]. This hypothesis is being tested prospectively as a scientific correlate embedded in the multi-center phase II and phase III studies of the GSK-Bio MAGE3 vaccine in patients with melanoma.

While there is not 100% overlap of the specific genes identified in these three clinical experiments, there is remarkable similarity in the categories of genes differentially expressed. In tumors from favorable clinical outcome patients, transcripts encoding various T lineage-specific markers, chemokines that can contribute to effector T cell recruitment, and innate immune cell molecules have been observed. Perhaps paradoxically, those tumors also appear to have the highest expression of immune inhibitory mechanisms, including indoleamine-2,3-dioxygenase (IDO), PD-L1, and FoxP3+ regulatory T cells (Tregs). In contrast, tumors from clinically resistant patients have shown poor expression of that gene set and in contrast show the highest expression of angiogenesis-associated factors, indicators of Notch and/or β-catenin pathway signaling, and serine protease inhibitors. These observations have pointed towards specific biologic pathways that might be manipulated in order to improve immune-mediated tumor destruction in vivo.

**Barrier 1: T cell trafficking**

It might be envisioned that cancer vaccines might not be effective unless activated tumor antigen-specific T cells can successfully home to tumor sites. Trafficking of activated T cells into target tissue sites is driven, in part, by the presence of specific chemokines which are likely induced through local tissue inflammation. Consistent with this notion, the gene expression profiles from pre-treatment melanoma biopsies indeed revealed expression of chemokine transcripts in the favorable clinical outcome group. This was also associated with the presence of T cell markers [5]. In vitro analysis of naïve versus effector CD8+ T cells demonstrated upregulated expression of CCR1, CCR2, CCR5, and CXCR3 in the effector stage. Based on known receptor/ligand interactions, these data suggested a potential role for 6 chemokines that might mediate attraction of CD8+ effector T cells: CCL2, CCL3, CCL4, CCL5, CXCL9, and CXCL10. Using a transwell system in vitro, each of these chemokines was sufficient to recruit CD8+ effector T cells, suggesting some redundancy [5]. The MAGE-A3 vaccine trial independently confirmed high expression of CCL5, CXCL9, and CXCL10 in favorable clinical outcome patients.

Analysis of a series of human melanoma cell lines revealed a small number of cell lines capable of spontaneously secreting these chemokines, suggesting that at least in some cases, differential presence of the relevant chemokines might be driven by biologic differences in the tumor cells themselves. These chemokine-producing melanoma cells successfully recruited activated CD8+ T cells in a reconstituted mouse xenograft setting in vivo [5]. Together, these results suggest that a subset of melanomas may have distinct properties that allow the expression of immunologically relevant molecules such as chemokines. A major implication of these results is that the ability of tumors to support recruitment of activated T cells into the tumor microenvironment may influence whether a given patient develops a clinical response following treatment with cancer vaccines. Absence of such chemokines likely represents the major barrier to vaccine efficacy in a subset of patients.
Barrier 2: Negative regulatory mechanisms in the tumor microenvironment

The tumors expressing abundant chemokines also contain variable numbers of activated CD8\(^+\) T cells. In a subset of HLA-A2\(^+\) patients from whom sufficient fresh tissue has been available, it has been possible to show that among these T cells are those recognizing defined melanoma antigen epitopes [6,7]. This observation raises the important question of why those tumors are not spontaneously rejected. In fact, functional analysis of such cells has revealed poor cytokine production and granzyme B expression upon analysis directly ex vivo [6,8]. These results suggest that inactivation of CD8\(^+\) T cells might occur following their recruitment into the tumor microenvironment. Multiple immune suppressive mechanisms that may inhibit T cell function in the cancer context have been described in the past several years [9–12]. Indeed, analysis of the melanoma metastasis gene array data revealed that tumors which contained T cell transcripts also contained high expression of the mRNA encoding indoleamine-2,3-dioxygenase (IDO). IDO has been shown to be involved in the induction of peripheral immunologic tolerance in other model systems [13]. Analysis of other candidate immune suppressive mechanisms revealed that those inflamed tumors also contained high levels of transcripts for PD-L1 (a ligand for the inhibitory receptor on activated T cells PD-1, [9]) and FoxP3 (a transcription factor characteristic of regulatory T cells (Tregs), [14]). In addition, analysis for expression of the costimulatory ligands B7-1 and B7-2 revealed absence of expression in the majority of tumors, suggesting that the melanoma microenvironment is also likely pro-anergy [15]. These results argue that a second major category of barrier to successful immune-mediated tumor destruction is the dominant action of at least four immune regulatory mechanisms operating within the tumor microenvironment, thus allowing tumor escape.

Mechanistic experiments in mouse models from multiple laboratories have indicated that each of the four negative regulatory pathways described above can be relevant for tumor resistance to immune destruction in various models in vivo [16–20]. Interference with PD-L1/PD-1 interactions, inhibition of IDO activity, depletion of Tregs, and reversal of T cell anergy through exposure to homeostatic cytokines, all have been efficacious in selected tumor models. Importantly, because multiple inhibitory mechanisms appear to operate in concert, one may envision emergence of compensation unless two or more pathways are blocked concurrently. Indeed, depletion of Tregs combined with homeostatic proliferation to uncouple T cell anergy have been shown to be potently synergistic in vivo [17]. Recently, we also have observed that PD-1 deficiency plus homeostatic proliferation also are synergistic (unpublished data). Such combinatorial approaches will ultimately be attractive to apply clinically.

T cell anergy is the most challenging of the above 4 immune inhibitory pathways to successful uncouple in vivo. Currently, induction of proliferation to homeostatic cytokines, most easily achieved through adoptive transfer into a lymphopenic recipient, is the strategy most amenable to clinical translation [16]. However, a better understanding of the molecular underpinnings of T cell anergy might reveal targets amenable to drug manipulation with the goal of restoring T cell function. Recent work from our group and others has indicated that diacylglycerol kinases (DGK), which phosphorylate diacylglycerol and deplete its availability for activating Ras and other pathways in response to TCR/CD28 ligation, are upregulated in anergic T cells [21,22]. Inhibition of DGKs in vitro can restore a large proportion of cytokine production by anergic T cells. Analysis of the promoter of DGKs followed by careful mechanistic studies have identified EGR2 as the central transcriptional regulator for expression of DGKs and other anergy genes in the anergic state (Zheng et al. manuscript submitted). Consistent with this notion, conditional deletion of the EGR2 gene can render peripheral T cells resistant to anergy induction. Because of the importance of this pathway, our group is currently screening a set of chemical libraries to phenocopy the
EGR2-loss phenotype through pharmacologic means. It is hoped that this approach could ultimately identify small molecule compounds that can improve T cell function in the cancer context in vivo.

**Innate immune “sensing” of tumor cells**

The observation that a subset of patients with metastatic melanoma appears to be able to prime a spontaneous anti-tumor CD8\(^+\) T cell response, all the way through the level of homing of effector T cells into the tumor microenvironment, raised a critical new question. Specifically, how is it possible for a tumor such as melanoma to activate the innate immune signals necessary to trigger a productive adaptive immune response? During infection, this frequently involves engagement of Toll-like receptors (TLRs) by conserved ligands derived from the pathogenic organism, including bacterial wall products (such as LPS) or hypomethylated CpG DNA sequences. Alternatively, cytosolic nucleic acid sensors trigger innate immune activation to many viruses [23,24]. Downstream, these events trigger activation of APCs to upregulate expression of costimulatory ligands and produce cytokines such as IL-12. A subset of ligands can also activate the inflammasome to trigger processing of IL-1, and also to produce innate factors such as type I IFNs [25,26]. However, it is not immediately evident which innate immune receptor systems might be involved in the context of “sensing” melanoma.

Interrogation of the melanoma metastasis gene expression profiling data for candidate innate immune genes revealed that tumors having a lymphocyte signature also showed a transcriptional profile characteristic of type I IFN signaling. Based on this observation, mechanistic studies were performed in mice to determine whether host type I IFNs are required for spontaneous CD8\(^+\) T cell priming in tumor models. Using a series of well-defined transplantable tumor systems, early T cell priming in the draining lymph node can be detected within around 1 week following tumor implantation [17]. This was preceded by detectable production of IFN-\(\beta\) in the tumor-draining lymph node at around day 3. Studies utilizing host mice deficient in either the type I IFN-\(\alpha/\beta\)R or Stat1 showed defective spontaneous T cell priming against a tumor-associated antigen, demonstrating that host type I IFN responsiveness is necessary as an innate immune factor to bridge for priming an adaptive immune response to tumors. Using bone marrow chimera and adoptive transfer approaches, this defect was found to map to the level of the dendritic cell (DC) compartment. Interrogation of DC properties revealed an isolated defect in the accumulation of the CD8\(\alpha^+\) DC subset within the tumor microenvironment. The necessity of CD8\(\alpha^+\) dendritic cells for cross-priming of anti-viral CD8\(^+\) T cell responses supports a critical role for this DC subset in vivo [27]. In fact, the Batf3 knockout mouse which has a selective defect in CD8\(\alpha^+\) DC development, showed an identical defect in priming CD8\(^+\) T cells to tumor. Therefore, host type I IFN signals are required for the priming of anti-tumor CD8\(^+\) T cells via CD8\(\alpha^+\) dendritic cells in vivo (Fuertes and Gajewski, manuscript submitted). This model system is similarly being utilized to identify the tumor-derived factors that induce type I IFN production from host dendritic cells, and which receptor system is responsive to those tumor-derived factors.

While host type I IFN production is necessary for the endogenous immune response that arises after tumor implantation in vivo, that immune response is not sustained. T cell dysfunction eventually develops in these models, which is associated with tumor outgrowth [26]. This finding raised the question of whether sustained presence of type I IFNs in the tumor site might improve tumor control. Indeed, preliminary data have indicated that expression of either IFN-\(\alpha\) or IFN-\(\beta\) by retroviral transduction can lead to total rejection of B16 melanoma in vivo (Spaapen and Gajewski, unpublished observations). While the mechanism of this potent therapeutic effect is being elucidated, these results already suggest
that introduction of sustained type I IFN preparations into tumor sites is an attractive approach to examine clinically, which might result in more potent tumor control than systemic administration.

**Hypothetical molecular differences to account for distinct tumor microenvironments**

The observation of at least two major subsets of melanoma metastases, “inflamed” and “non-inflamed”, raises the question of what are the molecular pathways in the tumor cells that may lead to differential expression of immune regulatory genes such as chemokines. The vast majority of melanomas display constitutive activation of the Ras pathway, either through mutations in N-Ras or B-Raf, or more rarely through upstream activation of growth factor receptors. Therefore, it is unlikely that variable engagement of the Ras pathway could explain phenotypic differences between tumors. However, several other signaling pathways have been found to be variably activated in subsets of tumors. These include activation of Notch signaling [28], β-catenin stabilization [29], Stat3 phosphorylation [30], PI3 kinase [31], ErbB4 [32] and c-met [33]. Several of these pathways are candidates for downregulating expression of immunoregulatory genes in melanoma. In vitro, knockdown of Stat3 has direct anti-tumor activity, but also induces expression of chemokines that might mediate lymphocyte trafficking [34]. In addition, our melanoma gene expression profiling data have suggested a correlation between Notch pathway and/or β-catenin pathway activation and lack of an immune infiltrate. Based on these notions, it is attractive to evaluate the functional role of these or other signaling pathways in the melanoma cells themselves that might regulate their ability to productively interact with the host immune system. Such work, if successful, might lead to small molecule inhibitor approaches to alter the tumor microenvironment from within and thus lead to improved immune-mediated tumor control.

**Conclusions: Clinical therapeutic implications**

The information gleaned from gene expression profiling, combined with the preclinical experiments dissecting mechanisms of tumor escape, have come together to suggest therapeutic strategies to facilitate immune-mediated tumor rejection at the level of the tumor microenvironment. For the non-inflamed tumor phenotype, it may be possible to promote appropriate inflammatory signals to facilitate recruitment of activated T cells into the tumor microenvironment after vaccination or adoptive T cell transfer. As proof of concept, recombinant viral vectors could be employed to introduce selected chemokines, innate immune factors such as IFN-β, or molecules that induce chemokines from host cells. Murine experiments utilizing an adenoviral vector encoding LIGHT, a TNF superfamily member that induces chemokine production from stromal cells via engagement of the LTβ receptor, have revealed remarkable efficacy of this approach [35]. Additional non-specific interventions that may promote desirable inflammation include introduction of TLR agonists, such as imiquimod or CpG oligonucleotides, or administration of single fractions of high-dose radiation [35]. These are attractive strategies to consider for clinical translation.

For the inflamed tumor phenotype, approaches for clinical application are farther along in development. It is this subset that appears to include the clinical responders to our current melanoma vaccine strategies. However, even by using gene expression profiling as a predictive biomarker to enrich for patients with the inflamed phenotype, the clinical response rate would be estimated to be around 30%. Therefore, interfering with the immune suppressive mechanisms that appear to be dominant in the tumor microenvironment of these patients may be the most critical therapeutic intervention strategy to consider. Early phase clinical trials are ongoing to inhibit IDO, block PD-1/PD-L1 interactions, or deplete Tregs.
For IDO, at least two small molecule inhibitors are being investigated. These are attractive because of their oral bioavailability [19,36]. For the PD-1 axis, neutralizing mAbs against either PD-1 or PD-L1 are being tested. Interestingly, results of a phase I study of the anti-PD-1 mAb developed by Medarex and BMS were recently presented and revealed an approximate 30% response rate in patients with melanoma, non-small cell lung cancer, and renal cell carcinoma [37]. For Tregs, several approaches targeting CD25 are being explored. Early data using Ontak, an IL-2/diphtheria toxin fusion protein, have shown promise [38,39]. In addition, the anti-CD25 mAb daclizumab has been tested and appears to be able to deplete Tregs without adversely affecting T cell priming to a vaccine [40]. A second anti-CD25 mAb, basiliximab, is available but has not yet been investigated in this fashion for Treg depletion and vaccine combinations.

For T cell anergy, clinically testable strategies to support reversal have been more challenging to pursue. However, we have shown that homeostatic proliferation in a lymphopenic recipient is one approach in preclinical models that can support recovery of anergic T cell function and promote tumor rejection in vivo [16]. It is therefore attractive to consider that the adoptive T cell therapy approaches that involve conditioning the patient with lympho-depleting chemotherapy prior to adoptive transfer may be efficacious, in part, by countering T cell anergy [41,42]. It is conceivable that this could be integrated with other immunotherapeutic strategies, such as vaccination, Treg depletion, or blockade of PD-1 or IDO. Another attractive approach would be through exogenous delivery of the homeostatic cytokine IL-15 [43,44]. While clinical development of IL-15 had been stalled, the NCI has recently manufactured a clinical grade lot of IL-15 that is entering phase I clinical trial testing.

Ultimately, once the most effective of these new therapies have been successfully developed, it is not difficult to envision a personalized therapy approach in the near future. One could imagine performing an analysis of pre-treatment biopsy material to determine the major phenotype of melanoma tumor (inflamed versus non-inflamed) through gene expression profiling, along with an analysis of the immune suppressive pathways dominating in the inflamed lesions. In this way, the most critical rate-limiting barriers could be overcome through specific interventions assigned based on tissue analysis of an individual patient's tumor. Patients with non-inflamed tumors may need to receive systemic vaccination along with local application of inflammatory signals to promote T cell recruitment. Patients with inflamed lesions may most critically need blockade of inhibitory pathways, such as PD-1. Ultimately, such analyses could be integrated into the kinase mutation analysis that currently is being utilized to select patients for agents that target B-Raf [45] or c-kit [46].

References


contribution in tumor models, the work is noteworthy because it makes it possible to consider pharmacologic manipulation to restore T cell function.


42. Rosenberg SA, Dudley ME. Adoptive cell therapy for the treatment of patients with metastatic melanoma. Curr Opin Immunol. 2009; 21:233–240. [PubMed: 19304471] The NCI surgery branch has made great strides in advancing adoptive T cell therapy as a cancer therapeutic. This approach is most successful when T cells are transferred after administration of lymphodepleting chemotherapy, arguing for the importance of homeostatic proliferation of transferred T cells.


Figure 1.
Two categories of tumor with distinct mechanisms of resistance to immune-mediated destruction at the effector phase. A. The non-inflamed tumors appear to have high expression of vascular markers as well as macrophages and fibroblasts. They have low indicators of innate inflammation, show poor chemokine production, and have a paucity of lymphocytes. It is hypothesized that the main reason for tumor escape in this subset is poor effector cell trafficking. B. The inflamed tumors appear to have a rich presence of innate immune signals, chemokines for T cell recruitment, and variable presence of T cells. However, these tumors also contain important immune suppressive mechanisms. It is hypothesized that the main reason for tumor escape in this subset is through dominant effects of negative regulation.