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GM-CSF-secreting melanoma vaccines

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The development of biochemical and genetic schemes to characterize cancer antigens led to the recognition that malignant melanoma frequently evokes a host response. While the generation of brisk T-cell infiltrates in early stage disease is correlated with prolonged survival, host reactions in most cases are insufficient to impede tumor progression. One variable that may limit the potency of the host response against nascent melanoma is the mixture of cytokines present in the tumor microenvironment. In a murine melanoma model, we identified granulocyte–macrophage colony stimulating factor (GM-CSF) as the most potent molecule for augmenting tumor immunity following gene transfer into melanoma cells. Vaccination with irradiated melanoma cells engineered to secrete GM-CSF enhances host responses through improved tumor antigen presentation by recruited dendritic cells and macrophages. Melanoma-specific $CD4^+$ and $CD8^+$ T-cells, CD1d-restricted NKT-cells, and antibodies mediate tumor rejection. Initial testing of this immunization strategy in patients with metastatic melanoma revealed the consistent induction of cellular and humoral antitumor responses that provoked the extensive necrosis of distant metastases without significant toxicity.
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Introduction

Although humoral and cellular responses to malignant melanoma were first demonstrated more than 20 years ago (Old, 1981), the crafting of genetic and biochemical strategies to identify the molecular targets of these responses dramatically advanced the field of cancer immunology (Boon *et al*., 1994). These cloning strategies uncovered a surprisingly diverse array of gene products that stimulate immune recognition (Boon and van der Bruggen, 1996; Old and Chen, 1998). $CD4^+$ and $CD8^+$ T-cells that react to mutant or wild-type melanoma-associated proteins can be detected frequently in the blood, lymphoid tissues, and malignant lesions of patients (Jäger *et al*., 2000; Zeng *et al*., 2001). Aberrantly expressed melanoma antigens consistently elicit high-titers of specific IgG antibodies (Sahin *et al*., 1995). Natural killer cells and other participants in innate immunity recognize stress-related genes induced as a consequence of transformation (Bauer *et al*., 1999; Groh *et al*., 1999).

This impressive breadth of immune reactivity constitutes a powerful framework for pursuing detailed investigations of the antimelanoma response. Longitudinal clinical-pathologic studies underscore an intriguing association between some antimelanoma reactions and favorable clinical outcomes. Clark and Mihm established that the presence of dense T-cell infiltrates in the vertical growth phase of primary malignant melanoma is correlated with a diminished incidence of recurrent disease and a reduced mortality (Clark *et al*., 1989; Clemente *et al*., 1996). The generation of brisk T-cell infiltrates in regional lymph node melanoma metastases similarly confers an improved prognosis compared to tumors without infiltrates (Mihm *et al*., 1996). While these striking responses are relatively uncommon, the correlations nonetheless highlight the possibility that T-cell activities may modulate melanoma progression. A comparison of the gene expression profiles of primary tumors that elicit lymphocyte responses versus those tumors that lack infiltrates may provide new insights into the mechanisms that shape endogenous antimelanoma immunity.

Cytokines in the tumor microenvironment

One major determinant that restricts the immunogenicity of nascent melanomas is the mixture of cytokines in the tumor microenvironment (Mach and Dranoff, 2000). Forni *et al* (1985, 1988) revealed that modifying the cytokine milieu could alter the outcome of the host response. The peritumoral injection of particular cytokines, especially interleukin-2, stimulated tumor rejection through the coordinated functions of neutrophils, eosinophils, macrophages, natural killer cells, and lymphocytes. In some cases, the response also generated protective immunity against later tumor challenge. These important findings motivated many additional studies examining the antitumor effects of putative immunostimulatory molecules (Tepper and Mule, 1994). The use of high-efficiency gene transfer systems to accomplish the stable modification of tumor cells considerably advanced this work by improving the

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pharmacology of the delivered proteins (Dranoff and Mulligan, 1995).

To compare the relative abilities of different gene products to enhance antitumor immunity, we generated a large panel of high-titer retroviral vectors expressing a variety of cytokines, adhesion molecules, and costimulatory molecules (Dranoff *et al*., 1993). The MFG retroviral vector used in these studies exploits the Moloney murine leukemia virus long terminal repeat to regulate expression of both a full-length transcript (for encapsidation into viral particles) and a spliced transcript (analogous to env) containing the inserted cDNA. The high viral titers and high-level expression of the transferred gene with this system obviate the requirement for selecting transduced cells.

This array of recombinant viruses proved useful for identifying host factors that limit the response to melanoma (Dranoff *et al*., 1993). The B16 murine model was particularly informative for this analysis, since vaccination with irradiated wild-type cells fails to induce significant levels of protective immunity. The relative immunogenicities of various engineered B16 cell populations are shown in Table 1. In these studies, irradiated genetically modified B16 cells were injected subcutaneously into syngeneic C57Bl/6 mice and the ability of the vaccinated mice to reject a subsequent challenge of live, wild-type B16 cells was determined. Although several gene products increased protective immunity to varying degrees, GM-CSF was the most potent molecule.

The injection of irradiated, GM-CSF-secreting tumor cells stimulated an intense local reaction consisting of dendritic cells (DCs), macrophages, and granulocytes (Dranoff *et al*., 1993; Mach *et al*., 2000). The accumulation of large numbers of professional antigen-presenting cells suggested that one function of GM-CSF in this model involved the augmentation of tumor antigen presentation (Dranoff *et al*., 1993; Huang *et al*., 1994). Substantial evidence indicates that DCs mediate a crucial role in priming antigen-specific immune responses (Banchereau and Steinman, 1998). DCs are well suited for this purpose, as they express diverse receptors that mediate the acquisition of antigens in peripheral tissues, process this material efficiently into MHC class I and II pathways, upregulate costimulatory molecules upon maturation, and migrate to secondary lymphoid tissues (Banchereau *et al*., 2000). These specializations have engendered considerable interest in manipulating DCs to augment antitumor responses (Young and Inaba, 1996).

A more detailed understanding of DC biology has revealed an impressive plasticity that allows these cells to respond optimally to varying inflammatory conditions and pathogens (Banchereau *et al*., 2000). This heterogeneity of phenotype and function renders important the identification of specific characteristics of DCs that are most appropriate for tumor vaccination. Toward this end, we compared the biologic activities of B16 melanoma cells engineered to secrete GM-CSF or Flt3-ligand (FL) (Mach *et al*., 2000). Interestingly, both cytokines provoke a marked expansion of DCs locally and systemically, but GM-CSF stimulated greater levels of protective immunity. Several differences between the DCs elicited by GM-CSF and FL may account for the distinct vaccination abilities. First, GM-CSF elicited a subset of DCs that are superior for the phagocytosis of particulate material, such as apoptotic tumor cells (Pulendran *et al*., 1997; Shen *et al*., 1997). Second, compared to FL, GM-CSF evoked higher levels of costimulatory molecules, indicative of greater functional maturation. This enhanced activity results in more efficient T-cell stimulation, thereby broadening the profile of induced lymphocyte effector mechanisms (Murtaza *et al*., 1999). Third, GM-CSF promoted uniformly high levels of CD1d on DCs, in contrast to FL, which triggered heterogeneous expression. CD1d is a nonclassical MHC class I molecule that presents lipid antigens (Kawano *et al*., 1997); these likely include gangliosides that are present in the cell membranes of melanoma cells (Yamaguchi *et al*., 1990). The CD1d–lipid complex stimulates invariant NKT-cells, a transitional population of lymphocytes that display a restricted T-cell receptor usage and features of natural killer cells (Bendelac *et al*., 1997). Importantly, NKT-cells play crucial roles in both the endogenous and therapeutic response to tumors (Smyth *et al*., 2002).

To examine whether NKT-cells contribute to the vaccine response of GM-CSF-secreting B16 cells, we studied mice deficient in CD1d or Jα281 (the TCR expressed by invariant NKT-cells) (Gillessen *et al*., submitted). Immunization activity was abrogated in both NKT cell-deficient strains. Whereas vaccinated wild-type mice manifested a broad T-cell cytokine response to B16 cells (which included IFN-γ, IL-4, IL-5, IL-10, IL-13, and GM-CSF), immunized CD1d or Jα281 mice showed the impaired production of most cytokines. T-cell IFN-γ secretion and tumor-specific cytotoxicity were retained, however. This pattern of effector mechanisms is comparable to that of wild-type mice immunized with FL-secreting tumor cells, suggesting that differences in the abilities of GM-CSF and FL to stimulate CD1d-restricted invariant NKT-cells may be decisive for tumor protection.

Table 1 Immunization activity of irradiated, genetically modified B16 melanoma cells

90%	Protection	GM-CSF
40%	Protection	IL-3, IL-4, IL-6
20%	Protection	IL-1, IL-7, SCF, G-CSF, Flt3-ligand, eotaxin, cyclophilin
0%	Protection	IL-2, IL-5, IL-10, IL-12, IL-18, TNF-α, IL-1Ra, IFN-γ, B7-1, M-CSF, RANTES, MIP-1α, MIP-1β, ICAM, TGF-β, CD2, MIF, lymphotactin

Syngeneic C57Bl/6 mice were immunized subcutaneously with 5×10^5 irradiated, modified B16 cells and challenged subcutaneously 1–2 weeks later with 1×10^6 live wild-type B16 cells. The fraction of animals surviving wild-type B16 challenge is indicated

The broad cytokine production elicited by GM-CSF-secreting melanoma vaccines is consistent with the requirement for $CD4^+$ T-cells in this system (Dranoff *et al*., 1993; Hung *et al*., 1998). $CD4^+$ T-cells also contribute to the production of antitumor antibodies, which similarly play a pivotal role in GM-CSF-stimulated immunity (Reilly *et al*., 2001). This coordinated cellular and humoral response may be best suited to overcome the multiple apoptotic defects that characterize advanced melanoma cells (Hanahan and Weinberg, 2000).

The delineation of specific properties of DCs that elicit high levels of tumor immunity provides important guidelines for optimizing the therapeutic use of these cells for cancer immunotherapy. Many strategies under investigation involve loading cancer antigens onto *ex vivo* expanded DCs (Banchereau *et al*., 2000). In these approaches, monocyte-conditioned medium (or the equivalent) is required in order to expand functionally mature DCs (Reddy *et al*., 1997; Thurner *et al*., 1999). In this context, the vaccination sites of GM-CSF-secreting tumor cells disclose the recruitment of DCs in concert with large numbers of macrophages and granulocytes; indeed, the three hematopoietic lineages derive from a common precursor (Inaba *et al*., 1993). Thus, the ability of GM-CSF to activate DCs, macrophages, and granulocytes coordinately is likely linked with the optimal maturation of DCs *in vivo*. These findings suggest that appropriate pharmacologic delivery of GM-CSF may have broad utility for vaccination.

Phase I clinical trials

In order to determine whether this vaccination scheme could enhance antimelanoma immunity in humans, we conducted a phase I study in patients with metastatic melanoma (Soiffer *et al*., 1998). In the trial, surgically resected tumors were processed to single-cell suspension by collagenase and mechanical digestion and then introduced into short-term culture. Replicating melanoma cells were infected with replication-defective retroviruses expressing human GM-CSF, irradiated with 15000 cGy, and cryopreserved in liquid nitrogen. The transduced cells were certified to be free of replication-competent retrovirus, endotoxin, mycoplasma, and other microbial contaminants. Vaccines were produced for 29 of the 31 patients enrolled, and the GM-CSF secretion rates achieved ranged from 84 to 965 ng/10^6 cells/24 h. These values represented at least a two log increase over endogenous levels. Patients were immunized intradermally and subcutaneously with 10^7 irradiated tumor cells (per treatment) administered at 28-, 14-, or 7-day intervals. Rapid disease progression forced the early withdrawal of eight patients from study. No significant toxicities attributable to the treatment, including autoimmune reactions or viral syndromes, were observed.

Consistent with the murine studies, vaccination sites disclosed brisk infiltrates of DCs, macrophages, and eosinophils in all 21 evaluable patients. Immunohistochemistry revealed high levels of B7-1 on the recruited DCs, indicative of functional maturation (Mach *et al*., 2000). In response to immunization, all patients developed reactivity to injections of nontransduced autologous tumor cells. These responses consisted of T-cells, macrophages, and eosinophils. The number of circulating eosinophils was also increased, reflecting the vaccine-induced T-cell production of GM-CSF, IL-3, and IL-5.

The most convincing evidence that vaccination augmented antimelanoma immunity was revealed by pathologic examination of the host response to disseminated tumors. Whereas metastatic lesions resected before vaccination were minimally infiltrated with cells of the immune system in all patients, metastatic lesions resected after vaccination were densely infiltrated with T and B cells, and showed extensive tumor destruction (at least 80%), fibrosis, and edema in 11 of 16 patients examined. Characterization of the infiltrating cells by immunohistochemistry revealed the accumulation of $CD4^+$ and $CD8^+$ T-cells, $CD20^+$ B lymphocytes, and plasma cells secreting immunoglobulin. Consistent with the pathologic evidence of tumor necrosis, the lymphocytes purified from the infiltrated metastases manifested potent cytotoxicity and secreted a broad profile of cytokines in response to the autologous tumor cells. High-titer antibodies recognizing melanoma-associated antigens were present in postvaccination sera as revealed by FACS and Western analysis. An unexpected finding in four patients was the targeted destruction of the tumor vasculature by activated lymphocytes, eosinophils, and neutrophils. The mechanisms underlying the selective targeting of tumor blood vessels remain to be elucidated.

While this phase I trial illustrates the biologic activity and safety of GM-CSF-secreting autologous melanoma cells, the use of conventional retroviral vectors to engineer the vaccines presents a logistical impediment to more extensive clinical evaluation. Murine leukemia virus-derived vectors require replicating target cells for efficient gene transfer and are potential carcinogens (Mulligan, 1993). These properties necessitate the establishment of short-term tumor cultures and extensive safety testing. One approach to overcoming these hurdles is to use adenoviral vectors, which readily infect resting target cells and are associated with minimal toxicities when applied in *ex vivo* gene transfer strategies (Berkner, 1988; Shenk, 1996).

Based upon these considerations, we conducted a second phase I trial in metastatic melanoma patients in which adenoviral vectors were used to engineer autologous GM-CSF-secreting tumor cells (Soiffer *et al*., submitted). The ability to manufacture vaccines for 34 of 35 enrolled patients validates the high efficiency of the production scheme. No significant toxicities were observed in the trial, establishing the safety of this gene transfer approach. While some differences in the antimelanoma immune response were found compared to the retroviral approach, T- and B-cell infiltrates in distant metastases were consistently observed. These

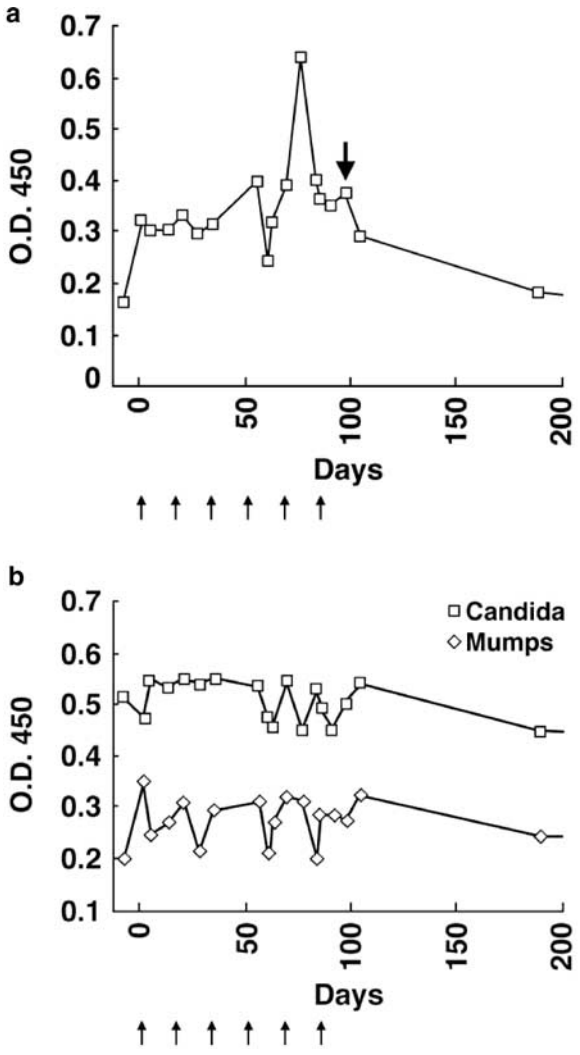

Figure 1 Vaccination augments antibodies to ATP6S1. (**a**) Longitudinal study of antibody titers to recombinant ATP6S1 in a long-term responding patient (sera 1 : 500 dilution). Small arrows denote vaccinations; bold arrow indicates time of subcutaneous metastasis resection. (**b**) Reactivity to mumps and candida antigens (sera 1 : 1000 dilution). Reproduced from Hodi *et al.* (2002)

encouraging results have motivated the initiation of adenoviral GM-CSF-based autologous tumor vaccine trials in patients with metastatic nonsmall cell lung or ovarian carcinoma and advanced myelodysplasia/acute myelogenous leukemia.

Target antigens

In order to identify the target antigens for the reactions against intact melanoma cells, we undertook a detailed analysis of a patient who manifested a long-term complete response to vaccination (ongoing disease-free survival at 6 years). A melanoma cell line was generated from a densely infiltrated metastasis and used to construct a cDNA expression library that was screened with postvaccination serum (Hodi *et al*., 2002). One of the gene products revealed by this work was ATP6S1, a putative subunit of the ubiquitous vacuolar H^+-ATPase complex (Supek *et al*., 1994). Longitudinal studies demonstrated that immunization provoked a 12-fold increase in antibodies to the protein, although reactivity to mumps and candida antigens was unaffected (Figure 1). Intriguingly, the peak reactivity to ATP6S1 was temporally associated with the development of erythema and hemorrhage in a subcutaneous metastasis. Further investigation disclosed an association between vaccine-stimulated humoral responses to ATP6S1 and tumor destruction in several other immunized metastatic melanoma patients (Hodi *et al*., 2002).

The immunogenicity of ATP6S1 was unexpected, since the gene product is broadly expressed in normal tissues and no tumor-associated mutations have been found. Nonetheless, a growing number of cancer antigens fail to show tumor-restricted expression (Scanlan *et al*., 1998, 1999), underscoring the inter-relations of tumor immunity and autoimmunity (Houghton, 1994). Perhaps transformation-induced alterations in the cell biology of ubiquitous proteins render them accessible to immune recognition.

Future prospects

Although some vaccinated metastatic melanoma patients achieved long-lasting clinical responses (currently up to 6 years), most patients eventually succumbed to disease progression. One mechanism that may limit the efficacy of GM-CSF-secreting melanoma vaccines is the attenuation of T-cell activation by cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) (Chambers *et al*., 2001). Whereas the binding of B7 family members to CD28 delivers an important costimulatory signal to T-cells, the engagement of CTLA-4 by these molecules results in cell cycle arrest and diminished effector function (Thompson and Allison, 1997; Doyle *et al*., 2001; Salomon and Bluestone, 2001). In the B16 melanoma model, the infusion of antibodies that block CTLA-4 function enhances the antitumor effects of GM-CSF-secreting tumor cell vaccines, albeit with the loss of tolerance to normal melanocyte differentiation antigens (van Elsas *et al*., 1999, 2001). Based upon this intriguing synergy, we have initiated clinical testing of the combination of a human blocking antibody to CTLA-4 and autologous, GM-CSF-secreting melanoma cells. These studies should provide important new insights into the mechanisms that limit the development of clinically efficacious antimelanoma immunity.

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