Published in final edited form as:

Cancer J. 2011 ; 17(5): 379–396. doi:10.1097/PPO.0b013e3182346320.

Multiple Vaccinations: Friend or Foe

Sarah E. Church^{1,2}, Shawn M. Jensen¹, Chris Twitty¹, Keith Bahjat^{1,2}, Hong-Ming Hu^{1,3}, Walter J. Urba¹, and Bernard A. Fox^{1,2}

¹Robert W. Franz Cancer Research Center, Earle A. Chiles Research Institute, Providence Cancer Center, Providence Portland Medical Center, Portland, Oregon 97213

²Departments of Molecular Microbiology and Immunology, Oregon Health and Science University, Portland, Oregon

³Cancer Research and Biotherapy Center, The Nanjing Second Hospital, 87 Ding Jia Qiao Road, Nanjing, Jiangsu 210009, P. R. China

Abstract

Few immunotherapists would accept the concept of a single vaccination inducing a therapeutic anti-cancer immune response in a patient with advanced cancer. But what is the evidence to support the “more-is-better” approach of multiple vaccinations? Since we are unaware of trials comparing the effect of a single vaccine versus multiple vaccinations on patient outcome, we considered that an anti-cancer immune response might provide a surrogate measure of the effectiveness of vaccination strategies. Since few large trials include immunological monitoring, the majority of information is gleaned from smaller trials in which an evaluation of immune responses to vaccine or tumor, before and at one or more times following the first vaccine was performed. In some studies there is convincing evidence that repeated administration of a specific vaccine can augment the immune response to antigens contained in the vaccine. In other settings multiple vaccinations can significantly reduce the immune response to one or more targets. Results from three large adjuvant vaccine studies support the potential detrimental effect of multiple vaccinations as clinical outcomes in the control arms were significantly better than that for treatment groups. Recent research has provided insights into mechanisms that are likely responsible for the reduced responses in the studies noted above, but supporting evidence from clinical specimens is generally lacking. Interpretation of these results is further complicated by the possibility that the dominant immune response may evolve to recognize epitopes not present in the vaccine. Nonetheless, the FDA-approval of the first therapeutic cancer vaccine and recent developments from preclinical models and clinical trials provide a substantial basis for optimism and a critical evaluation of cancer vaccine strategies.

Introduction

Traditional views regarding cancer vaccines hold that persistence of a therapeutic anti-tumor response would be best accomplished by providing “booster” vaccinations. This postulate is based in large part on a well-established tenet of immunology based on the success of vaccines to protect uninfected naïve individuals from subsequent exposure to specific infectious agents or their toxins. In these cases a priming vaccination is typically followed by a series of booster vaccines that expand the pool of memory B and T cells (1,2).

However, some vaccines for infectious disease provide protection with a single dose (influenza, smallpox). This is similar to many preclinical tumor vaccine studies where a single vaccination can prime tumor-specific immune responses that provide protection from a subsequent tumor challenge. In most models, the ability of a single vaccine to provide therapeutic immunity has correlated with a tumor-specific Type 1 immune response, where CD8 T cells secrete IFN- γ and/or TNF- α (3). Classical tumor immunotherapy studies frequently start with a single immunization with irradiated immunogenic tumor cells or tumor cells mixed with *Corynebacterium parvum*, followed by serial immunization with live tumor cells to generate “immune” mice(4-7). Immune responses in mice that reject tumor challenges are likely to be substantially different from mice receiving repetitive vaccinations with a vaccine that does not contain viable tumor cells. Recently, our group reported that T cells from thrice vaccinated mice were significantly less effective in adoptive transfer studies than T cells from mice receiving a single vaccination(8). A striking difference observed in multiply vaccinated animals was an increase in the number of regulatory T cells. Elimination of these regulatory cells during the second and third vaccinations resulted in a recovery of therapeutic efficacy. At the same time a number of large phase III clinical trials found that patients receiving multiple vaccines had significantly worse outcomes than control arms. This included two adjuvant studies where patients were randomized to receive a vaccine composed of three allogeneic melanoma cell lines plus BCG versus BCG alone (9,10). In one study 1,166 patients with stage III melanoma were enrolled. In a second, 496 patients with stage IV melanoma were enrolled. At the interim analysis both studies were halted due to significantly worse outcomes in the tumor vaccine arms (11). In another study, 1,314 stage II melanoma patients were randomized to observation or vaccination with a ganglioside vaccine (11,12). When an interim analysis was performed the vaccine arm exhibited a significantly worse survival than observation and the trial was stopped. These results moved us as well as many in the field to evaluate the rationale for repetitive vaccinations (8,10,12).

As noted above, one setting where multiple “booster” doses is effective is in the prevention of infectious disease. An obvious difference between vaccines for the prevention of infectious disease and the immunotherapy of cancer, is that in the setting of cancer, vaccines are not yet preventative and therapeutic vaccines are not administered to naïve individuals but to patients that have lived with their cancer for months to years and frequently have substantial tumor burden at the time of vaccination. Additionally, unlike vaccines for infectious disease, that contain foreign antigens to which the host is not immunologically tolerant, most cancer vaccines contain antigens normally expressed by the host, which provides an opportunity for the host to develop immunological tolerance. Finally, while tumor cells are continuously undergoing mutations that improve the chances of escaping an immune response, the success of many vaccines for infectious disease likely relates to the stability of the antigenic repertoire (14). We consider that these observations provide insight into why clinical trials of therapeutic cancer vaccination have been less effective than vaccines for prevention of infectious disease.

Protection against pathogens often requires multiple vaccinations

Given the success of booster vaccines for infectious diseases, a review of the literature of immune responses that correlate with protective immunity to pathogens is warranted. The FDA has approved over 70 vaccines for distribution in the United States; of these less than half protect with a single vaccination (influenza, smallpox, pneumococcal) and the majority require one or more boosting vaccinations (hepatitis, measles, encephalitis, tetanus)(15). The infectious disease field has a limited number of surrogates for protective immunity induced by vaccination. As an example the major correlate of protection for smallpox vaccination, which historically has been administered by scarification with a bifurcated needle, is

measured by the appearance of a primary vesicle at the vaccination site (16). This pustule has been one of the best correlates to vaccine protection, however it is highly specific for the route of vaccination on the skin's surface (1,17). Antibodies reduce disease by opsonization, antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) to neutralize pathogens and eliminate pathogen infected cells (18). Monitoring the antibody response can provide an indication of vaccine-induced protective immunity. For the yellow fever virus the induction of a yellow fever plaque neutralizing antibody of >0.7 log conferred protection in non-human primate models and is widely accepted as evidence of protective immunity (19). Antibodies developed following tetanus vaccination have been shown to be protective at levels as low as 0.01 IU/ml (2). However, neutralizing antibodies wane over time after vaccination with tetanus and hepatitis B and therefore booster vaccinations are suggested (1,20). Memory B cell responses do not seem to correlate with serum levels of vaccine-specific antibodies; however, they do correlate with protection from chronic viral infections (1). Due to the "chronic" nature of cancer immunity it is possible memory B cells could be an important aspect for both cancer vaccine monitoring and the therapeutic effects of immunotherapy. Certain viral infections are more likely to elicit vaccine-specific cellular responses than others. The efficacy of smallpox vaccination, which eradicated smallpox in 1980 is thought to be a result of vaccine-induced CD4 and CD8 T cells responses nearly identical to those observed following smallpox infection (21). This possibility has pushed the infectious disease field to develop vaccines that mimic cellular immunity to natural infection (22). Induction of polyfunctional T cells that produce IFN- γ , TNF- α , IL-2 and MIP-1 β correlates with viral protection, vaccinia vaccination and prime-boost regimens using attenuated vaccinia virus and therefore are another measure of vaccine efficacy (23). Thus, for many infectious diseases booster vaccines augment immune responses that correlate with protection from that disease. The human papilloma virus (HPV) vaccine is administered as a priming dose followed by two booster vaccinations and is one of the best examples of a multiply administered preventative vaccine that decreases incidence of cervical cancer (24). What is the evidence that administration of additional booster vaccinations augments the anti-tumor immune response in the therapeutic setting? To address this question we reviewed clinical trials that reported immunological monitoring at two or more time points. We included some reports that did not include monitoring but translated concepts or had some clinical outcome we felt contributed to the review. A table summarizing the effect of specified vaccination strategies on the anti-cancer immune response is provided (Table 1).

PEPTIDE VACCINES: gp100 peptide vaccine in metastatic melanoma

Melanoma differentiation antigens have been the subject for many of the peptide vaccine trials. Among this group, melanocyte lineage-specific antigen glycoprotein (gp)100 received a great deal of attention because its expression is limited to pigmented cells, it was a common target of tumor-infiltrating lymphocytes (TIL) cultures and the adoptive transfer of gp100-reactive TIL were associated with objective clinical responses (25-27). The largest number of gp100 studies were performed with the gp100 (209-2M) peptide, a peptide modified to express a methionine at the second position which increases the affinity for its MHC molecule and leads to superior T-cell activation. Vaccination with this altered peptide ligand could prime and expand T cells capable of recognizing native peptide and HLA-A2+ tumor cells that expressed gp100 (28). An analysis of published results from 8 trials using gp100 peptide alone to immunize 90 patients with metastatic melanoma identified one objective clinical response for an overall response rate of approximately 1% (29). Similarly in a recent study by Hodi et al., 1.5% of patients (2/136) had a PR to gp100 peptide treatment alone (30). A trial giving up to ten gp100(209-2M) peptide vaccinations, at 3-week intervals, showed that gp100-specific T cell frequency increased in all patients after 1 or 2 vaccinations and these patients had T cells expressing IFN- γ . Only one patient was analyzed

after additional vaccinations and that patient showed a diminished gp100-specific T cell frequency(31).

gp100 peptide vaccine (CD8 epitope) in the adjuvant setting

Given the possible immunosuppressive properties of metastatic disease, our group administered the gp100 (209-2M) peptide vaccine to resected stage I-III melanoma patients with no evidence of disease(32). An HPV peptide was included as a control. While the trial was not powered to detect an effect on survival, the effect of vaccination at 2 week or 3 week intervals on gp100-specific T cells was assessed. No patient had a detectable level of gp100-specific T cells prior to vaccination. At 6 months following the first vaccination, 96.5% of (28 of 29) vaccinated patients developed a detectable response. There was no significant difference between tetramer-binding T cells observed with vaccination at 2 or 3 week intervals. Evaluation of the response to the foreign HPV peptide showed that the median responses to gp100 and the HPV peptides were similar ($p<0.92$). While numbers were small, patients older than 60 had a significantly ($P<0.0055$) lower response to peptide vaccination than younger patients.

Peptide-specific T cell numbers increase with additional vaccine cycles

Two subsequent studies in patients with high risk of melanoma recurrence evaluated vaccination with two CD8 epitopes from tumor-associated antigens, gp100 (209-2M) and tyrosinase 368–376 (370D). In the first study the peptides were emulsified separately in IFA and injected s.c. in different extremities. Seventeen percent of patients exhibited strong responses to vaccination, defined as greater than 10% circulating tetramer+ CD8 T cells. In the second study the peptides were mixed together and injected at one site. In this case there were no tetramer responses to gp100, which was significantly ($p<0.01$) less than that observed for the preceding study. In contrast, T-cell responses against tyrosinase were increased; demonstrating the complexity of the immune system's response to these altered peptide ligands (33). In both studies the authors performed a careful kinetic analysis of peptide-reactive T cell frequencies over time. While gp100 tetramer-binding T cells were detected in some patients in all cohorts after a single vaccine cycle, the mean number of peptide-reactive T cells, identified by ELISpot or tetramer, continued to increase with each vaccine. In this setting, multiple vaccinations with class I binding epitopes in IFA augmented the priming and expansion of vaccine-specific T cells. Patients receiving a gp100 class I-restricted CD8+ epitope alone generated long-term memory T cells that were reactivated by booster vaccines. Unfortunately, a high frequency of peptide-specific T cells against a single epitope was not protective as tumors progressed even in patients with greatly levels elevated peptide-specific T cells (34). The evolution of escape mutants that lost antigen, HLA or had defects in antigen processing pathways was not ruled-out, however other possibilities could be responsible for tumor progression; including a failure of T cells to traffic to tumor sites and/or retain tumor destructive function.

Vaccination with tumor-associated CD4 peptide epitopes reduces the CD8 response

Another possibility, supported by preclinical studies, is that CD8 T cells primed with class I epitopes, in the absence of CD4 T cell help, may be less effective mediators of long-term anti-cancer immunity (35). A possible approach to rectify this is to include class II peptide epitopes recognized by tumor-reactive CD4 T cells. One report analyzed results from two consecutive, nonrandomized studies comparing the immune response to vaccination with a class I-restricted peptide of gp100 versus immunization with both a class I- and a class II-restricted peptide from the same antigen. Two vaccinations with the class-I restricted peptide alone immunized 95% of patients (21 of 22) to the specific peptide. In contrast, vaccination with both class I and class II-restricted gp100 peptides immunized only 50% of patients ($p<0.005$) and the degree of peptide-specific immunity was reduced by including the class II

peptide ($p < 0.01$)(36). A recent 168 patient randomized multi-center study used a vaccine consisting of 12 class I major histocompatibility complex-restricted melanoma peptides (12MP) in combination with either non-specific tetanus helper peptide or six melanoma associated helper peptides (6MHP). In addition, these patients were randomized to receive pre-vaccine treatment with 300 mg/m² cyclophosphamide or no chemotherapy. Similar to the study by Phan et al., patients receiving both the CD8 and CD4 melanoma-specific peptides had significantly weaker CD8 T cell responses than patients receiving the “non-tumor-specific” tetanus help. However, patients, receiving the class II-restricted melanoma peptide did generate CD4 T cell responses to those epitopes. The dose and schedule of cyclophosphamide had no effect on T cell responses to vaccine or on overall survival at 3 years (37).

Why did the presence of tumor-specific help result in significantly weaker CD8 T cell responses to vaccine? While there is a possibility that the CD4 epitopes increased regulatory T cells (Tregs), pilot studies in Phan et.al. found no differences in Treg numbers pre and post vaccination. However, this does not rule out skewing of the Treg pool to include more vaccine antigen-specific Treg cells. Another possibility is that the presence of tumor-specific/associated CD4 epitopes supports the spreading of the immune response to epitopes or antigens other than those included in the vaccine. This epitope spreading would not be picked-up by the peptide-specific assays employed to monitor the immune response. However, there was no difference in overall survival between the two groups at 3 years, suggesting that there was not a striking difference in the efficacy of either strategy.

LONG PEPTIDES: Composition influences booster vaccine effectiveness

An alternative vaccine strategy is to use peptides that are longer (25-35 amino acid) than the length of the MHC binding groove. One example of this is a trial that employed 13 amino acid (aa) long peptides that covered the entire sequence of HPV E6 and E7 proteins to vaccinate end-stage cervical cancer patients. Groups included patients receiving both E6 and E7 peptides at one site versus E6 peptides in one limb with E7 in a second limb. While the median immune response to HPV E6 was not different for either group, the median response to HPV E7 was substantially augmented by separating the vaccines. When peptides for both HPV E6 and E7 were combined, three or four vaccines uniformly resulted in a lower frequency of peptide-specific IFN- γ producing T cells than observed after only 2 vaccines. This was not true for patients who were vaccinated with E6 and E7 peptides in different limbs. In this setting three or four vaccinations increased the number of tumor-specific functional T cells over that observed pre-treatment or following two vaccinations (38). There is not a clear understanding of why the responses developed as they did, and given the low objective response rate, there was no opportunity to correlate immunological effects with clinical response. One thing is clear, numerous studies have shown that vaccination with a single peptide or pools of class I or II binding peptides in montanide does not generate therapeutic immune responses.

Her2/neu helper epitope vaccines with GM-CSF

Will addition of an adjuvant increase the immune response to vaccination? Early gene transfer experiments in mice identified GM-CSF as the “optimal” single cytokine for eliciting an anti-tumor immune response when comparing vaccination with a whole panel of cytokine secreting tumor cell lines (39). Based on these observations, and the availability of GM-CSF, it has been used as an adjuvant in numerous clinical trials. Peptides derived from Her2/neu, a self-protein over expressed by many human adenocarcinomas, are immunogenic in humans. Disis and colleagues, enrolled 64 patients with Her2/neu+ breast, ovarian or non-small-cell lung cancer (NSCLC) onto a three arm trial. Only patients with no evidence of disease or stable disease were eligible for this innovative design that included class I binding

motifs “inside” the class II motif sequences. If patients were HLA-A2+ they received a vaccine consisting of three 14-18 aa helper epitopes that also contained HLA-A2 binding motifs. Patients who were HLA-A2 negative were randomized to receive helper epitopes derived from either the intracellular domain (ICD) or the extracellular domain (ECD) of Her2/neu. All vaccines were mixed with 100-125 ug granulocyte macrophage-colony stimulating factor (GM-CSF) and administered intradermally monthly for 6 months to the same regional area. No montanide was used. The immunological monitoring of the patients on this trial was extensive with blood draws occurring at monthly intervals for one year (40-44). While ninety-two percent of patients generated a T cell response to Her2/neu, and the estimated probability of developing a T cell response to Her2/neu peptide at some time point increased with each additional vaccination; the magnitude of the proliferative response frequently peaked early and diminished while still receiving vaccinations. However, some level of immunity was maintained long-term following vaccination. One notable observation was that patients who responded early to vaccination (vaccine 1-3) tended to exhibit stronger stimulation indices to peptide stimulation than those responding after 4-6 vaccines. The probability of broadening the immune response to protein-specific immunity took more time. This broadening of the immune response to include components of the protein not included in the vaccine, frequently defined as epitope spreading, occurred in the 6 month follow-up period when no vaccines were given.

Melanoma HLA class I restricted (CD8 epitopes) with GM-CSF and/or IFN α 2b

In a study similar to that reported above, 121 patients with resected melanoma stage IIB-IV were randomized to receive 12 melanoma peptides (12MP) in combination with a tetanus helper peptide in montanide with or without GM-CSF. In this study 110 μ g of GM-CSF was mixed into the montanide with the intent to provide slow continuous release of cytokine at the vaccine site. Surprisingly, the GM-CSF group had significantly fewer patients with CD8+ T cell responses to 12MP (34% vs 73%, $p < 0.001$) and fewer patients with CD4 response to tetanus. Overall survival at three years was higher in the group not receiving the very low dose GM-CSF (76% vs 52%), but this was not significantly different (45). In another melanoma study, 3 HLA-class I restricted peptides, tyrosinase, gp100 and MART-1, were used as a vaccine in HLA-A2 positive patients who received peptides alone or in combination with GM-CSF (250 ug/d x 14d), at a higher dose than that used above, and/or IFN α 2b. Overall immune response as measured by ELISpot after 3 vaccinations was 21.2% (14 of 66) to any of the vaccine peptides. However, at a later time point when patients had received more vaccinations the percentage of patients with detectable peptide-specific responses increased to 37% (20 of 54). In contrast to the report noted above, in this study treatment with GM-CSF or IFN slightly increased the immune response rate, but these differences were not significant. Analysis of the 115 patients enrolled in this study indicated patients with an immune response to the vaccine had significant improvement in median overall survival compared to patients that did not have an immune response, 21.3 versus 13.4 months ($p = 0.046$) (46). There were no significant differences in overall survival between patients receiving GM-CSF and/or IFN α 2b. While the GM-CSF dose used in the study of Slingluff et al., was associated with a significantly reduced immune response rate to the 12MP, the total dose of GM-CSF was very small (110 μ g) (45). While their intent was to provide slow release of GM-CSF at the vaccine site, we raise the possibility that insufficient GM-CSF was released and therefore failed to provide an adjuvant effect. But that doesn't explain why the response rate was inferior to that of montanide alone. Nonetheless, patients receiving a substantially higher dose of GM-CSF each day for two weeks following vaccination, did not exhibit a reduced T cell response to vaccination.

WHOLE CELL VACCINES: GM-CSF-gene modified autologous tumor vaccines

Anti-tumor T cell cytokine responses were evaluated in 10 patients among 29 patients vaccinated with autologous melanoma cells engineered, using a retroviral vector, to secrete GM-CSF (range GM-CSF secretion 84 to 965 ng/10⁶/24hr)(47). While not fully controlled for tumor-specificity, results from 9 patients suggest that repeated vaccinations augmented the IL-3, IL-4, IL-5 and GM-CSF cytokine responses to autologous tumor. IFN- γ and TNF- α responses were not observed. While a pattern of dense lymphocytic infiltrates was observed in post-treatment tumor biopsies, no objective clinical responses were observed. The augmentation of type 2 (IL-3, IL-4 and IL-5), cytokine responses, might be considered a negative indication for performing multiple vaccines, since preclinical trials have generally found that vaccines inducing a type 2 cytokine response were non-therapeutic. A subsequent trial of 33 metastatic melanoma patients treated with irradiated autologous melanoma cells transduced with an adenoviral vector encoding GM-CSF (median GM-CSF secretion 534 ng/10⁶/24 hrs), resulted in successful immunization of 29 patients. One complete, one partial and one mixed-response were observed and at 36 months following vaccination 29% of patients (10 of 35) were alive and 4 had no evidence of disease. All patients had a substantial number of dendritic cells, macrophages, eosinophils, B and T cells at the site of vaccination, which correlated with tumor destruction. An interesting observation was the apparent coordinated T and B cell response with large numbers of plasma cells secreting antibody in the post vaccine biopsies. While all patients developed antibodies reactive with the adenovirus used for vaccine transduction, the possible anti-tumor reactivity of antibody was not discussed and tumor-specific T cell responses were not assessed (48). One difference between these two trials was the use of different viral vectors. In the first trial a retroviral vector was used, while the second trial, where clinical responses were observed, used an adenoviral vector.

An adenoviral vector was also used to transduce a GM-CSF construct into autologous non-small cell lung cancer (NSCLC) cells for use as a vaccine. In this study three complete responses (CR) were reported for 33 patients vaccinated(49). In each case these patients were heavily pretreated. Post hoc analysis of these data showed a significant correlation ($p < 0.03$) with the capacity of GM-CSF vector-modified autologous tumor vaccine to secrete a modest level of GM-CSF (> 40 ng/24 hr/10⁶ cells) and increased patient survival (49). Analysis of anti-tumor responses was not reported. The 9% CR rate in this small group of patients provided some measure of enthusiasm for this approach, but generating autologous vaccines is technically difficult and while within the scope of some academic medical centers, is not an easily commercialized product. For these reasons allogeneic GM-CSF-secreting vaccines were considered an attractive candidate for treatment due to standardized transduction efficacy and off-the-shelf availability.

Allogeneic gene-modified cells used as a vaccine

Based on the complete remission seen after vaccination with an autologous NSCLC vaccine, a second NSCLC study that employed an allogeneic K562 cell line, genetically engineered to secrete GM-CSF, as a bystander source of GM-CSF was begun. The vaccine comprised a mixture of isolated autologous tumor cells and allogeneic GM-CSF secreting bystander cells (K562). The idea being that the bystander cells would produce GM-CSF at the site of the vaccine without having to gene-modify the autologous tumor. There was no objective clinical responses in 49 vaccinated patients. Besides the addition of K562 bystander cells, another difference in these two trials was the amount of GM-CSF secreted which, on average, was 25-times higher with the K562 bystander cells than in the original trial with 3 CR (50). No tumor-specific immunological monitoring was performed in these studies.

An alternative to mixing bystander cells that secrete GM-CSF with autologous tumor cells to generate a vaccine, is to transduce GM-CSF expression vectors, or the cytokine of interest into allogeneic tumor cells of the same histology as the patient to be treated. These cells would presumably share antigens with the patient's tumor cells but could be used where it is difficult or impossible to obtain autologous tumor. Prostate cancer is a good candidate for this off-the-shelf approach as it metastasizes to the bone and it is virtually impossible to isolate sufficient tumor cells for autologous vaccine production. Prostate GVAX is composed of two allogeneic yet histologically distinct prostate tumor cell lines, PC3 and LNCAP, both of which are transduced to secrete GM-CSF. Phase I/II trials in patients with advanced disease were performed with prostate GVAX vaccine administered at a low (100×10^6 cells $28d \times 6$), medium (200×10^6 $14d \times 12$) or high (300×10^6 $14d \times 12$ and $500 \times 10^6 \times 1$) dose with corresponding survival of 23, 20 or 34.9 months, respectively (51). In another trial patients were treated with a 500×10^6 cell priming dose and 12 booster vaccinations with 100×10^6 or 300×10^6 GVAX cells, biweekly for 6-months. Progression-free survival assessed by bone scans was 2.8 and 5 months with low dose and high dose vaccines, respectively (52). Despite promising phase II results, phase III trials comparing chemotherapy (docetaxel and prednisone) to GVAX were prematurely terminated based on the results of a previously unplanned futility analysis which determined that the study had less than a 30% chance of meeting its predefined primary endpoint of improvement in overall survival. Higano and colleagues reported in early 2009 that while the median survival was similar in both groups, the follow-up Kaplan-Meier (KM) survival curve shows that patients receiving GVAX were crossing above patients treated with docetaxel and prednisone at approximately 22 months. While the follow-up is ongoing this separation in the curves raises the possibility that the GVAX vaccine strategy could have benefit for a subset of patients. No T cell monitoring was performed on these patients.

Whole cell vaccination with GM-CSF protein had varied response

Other studies have combined GM-CSF protein with whole cell vaccines. In a study by Faries et.al., patients were vaccinated with Canvaxin, a whole cell vaccine comprising three allogeneic melanoma cell lines and administered with bacille Calmette-Guérin (BCG) for the first two vaccines, and randomized to receive no additional treatment or $200 \mu\text{g}/\text{m}^2/\text{d}$ GM-CSF on the day of vaccination followed by daily doses for 5 more days. Patients receiving both BCG and GM-CSF had an increased immune response by TA90 IgM antibody titer and increased TA90 immune complexes, but had a diminished DTH response to melanoma cells and a trend towards worse survival (53). It should be noted that at two years the survival of the group receiving the vaccine with BCG, but without GM-CSF, was significantly better ($p < 0.002$), but with longer follow-up the difference was no longer significant ($p = 0.097$). Another phase I trial with an allogeneic breast cancer cell line, MDA-MB-231 expressing CD80, compared two doses of GM-CSF at $100 \mu\text{g}$ or $50 \mu\text{g}$ twice daily for 5 days versus BCG (54,55). Four of nine evaluated patients developed vaccine-specific T cell responses and this was split evenly between patients receiving GM-CSF and BCG. While the number of evaluable patients was small, the patients receiving GM-CSF developed stronger type I (IFN- γ) vaccine-specific responses, while those receiving BCG developed stronger type 2 (IL-5) responses. In addition, a 2-fold increase in tumor-specific antibody IgG levels was observed in 25% of (6 of 24) patients, three in each of the adjuvant groups. However, 83.3% (5 or 6) of patients that developed antibody responses were in the groups receiving the largest number of vaccinations (median of 6 vaccines per group) (56). One patient in the cohort receiving high dose vaccine and GM-CSF had 5% of circulating CD8 T cells capable of specifically producing IFN- γ when stimulated with the vaccine. This patient maintained this population for more than three years following the last vaccine and has remained disease free for more than 10 years (54).

The observation of BCG generating a dominant type 2 cytokine response (54,55) may provide a possible explanation for the study of Faries et.al., where a worse outcome was observed with patients receiving both BCG and GM-CSF (53). We postulate that the addition of GM-CSF may have augmented the priming of tumor-specific T cells destined for a non-destructive cytokine (IL-5) profile. Other studies have employed daily s.c. injections of GM-CSF at the vaccine site. Given the rapid diffusion of GM-CSF away from the vaccine site, this is unlikely to recapitulate the activity observed with GM-CSF gene-modified tumor cells that provide sustained slow release of cytokine. A number of alternative approaches have been explored to provide local sustained release of GM-CSF, including mini (insulin) pumps, micro beads, polymeric microspheres and liposomes (57,58). In each case where tested, the development of vaccine-induced T cell responses and anti-tumor immunity is superior with slow release GMCSF when compared to free GM-CSF injected at the vaccine site. Several clinical trials are currently underway and others are planned using slow sustained release of low-dose GM-CSF(59). While the addition of GM-CSF to the vaccine may help cross-priming and expansion of an anti-tumor response, the high doses and routes explored above were not sufficient to induce a strong anti-tumor immune response in a majority of patients studied.

Evidence to support GM-CSF with TLR signals

In addition to its positive affects on antigen presenting cells (APC), GM-CSF, particularly at high doses, can also have negative effects on immune response generation by inducing myeloid derived suppressor cells (MDSC)(60). GM-CSF also can induce milk fat globulin epithelial factor 8(MFG-E8), which enhances tumor cell survival, invasion, angiogenesis and augments local immune tolerance(61,62). Experiments utilizing GM-CSF^{-/-} mice showed that GM-CSF mediates upregulation of MFG-E8 on APCs. MFG-E8-overexpressing APC exposed to apoptotic cells secreted more TGF- β and less IL-12, resulting in generation of Tregs and inhibition of the Th1 and Th17 immune response(61,62). This mechanism identified in preclinical models provides insights into how multiple dosings of a GM-CSF based vaccine could act as a double-edged sword, promoting recruitment of APC but also limiting the development of a therapeutic immune response. Immuno histochemical analysis of human tumor biopsies has identified MFG-E8 at tumor sites supporting the development of strategies to block this potentially suppressive agent(62). Toll-like-receptor (TLR) agonists have been shown to decrease MFG-E8 expression on CD11b+ cells and reduce suppressive cytokines TGF- β and IL-6. Therefore, TLR-agonists are likely to be important components for GMCSF-based vaccination strategies(61). In a pre-clinical melanoma model, a copolymer of D, L-lactide and glycolide (PLG) matrices was mixed with tumor lysate and GM-CSF to provide continuous release of GM-CSF and tumor antigen at the vaccine site. A ~3.5 mg/kg dose of GM-CSF reduced recruitment of dendritic cells (DC) in tissue compared to a lower GM-CSF dose (~1.5 mg/kg). Importantly when the low dose of GM-CSF was combined with *in situ* TLR9 agonist (CpG) DC recruitment increased resulting in enhanced CTL activity and improved protection against tumor, compared to either treatment alone (63). Adding a CpG agonist to clinical vaccine trials has also improved immune responses in patients with cancer. In a phase II trial for cutaneous melanoma with MAGE-A3vaccination, 75 patients were randomized to receive MAGE-A3 combined with either CpG, MPL and QS21 (AS15) or MPL and QS21 (ASO2B). The AS15 (with CpG) vaccine formulation resulted in an objective clinical response in 3 patients (11-24months) and 1 partial response (5 months) compared to only 1 partial response with MAGE-A3 combined with MPL and QS21 without CpG (ASO2B)(64). The CpG cohort saw increased CD4 T cell responses in 72% of patients compared to only 36% of patients when the vaccine lacked CpG. The CpG cohort also developed higher anti-MAGE-A3antibody titers. In another vaccine trial with 18 patients, the combination of CpG with NY-ESO-1 protein in montanide resulted in elicitation of humoral, CD4 and CD8 T cell responses. Immunological

monitoring used NY-ESO-1 peptide pools to determine the percentage of IFN- γ ⁺ NY-ESO-1-specific CD4 and CD8 T cells; 94.4% of patients (17 of 18) had a CD4 T cell response and 50% had a CD8 T cell response (9 of 18). The numbers were small, but after 3 to 4 vaccines many patients had a steady increase in the percentage of CD8 precursors in the PBMC, that following in vitro sensitization (IVS), were capable of secreting IFN- γ when restimulated with NYESO-1 peptides. This effect appeared a bit stronger for CD4 T cells. Even more striking was the increase in NY-ESO-1-specific antibody response with each subsequent vaccination(65). In this setting, multiple vaccinations with CpG and antigen in montanide continued to provide a boosting effect that was observed following IVS. We find these data convincing, but this monitoring strategy is substantially different than fresh ex vivo monitoring of vaccine-specific immune responses and suggests that the magnitude of the immune response in this study is not as strong as that observed for the study that combined a TLR 9 signal (CpG) and a TLR 4 signal (MPL) with antigen and saponin. The data from this trial are largely unpublished and have not been subjected to formal peer review; however, our conclusion from the available data is that multiple booster vaccines, particularly when combined with saponin, TLR 4 and 9 signals may augment the anti-tumor immune response.

Epitope spreading and heterologous prime-boost

Disis and colleagues have evaluated epitope spreading in a number of trials (40,44,66). One notable example the development of immune responses to HER-2/neu epitopes that were not present in the vaccine was in a trial combining trastuzumab with a HER-2/neu helper peptide vaccine. Epitope spreading correlated with an increase in a vaccine-specific Th1 response and a decrease in serum TGF- β . Multiple trials have reported a relationship between humoral and/or T cell tumor antigen epitope spreading and clinical outcome(67-72). These studies have reported epitope spreading both within the protein immunogen, as well as, interepitope spreading, to peptides from entirely different proteins. One method to maximize and focus the immune response to the tumor/tumor-associated antigen of choice while avoiding the deleterious effects of vector-specific immunity is through heterologous prime-boost strategies. Heterologous prime-boost provides a priming vaccination with one vector and comes back with a second “type” of vector that shares the same target antigen. This provides a way to maximize and focus the immune response to the tumor/tumor-associated antigen of choice while avoiding the deleterious effects of vector-specific immunity. The augmented immune response to the target antigen and the inflammatory response would also support the development of interepitope spreading. In the case of viral vaccine vectors, the humoral response to viral proteins prevent viral entry and infection during secondary vaccination.

One approach to increase the magnitude of the tumor-specific T cell response is to use a microbial-based vaccine vector expressing one or more tumor-associated antigens contained within the irradiated tumor cell vaccine or separately in a heterologous prime-boost combination. Priming with whole cell vaccine elicits a broad T cell response of limited magnitude, which when boosted by a microbial-based vaccine expressing a defined tumor-associated antigen, can promote exceptional expansion of T cells specific for the shared antigen(73).

Live-attenuated vectors based on the intracellular bacterium *Listeria monocytogenes* (Lm) have performed remarkably in this capacity by introducing the inflammatory environment to induce memory T cell persistence (74). In preclinical studies, mice immunized with the whole cell vaccine, CT26-GVAX, developed primed AH1-vaccine-specific CD8⁺ T cells. However, this CD8 T cell response typically comprised only a fraction of a percent of the total CD8⁺ T cells (0.1-0.3%). Boosting this response with a live-attenuated LM vaccine

expressing AH1 expands the AH1-specific CD8 T cell population nearly 1000-fold, to 17-21% of the total CD8+ T cell population (unpublished data). One potential caveat of this approach is that T cells specific for epitopes encoded by the boosting vaccine may expand at the expense of T cells recognizing other tumor-associated antigens (75). In this scenario, the breadth of the T cell response could be lost and the benefits of a whole cell vaccine minimized.

Viral vectors have also been used successfully as vaccines. A recent study using a heterologous s.c. prime with rhesus cytomegalovirus (rhCMV) vector expressing simian immunodeficiency virus (SIV) followed by heterologous boost with adenovirus 5 (Ad5) expressing SIV antigens protected against SIV infection by increasing viral-specific memory CD8 and CD4 T cells (76). In this instance the heterologous prime-boost strategy did not add to the strong and persistent immune response induced by the rhCMV alone, suggesting that CMV may provide unique advantages as a vector for delivering cancer vaccines.

Prostate-specific antigen (PSA)-PROSTVAC is a prostate vaccine consisting of a priming vaccination with recombinant vaccinia expressing 4 PSA genes followed by boosting vaccination with a fowl pox vector expressing the same genes. Phase II clinical trials using PSA-TRICOM in metastatic prostate cancer improved median overall survival by 8.5 months compared to patients treated with empty vector (77). A two-fold increase in PSA-specific IFN- γ production was seen in 44.8% of the patients post vaccination and patients with strong IFN- γ responses had >6-fold increase in survival when compared to those that did not make a response ($p=0.055$) (78). Another prime-boost strategy was recently reported by Ribas et al., in this case the priming vaccination included 4 intra-lymph node injections of a plasmid encoding 4 epitopes for melan-A and tyrosinase with two subsequent boosts with peptide analogs. A high (300 μ g) and low (100 μ g) dose of boosting peptide were studied with similar frequency of antigen-specific responses observed in the evaluable patients (75% vs 67%) (79). Although the numbers of patients evaluated were small, the immune response rate was twice that when using only intranodal peptide (80). Heterologous prime-boost with the proper adjuvants may expand an existing immune response and potentially generate a *de novo* response; however, determining timing, location and type of prime-boost vaccination may be important for inducing persistent anti-tumor immunity.

Immunogenic death enhances vaccine efficacy

Immunogenic cell death is important to elicit TLR signaling. This type of death is considered “immunogenic” because it supports cross-presentation of tumor-antigens to APCs by inducing proteins and chaperones that deliver dying cells to APC for phagocytosis (81,82). As an example the protein high mobility group box 1 (HMGB1) is expressed upon immunogenic tumor death and interacts with TLR4 receptor on APCs. Interestingly, a polymorphism in the TLR4 (N299G) gene decreases responsiveness to HMGB1 activation of APCs and this loss-of-function corresponds to an increased likelihood of drug-resistant relapse for patients with breast cancer (82). Screening for this mutation before vaccination could signal a requirement to add other TLR agonists or adjuvants to the vaccine. Immunogenic cell death also stimulates natural killer and cytotoxic lymphocytes by increasing expression of natural killer group 2, member D (NKG2D). However, tolerogenic cellular death induced by certain types of DNA damage can lead to shedding of the NKG2D ligand major histocompatibility complex class I chain-related protein A (MICA)(81,83). Activation of NKG2D on natural killer and CD8 T cells induces tumor destruction, but when NKG2D ligands, such as MICA are shed, they downregulate NKG2D and induce immune suppression (84). Serum levels of soluble MICA ligand have been associated with worse prognosis in patients with multiple myeloma and decreased responsiveness to ipilimumab treatment in patients with advanced melanoma (73,85,86). Melanoma patient receiving six vaccinations with autologous tumor cells transduced to secrete GM-CSF followed by

ipilimumab treatment exhibited significant humoral response to MICA. These anti-MICA antibodies functionally destroyed tumors via complement fixation and opsonization. This suggests vaccination with MICA may be a candidate for combination therapy with cytotoxic agents and vaccination (86).

Chemotherapy and radiation in combination with vaccination

Chemotherapy and radiotherapy, by eliciting immunogenic cell death, are important tools for combination with immunotherapy. The cytotoxic agents, oxaliplatin, irinotecan, docetaxel, doxorubicin and irradiation have all been shown to induce immunogenic cell death in preclinical models (81). In addition to direct anti-tumor effects that may provide a source of antigen for cross-presentation, they can reduce Tregs and MDSCs, mediate release of TLR signals from the gut, and induce lymphopenia, creating space for improved antigen-driven expansion of vaccine-reactive T cells(87). Jaffee and colleagues administered a vaccine composed of two allogeneic pancreatic cancer cell lines, PANC10.05 and 6.03, genetically modified to secrete GM-CSF, prior to initiation of chemoradiation therapy. Booster vaccines were administered at monthly intervals, 8weeks following chemoradiotherapy. Twenty-one percent (3 of 14) of patients remain disease-free 10 years following vaccination. The 3 surviving patients all exhibited a DTH response to autologous tumor (88). Recently a single institution phase II trial using the same strategy for 60 patients included further anti-tumor immune monitoring by measuring the anti-mesothelin response. The overall population had mesothelin-specific CD8 T cells following 1 vaccination, but this response waned following 5 vaccinations. Patients who were HLA-A1 and - A2, maintained a substantially stronger anti-mesothelin response following 5 vaccinations, which correlated with disease-free survival (89), suggesting people with certain HLA-types respond differently to multiple vaccinations.

Another trial looked at the effect of combination treatment with cyclophosphamide, which is known to induce immunogenic death, and vaccination with an allogeneic GM-CSF-secreting vaccine for pancreatic cancer (CG8020/CG2505). Survival with vaccination alone was 2.3 months, while cyclophosphamide (250 mg/m²) given one day prior to vaccination increased survival to 4.3 months (90). Some of the most successful trials with allogeneic GM-CSF vaccination have been seen with combination therapy for liquid tumors. In the case of imatinib-resistant chronic myeloid leukemia, four vaccinations with the allogeneic K562/GM-CSF cell line, given at 3-week intervals with or without imiquimod adjuvant, decreased BCR-Abl expression; 7 patients had undetectable disease by PCR(91). In addition, K562/GM-CSF vaccination in combination with autologous stem cell transplant (ASCT) for treatment of acute myeloid leukemia induced a DTH response and demonstrated a 3-year relapse free survival of 61.8%. Patients also received cytarabine, doxorubicin, busulfan and cyclophosphamide, which may also have contributed to the lasting anti-tumor immune response, evidenced by IFN- γ and granzyme B production(92).

A phase I dose escalation study used HER2-positive allogeneic breast cancer lines transduced to secrete GM-CSF as a vaccine, alone or combined with simultaneous administration of low or high dose cyclophosphamide and doxorubicin. Although the higher dose chemotherapy eliminated the humoral immune response, the low dose chemotherapy augmented the anti-HER2 antibody responses (93). In another study, pretreatment with 300 mg/m² cyclophosphamide reduced progression of breast carcinoma when used in conjunction with a vaccine against mucin antigen, sialyl-Tn, with a projected median survival of 19.7 versus 12.6 months with vaccine alone ($p=0.0176$) (94). In a pilot study for treating prostate cancer with a combination therapy of radiation, GM-CSF and IL-2, with or without vaccination with a fowl pox viral vector containing PSA and co-stimulatory molecules, 76% of patients (13 of 17) vaccinated had a PSA-specific T response, with no detectable PSA-specific T cells in the non-vaccinated group (95).

Additional strategies to augment the immune response to booster vaccines

Vaccination regimens also have the capacity to induce immunosuppressive mechanisms including Tregs, MDSC, IL-10 producing B cells (B10) and an anti-inflammatory cytokine environment, which work against the goal of immune response induction.

Regulatory T cells—A high frequency of circulating Tregs have been seen in patients with lung, ovarian, breast, colorectal, oesophageal, gastric, hepatocellular, leukemia, lymphoma, melanoma and pancreatic cancers (reviewed by Zou 2006)(96). This increase in Tregs, particularly within the tumor, has been associated with poor prognosis (97). Pre-clinical and clinical trials have tried to eliminate Tregs in combination with vaccination. In a phase I trial 3 vaccinations with an NY-ESO-1 DNA vector were administered every 4 weeks; 93% of patients (15 of 16) developed a vaccine-specific response and *in vitro* data suggested this tumor-specific response was inhibited by Tregs (98). In preclinical studies, three vaccinations with a whole cell vaccine secreting GM-CSF decreased therapeutic efficacy and increased Treg numbers. When CD4 cells were partially depleted prior to the 2nd and 3rd vaccination antitumor immunity was restored identifying CD4-depletion as a method to decrease Tregs (8). This could be rapidly translated to clinical trials, as there is a humanized CD4-depleting antibody zanolimumab (Hu-max-CD4), which has been used to treat cutaneous T-cell lymphoma. This antibody is currently being used in combination with interleukin-2 (IL-2) in a phase II clinical trial to reduce Tregs (99,100). In preclinical models CD4-depletion has increased anti-tumor immune responses; however, some level of CD4 T cell help is likely critical for priming and maintenance of memory CD8 T cells and depletion of the beneficial CD4 T cells could be detrimental to long-term immunity (101,102). Other methods have been used to target Tregs, including CD25-blockade or depletion and small molecule inhibitors of TGF- β . Human Tregs express high levels of the IL-2R α , CD25. Two types of CD25 targeted antibodies have been used to reduce Treg numbers in cancer patients. The humanized monoclonal CD25-blocking antibody, daclizumab, has been used to reduce Treg function in multiple clinical trials. The best results were seen in a trial where patients received daclizumab one week prior to five vaccinations with hTert or survivin peptides plus GM-CSF, which resulted in an antigen-specific CTL response (103).

Another CD25-targeted therapy, the immunotoxin denileukin diftitox (ONTAK), is a fusion protein of IL-2 coupled with the active enzyme of diphtheria toxin. ONTAK was originally developed for treating T cell lymphoma, however it is currently being used in combination with vaccination as a method to reduce Tregs in ovarian, breast, lung and renal carcinoma (104-106). Renal cell carcinoma patients that were pre-treated with ONTAK followed by vaccination with DCs transfected with tumor-RNA exhibited a 7.2 and 7.9 fold median increase in CD4 and CD8 T cell responses against RNA transfected DC, respectively (104). There are a number of pitfalls to CD25-depletion strategies. First, CD25 is not only expressed on Tregs, but also activated CD4 and CD8 effector T cells and eliminating these cells can eliminate important tumor-reactive T cells; second these CD25-depletion strategies do not always significantly reduce Treg numbers when confirmed by FOXP3 expression (107-109). Finally, CD25 is expressed on APCs involved in IL-2 signaling to T cells and depletion/blocking of these APCs decreases T cell activation (110). Furthermore the role of CD25 might be drastically different in mice and humans. In mice, CD25 depletion results in loss of tolerance whereas in humans it causes immunodeficiency, indicating mouse CD25-depletion models may not predict the clinical outcome of CD25-blockade (111-113). Supporting this concept, a phase I/II trial combining vaccination with tumor-antigen pulsed DCs with prior blockade using daclizumab found no difference in progression free-survival compared to vaccination alone (107). Treg suppression may be reduced by targeting induction of new Tregs. Transforming-growth-factor- β (TGF- β), which is secreted by many tumors suppresses effector T cells and induces Tregs and tumor-activated

macrophages (114). SM16, a small-molecule inhibitor of TGF- β type I receptor (ALK5) kinase, has been shown to decrease mesothelioma recurrence and decrease metastatic breast cancer pathology by altering anti-tumor immunity(115,116). These studies suggest that systemic TGF- β inhibition may be a promising addition to combination immunotherapy strategies.

Myeloid Derived Suppressor Cells—The tumor environment induces the development of MDSC, which have been shown to downregulate IFN- γ expression, increase Tregs and decrease anti-tumor immunity(117,118). GM-CSF secreting vaccines also induce MDSC accumulation and induction in both the tumor microenvironment and periphery (60). In pre-clinical models and *in vitro* human studies all-trans-retinoic acid (ATRA) increases anti-tumor immunity to vaccination by directly reducing myeloid suppressor cells (118,119). Phosphodiesterase-5 (PDE-5) inhibitors also inhibit MDSC function. When PDE-5 blockade was administered the same day as tumor challenge it prevented 50-70% of tumor growth in a number of different pre-clinical tumor models (120). A phase I clinical trial combining telomerase vaccination with GM-CSF and tadalafil will examine the role of PDE5-inhibitors in combination therapy for pancreatic cancer(99).

IL-10-producing B cells (B10)—The role of B10 cells in the anti-tumor response is not completely clear, although evidence suggests tumor-induced B cells inhibit CD8 T cell proliferation, reduce IFN- γ and IL-2 production and induce Treg cells causing progression of metastatic breast cancer(121,122). B-cell-deficient mice have reduced Tregs and increased anti-tumor immunity; however, preclinical studies have shown that depletion of B cells with anti-CD20 antibodies can also decrease effector T cell number and function (123,124). One possible explanation for this effect is the depletion of antigen-presenting B cells that may be beneficial to antitumor immunity(123,125). Rituximab, the FDA-approved B-cell depleting antibody is certainly a contender for B10 depletion studies, however new therapies designed to target B10 producing cells specifically would be useful.

Cytokine administration can improve antitumor inflammatory conditions

The cytokine environment in the tumor can vastly alter the immune response. Therefore, exogenous treatment with immune-stimulating cytokines is an attractive avenue of combination treatment with vaccination. A recent phase III clinical trial comparing IL-2 treatment plus gp100 peptide vaccination to IL-2 treatment alone for metastatic melanoma reported improved overall response rates, as well as, increased progression-free survival (126). While the mechanisms responsible for this effect are unclear, it suggests additional strategies to improve vaccine efficacy.

Interleukin-7 (IL-7) is important for homeostasis of naïve and memory T cells(127). Treatment with IL-7 has been shown to protect against viral infection and increase CD8 T cell numbers(128). We recently demonstrated its ability to enhance antigen-driven proliferation of tumor-specific T cells and inhibit the suppressive effect of CD8+CD122+ regulatory T cells (129). Initial toxicity trials have shown very little toxicity in patients with refractory cancer and in combination with MART-1 and gp100-peptide vaccination, IL-7 increased the number of CD4 and CD8 T cells and reduced the numbers of Tregs, in a dose-dependent manner(130,131).

Interleukin-15 (IL-15) is an important cytokine for activation and proliferation of NK and T cells, as well as, increasing memory T cell response during vaccination (132,133). T cells activated *ex vivo* in the presence of IL-15 prior to adoptive immunotherapy exhibit enhanced therapeutic efficacy in a number of pre-clinical tumor models (134-136). Therefore, administration of recombinant IL-15 has potential combined with vaccination and

is currently being tested in phase I clinical trials. Furthermore, chimeric constructs of IL-15R α -IgG-Fc, which stabilize IL-15 signaling, enhance anti-tumor activity and are being developed for clinical trials (137).

Interleukin-21 (IL-21) is also an attractive candidate for combination therapy due to its ability to induce memory stem cells (138). Administration of IL-21 decreases Tregs in the tumor microenvironment and suppressor Treg function (139-141). Fourteen stage IV melanoma patients treated with human rIL-21 at 30 ug/kg/day for 5-cycles showed one complete response, one partial response and experienced very little toxicity (142). This early study underscores the potential rIL-21 has for combination therapy.

New sources of antigens for tumor vaccination

Whole tumor cell vaccines contain the full spectrum of unique and shared antigens that the immune system might recognize in the development of a therapeutic immune response. In reality, whole cell vaccines likely result in recognition of only long-lived proteins that survive long enough to be cross-presented to the immune system by APC (143). It has been postulated that as a consequence of their being long-lived, many of these self-proteins have already been cross-presented under non-inflammatory conditions, resulting in the induction of immunological tolerance to these antigens in the periphery. In contrast, short-lived proteins (SLiPs) or defective ribosomal products (DRiPs), because they are so rapidly degraded by the proteasome and shuttled to the membrane in class I molecules, represent excellent tumor targets, but are not available to be cross-presented by the APC (144). Thus peripheral tolerance is less likely induced against these SLiPs. While at the same time, these SLiPs and DRiPs may comprise the majority of targets being presented by MHC (145). Li et al., pioneered a strategy that exploits proteasome blockade, which leads to accumulation of SLiPs and DRiPs in autophagosomes, to develop a new vaccine with therapeutic efficacy superior to GM-CSF-secreting vaccines (146). Subsequently, Twitty et al., documented that this autophagosome vaccine strategy could challenge a well-established paradigm in tumor immunology that has stood for 50 years. Specifically, they showed that vaccination with a unique chemically-induced sarcoma protected animals against a challenge with the sarcoma used for the vaccine, but not from a challenge with other related sarcomas (147). In contrast, when autophagosomes were used for vaccination a proportion from one tumor protected not only against the immunizing tumor but also provided significant cross-protection from a challenge with other sarcomas in 8 of 9 combinations tested. Whole tumor cells provided no significant cross-protection in any of the same 9 combinations. It will be interesting, and potentially clinically relevant, to identify the SLiPs that serve as cross-reactive antigens in these models. These promising preclinical results highlight the potential that new sources of “non-tolerant” shared antigens may have on the effectiveness of cancer vaccines. A clinical trial of this strategy has been initiated in patients with NSCLC.

Discussion

The observation that high frequencies (>5%) of tumor-specific T cells in the peripheral blood correlate with objective clinical response in adoptive immunotherapy studies has provided a bar for proof-of-concept to establish what might be required to achieve objective clinical responses using cancer vaccines (148). However, the majority of clinical trials never identify this level of T cell response in vaccinated patients. In these studies it is an order of magnitude less. Does this explain the limited success of cancer vaccines? Is it possible that current vaccine strategies that include boosting actually attenuate the anti-tumor immune response?

It is interesting that the first FDA-approved therapeutic vaccine recommends only three vaccinations (149). While detailed immunological monitoring results from this novel

vaccine strategy have not been published, data presented at the SITC-NCI workshop and available on the SITC web site, document a significant correlation ($p < 0.049$) between a positive ELISpot response to the fusion protein target antigen (PSMA) contained in the vaccine and overall survival (<http://www.sitcancer.org/meetings/am10/biomarkers10/65>). While it is unclear how an immune response to a novel fusion protein impacts the anti-cancer immune response, it seems plausible that a strong immune response to the foreign fusion protein may have led to spreading of an immune response against other relevant tumor targets.

Regarding the question of whether multiple vaccinations are friend or foe, a review of the clinical trials summarized in table 1 provides a mixed picture. In some cases, there was evidence that boosting augmented an antigen-specific immune response (26,41,46,47,65,150-153). Other studies with altered peptide ligands induced strong immune responses to a single epitope, but did not provide protection from tumor recurrence (34). Even in studies where large percentages of patients generated vaccine-specific responses, the magnitude of response frequently decreased, sometimes even despite additional vaccinations (38,43,45,53,70,78). Combining tumor-specific CD4 epitopes did not uniformly help and in some cases significantly hindered tumor-specific response (36,154). Explanations for the limited effects of cancer vaccine boosters are unclear, but numerous possibilities exist. To start, we acknowledge that in 2011 the immunotherapy community is still uncertain about the immune parameters that should be viewed as correlates with clinical response. A recent report from the iSBTC-SITC/FDA/NCI Workshop on Immunotherapy biomarkers recognized that “Despite substantial efforts from many groups, we do not know which parameters of immune responses, and which assays used to assess these parameters, are optimal for efficacy analysis. Indeed, the tumor-specific cellular immune response promoted by immunization often has not correlated with clinical cancer regression despite the induced cytotoxic T cells detected in in vitro assays” (155). Another limitation to identifying immune biomarkers that correlate with clinical response is the low response rate to current cancer vaccine strategies. The recent report of Kirkwood, et al., showing a statistically significant correlation between development of a vaccine-induced immune response and increased overall survival provides encouragement that it will be possible to identify, in the peripheral blood, a clinically relevant biomarker for response.

The strongest support for booster vaccines augmenting anti-cancer immune responses is found in trials where immunologically active or complementary treatments were combined with the vaccine strategy, but even here, augmentation of the anti-cancer immune response was not universal (55,64,65,68,93,94,103,104). The immunological status at the host-cancer interface may provide an explanation for these results. Studies by Galon et al., reported that intra-tumor CD3⁺ T cells and cells at the invasive margin correlated with tumor stage and patient outcome, moreover, this was correlated with CD45RO expression, a marker associated with memory T cells (156). Galon’s group also reported that a Th1^{hi} (Tbet, IRF1, IL12Rb2, STAT4) Th17^{low} (RORC, IL17A) gene profile correlated with disease-free survival (157). Identifying immunological “signatures” associated with favorable prognosis is an active area of investigation and may identify patients most likely to benefit from a vaccination strategy that ultimately boosts their endogenous response. In addition to colorectal cancer, disease-survival, in women with epithelial ovarian cancer has also been positively associated with intratumoral expression of CD8, CD3, FoxP3, TIA-1, CD20, MHC class I and II (158). Furthermore ER-negative breast cancer patients with an 8-gene mRNA signature of CD19, CD3D, CD48, GZMB, LCK, MS4A1, PRF1 and SELL within TIL had improved disease-free survival following treatment with adjuvant anthracycline-based therapy (West, N et al. written correspondence). Melanomas that express the chemokines, CCL2, CCL3, CCL4, CCL5, CXCL9 and CXCL10, had increased infiltration

of CD8 T cells indicating tumor chemokine expression is associated with lymphocyte infiltration (159).

These immune characterizations have moved from retrospective analyses and are now integrated into the original clinical trial design as prospective biomarkers. One phase II trial in non-small cell lung cancer (NSCLC) selected patients by gene expression profiling before comparing vaccination with MAGE-A3 plus adjuvant and placebo. Patients with a predictive signature exhibited improved clinical outcome (160). This trial has moved to phase III and the signature is being evaluated in metastatic melanoma patients. We hypothesize that patients with positive immune signatures respond significantly better to vaccination because they do not require the priming of a new anti-cancer immune response; simply a boosting of the existing response (Figure 1A). On the contrary, patients who have not generated an anti-cancer immune response, as measured by an absence of immune effector and suppressor cells (Figure 1B), do less well clinically because the current vaccine strategies are not effective at priming of a *de novo* anti-cancer immune response. In contrast, patients with an immunosuppressive signature (Figure 1C) would require combination therapies that first target the culprits of negative regulation, in addition to providing effective priming and co-stimulatory signals. While Figure 1 separates the immune signatures into three groups, we appreciate that in most patients there is likely to be a continuum of immune response over time and/or that all three might be represented at different metastatic sites in the same patient.

Our interpretation of the immune signature data is that failure to consider innovative combination approaches in the cohort of patients identified in panels B and C (Figure 1) will lead to limited success. While controversial, we consider the goal of inducing a 5% level of cancer-reactive circulating T cell to be an obtainable benchmark for cancer vaccines. To obtain this magnitude of response in a large fraction of patients will require tailoring the therapy to the patient based on their immunological signature as well as their genetic profile. The availability of a wide range of novel immunologically active agents provides many possibilities. Improved understanding of the complex mechanism of action of adjuvants, such as GM-CSF, have provided insights into how they might be administered more effectively and how and with what they might be combined to optimize anti-cancer immune stimulating activities while blocking negative regulators. The discovery of classes of tumor antigens that are not typically cross-presented and thus less likely to have established peripheral immune tolerance may provide a novel source of targets for a next generation of cancer vaccines. The availability of a host of agents that interfere with suppressive activities, provide agonistic costimulatory or checkpoint blockade must also play a critical role in the next generation of cancer immunotherapies. The cancer immunotherapy field recognizes that combination therapies offer much promise for improving patient outcomes and have identified the inability to obtain reagents to perform combination immunotherapy clinical trials as one of the leading hurdles to successful immunotherapy (161). An international consortium of 11 societies and associations have joined together as the World Immunotherapy Council to begin addressing these hurdles that include the accessibility of agents from commercial sources, funding for performance and monitoring of clinical trials and developing the next generation of translational research teams that will integrate these strategies into a comprehensive personalized approach for patients with cancer (161). The Cancer Immunotherapy Network (CITN) is another recently established force, supported by the National Cancer Institute, that is diligently working to initiate clinical trials of combination immunotherapy (162-164).

In conclusion, the immunological monitoring data obtained from clinical trials to date have been too limited to determine whether multiple vaccinations are beneficial or not. Nonetheless, it is clear that the current vaccines strategies have been generally ineffective;

they lead to weak immune responses and low response rates. The availability of immunologically active agents and the appreciation that vaccines may, via epitope spreading, induce immune responses to targets not included in the vaccine, coupled with novel technologies to monitor the development of immune responses to those unknown targets, promise to guide the future development of clinical immunotherapy strategies(165). We suggest that characterizing the immune signature of each patient's cancer coupled with the utilization of improved methods to assess the vaccine-induced immune response will help distinguish the benefits and detriments of multiple vaccinations. Ultimately, these refinements coupled with access to novel immunologically active agents will lead to stronger immune responses to cancer vaccines and substantial improvements in the outcomes of patients with cancer.

References

1. Amanna IJ, Carlson NE, Slifka MK. Duration of humoral immunity to common viral and vaccine antigens. *N. Engl. J. Med.* 2007; 357:1903–1915. [PubMed: 17989383]
2. Amanna IJ, Messaoudi I, Slifka MK. Protective immunity following vaccination: how is it defined? *Hum Vaccin.* 2008; 4:316–319. [PubMed: 18398296]
3. Winter H, Hu HM, McClain K, et al. Immunotherapy of melanoma: a dichotomy in the requirement for IFN-gamma in vaccine-induced antitumor immunity versus adoptive immunotherapy. *J. Immunol.* 2001; 166:7370–7380. [PubMed: 11390488]
4. Basombrío MA. Search for common antigenicities among twenty-five sarcomas induced by methylcholanthrene. *Cancer Research.* 1970; 30:2458–2462. [PubMed: 4097428]
5. Foley EJ. Antigenic properties of methylcholanthrene-induced tumors in mice of the strain of origin. *Cancer Research.* 1953; 13:835–837. [PubMed: 13116120]
6. Klein G, Sjogren HO, Klein E, et al. Demonstration of resistance against methylcholanthrene-induced sarcomas in the primary autochthonous host. *Cancer Research.* 1960; 20:1561–1572. [PubMed: 13756652]
7. Shu SY, Rosenberg SA. Adoptive immunotherapy of newly induced murine sarcomas. *Cancer Research.* 1985; 45:1657–1662. [PubMed: 3872168]
8. LaCelle MG, Jensen SM, Fox BA. Partial CD4 Depletion Reduces Regulatory T Cells Induced by Multiple Vaccinations and Restores Therapeutic Efficacy. *Clin. Can. Res.* 2009; 15:6881–6890.
9. Morton D, Mozzillo N, Thompson J, et al. An international, randomized, phase III trial of bacillus Calmette-Guerin (BCG) plus allogeneic melanoma vaccine (MCV) or placebo after complete resection of melanoma metastatic to regional or distant sites. *J of Clin Oncol.* 2007; 25(suppl):18. Abstract 8508.
10. Eggermont AMM. Immunostimulation versus immunosuppression after multiple vaccinations: the woes of therapeutic vaccine development. *Clin. Cancer Res.* 2009; 15:6745–6747. [PubMed: 19903779]
11. Eggermont AM, Suci S, Ruka W, et al. EORTC18961: Post-operative adjuvant ganglioside GM2-KLH21 vaccination treatment vs observation in stage II (T3-T4 N0M0) melanoma: 2nd interim analysis led to an early disclosure of the results. *J Clin Oncol.* 2008; 26(suppl) Abstract 9004.
12. Eggermont AM, Suci S, Rutkowski, et al. Randomized phase III trial comparing postoperative adjuvant ganglioside GM2-KLH/QS-21 vaccination versus observation in stage II (T3-T4N0M0) melanoma: Final results of study EORTC 18961. *J Clin Oncol.* 2010; 28(suppl) Abstract 8505.
13. Eggermont AMM. Therapeutic vaccines in solid tumours: can they be harmful? *Eur. J. Cancer.* 2009; 45:2087–2090. [PubMed: 19477117]
14. Rappuoli R, Aderem A. A 2020 vision for vaccines against HIV, tuberculosis and malaria. *Nature.* 2011; 473:463–469. [PubMed: 21614073]
15. [Accessed: June 2011] Vaccines Licensed for Immunization and Distribution in the US with Supporting Documents. <http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm093830.htm>
16. Frey SE, Couch RB, Tacket CO, et al. Clinical responses to undiluted and diluted smallpox vaccine. *N. Engl. J. Med.* 2002; 346:1265–1274. [PubMed: 11923490]

17. McElrath MJ, Haynes BF. Induction of immunity to human immunodeficiency virus type-1 by vaccination. *Immunity*. 2010; 33:542–554. [PubMed: 21029964]
18. Clynes RA, Towers TL, Presta LG, et al. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nat Med*. 2000; 6:443–446. [PubMed: 10742152]
19. Mason RA, Tauraso NM, Spertzel RO, et al. Yellow fever vaccine: direct challenge of monkeys given graded doses of 17D vaccine. *Appl Microbiol*. 1973; 25:539–544. [PubMed: 4633476]
20. Lu JJ-Y, Cheng C-C, Chou S-M, et al. Hepatitis B immunity in adolescents and necessity for boost vaccination: 23 years after nationwide hepatitis B virus vaccination program in Taiwan. *Vaccine*. 2009; 27:6613–6618. [PubMed: 19698812]
21. Hammarlund E, Lewis MW, Hanifin JM, et al. Antiviral Immunity following Smallpox Virus Infection: a Case-Control Study. *J. Virol*. 2010; 84:12754–12760. [PubMed: 20926574]
22. Bachmann MF, Jennings GT. Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. *Nat Rev Immunol*. 2010; 10:787–796. [PubMed: 20948547]
23. Precopio ML, Betts MR, Parrino J, et al. Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8(+) T cell responses. *J. Exp. Med*. 2007; 204:1405–1416. [PubMed: 17535971]
24. FUTURE II Study Group. Quadrivalent vaccine against human papillomavirus to prevent high-grade cervical lesions. *N. Engl. J. Med*. 2007; 356:1915–1927. [PubMed: 17494925]
25. Bakker AB, Schreurs MW, de Boer AJ, et al. Melanocyte lineage-specific antigen gp100 is recognized by melanoma-derived tumor-infiltrating lymphocytes. *J. Exp. Med*. 1994; 179:1005–1009. [PubMed: 8113668]
26. Kawakami Y, Dang N, Wang X, et al. Recognition of Shared Melanoma Antigens in Association With Major HLA-A Alleles by Tumor Infiltrating T Lymphocytes From 123 Patients With Melanoma. *J. Immunother*. 2000; 23:17. [PubMed: 10687134]
27. Seiter S, Monsurro V, Nielsen M-B, et al. Frequency of MART-1/MelanA and gp100/PMel17-specific T cells in tumor metastases and cultured tumor-infiltrating lymphocytes. *J. Immunother*. 2002; 25:252–263. [PubMed: 12000867]
28. Rosenberg SA, Yang JC, Schwartzentruber DJ, et al. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat Med*. 1998; 4:321–327. [PubMed: 9500606]
29. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. *Nat Med*. 2004; 10:909–915. [PubMed: 15340416]
30. Hodi FS, O'Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N. Engl. J. Med*. 2010; 363:711–723. [PubMed: 20525992]
31. Lee KH, Wang E, Nielsen MB, et al. Increased vaccine-specific T cell frequency after peptide-based vaccination correlates with increased susceptibility to in vitro stimulation but does not lead to tumor regression. *J. Immunol*. 1999; 163:6292–6300. [PubMed: 10570323]
32. Smith JW, Walker EB, Fox BA, Haley D, Wisner KP, Doran T, et al. Adjuvant immunization of HLA-A2-positive melanoma patients with a modified gp100 peptide induces peptide-specific CD8+ T-cell responses. *J. Clin. Oncol*. 2003; 21:1562–1573. [PubMed: 12697882]
33. Rosenberg SA, Sherry RM, Morton KE, et al. Altered CD8(+) T-cell responses when immunizing with multipeptide peptide vaccines. *J. Immunother*. 2006; 29:224–231. [PubMed: 16531823]
34. Rosenberg SA, Sherry RM, Morton KE, et al. Tumor progression can occur despite the induction of very high levels of self/tumor antigen-specific CD8+ T cells in patients with melanoma. *J. Immunol*. 2005; 175:6169–6176. [PubMed: 16237114]
35. Hu HM, Winter H, Urba WJ, et al. Divergent roles for CD4+ T cells in the priming and effector/memory phases of adoptive immunotherapy. *J. Immunol*. 2000; 165:4246–4253. [PubMed: 11035058]
36. Phan GQ, Touloukian CE, Yang JC, et al. Immunization of patients with metastatic melanoma using both class I- and class II-restricted peptides from melanoma-associated antigens. *J. Immunother*. 2003; 26:349–356. [PubMed: 12843797]
37. Slingluff CL, Petroni GR, Chianese-Bullock K, et al. Randomized Multicenter Trial of the Effects of Melanoma-Associated Helper Peptides and Cyclophosphamide on the Immunogenicity of a Multi-peptide Melanoma Vaccine. *J. Clin. Oncol*. 2011; 29:2924–2932. [PubMed: 21690475]

38. Kenter GG, Welters MJP, Valentijn ARPM, et al. Phase I Immunotherapeutic Trial with Long Peptides Spanning the E6 and E7 Sequences of High-Risk Human Papillomavirus 16 in End-Stage Cervical Cancer Patients Shows Low Toxicity and Robust Immunogenicity. *Clin. Can. Res.* 2008; 14:169–177.
39. Dranoff G, Jaffee E, Lazenby A, et al. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc. Natl. Acad. Sci. U.S.A.* 1993; 90:3539–3543. [PubMed: 8097319]
40. Disis ML, Grabstein KH, Sleath PR, et al. Generation of immunity to the HER-2/neu oncogenic protein in patients with breast and ovarian cancer using a peptide-based vaccine. *Clin. Cancer Res.* 1999; 5:1289–1297. [PubMed: 10389911]
41. Disis ML, Schiffman K, Guthrie K, et al. Effect of dose on immune response in patients vaccinated with an her-2/neu intracellular domain protein--based vaccine. *J. Clin. Oncol.* 2004; 22:1916–1925. [PubMed: 15143085]
42. Disis M, Delarosa C, Goodell V, et al. Maximizing the retention of antigen specific lymphocyte function after cryopreservation. *J. Immunol. Meth.* 2006; 308:13–18.
43. Salazar LG, Coveler AL, Swensen RE, et al. Kinetics of tumor-specific T-cell response development after active immunization in patients with HER-2/neu overexpressing cancers. *Clin. Immunol.* 2007; 125:275–280. [PubMed: 17913588]
44. Knutson KL, Disis ML. Expansion of HER2/neu-specific T cells ex vivo following immunization with a HER2/neu peptide-based vaccine. *Clin. Br. Can.* 2001; 2:73–79.
45. Slingluff CL, Petroni GR, Olson WC, et al. Effect of granulocyte/macrophage colony-stimulating factor on circulating CD8+ and CD4+ T-cell responses to a multi-peptide melanoma vaccine: outcome of a multicenter randomized trial. *Clin. Cancer Res.* 2009; 15:7036–7044. [PubMed: 19903780]
46. Kirkwood JM, Lee S, Moschos SJ, et al. Immunogenicity and Antitumor Effects of Vaccination with Peptide Vaccine +/- Granulocyte-Monocyte Colony-Stimulating Factor and/or IFN-2b in Advanced Metastatic Melanoma: Eastern Cooperative Oncology Group Phase II Trial E1696. *Clin. Cancer Res.* 2009; 15:1443–1451.
47. Soiffer R, Lynch T, Mihm M, et al. Vaccination with irradiated autologous melanoma cells engineered to secrete human granulocyte-macrophage colony-stimulating factor generates potent antitumor immunity in patients with metastatic melanoma. *Proc. Natl. Acad. Sci. U.S.A.* 1998; 95:13141–13146. [PubMed: 9789055]
48. Soiffer R, Hodi FS, Haluska F, et al. Vaccination with irradiated, autologous melanoma cells engineered to secrete granulocyte-macrophage colony-stimulating factor by adenoviral-mediated gene transfer augments antitumor immunity in patients with metastatic melanoma. *J. Clin. Oncol.* 2003; 21:3343–3350. [PubMed: 12947071]
49. Nemunaitis J, Serman D, Jablons D, et al. Granulocyte-Macrophage Colony-Stimulating Factor Gene-Modified Autologous Tumor Vaccines in Non-Small-Cell Lung Cancer. *J Nat Cancer Inst.* 2004; 96:326–331. [PubMed: 14970281]
50. Nemunaitis J, Jahan T, Ross H, et al. Phase 1/2 trial of autologous tumor mixed with an allogeneic GVAX® vaccine in advanced-stage non-small-cell lung cancer. *Cancer Gene Ther.* 2006; 13:555–562. [PubMed: 16410826]
51. Higano CS, Corman JM, Smith DC, et al. Phase 1/2 dose-escalation study of a GM-CSF-secreting, allogeneic, cellular immunotherapy for metastatic hormone-refractory prostate cancer. *Cancer.* 2008; 113:975–984. [PubMed: 18646045]
52. Small EJ, Sacks N, Nemunaitis J, et al. Granulocyte macrophage colony-stimulating factor--secreting allogeneic cellular immunotherapy for hormone-refractory prostate cancer. *Clin. Cancer Res.* 2007; 13:3883–3891. [PubMed: 17606721]
53. Faries MB, Hsueh EC, Ye X, et al. Effect of granulocyte/macrophage colony-stimulating factor on vaccination with an allogeneic whole-cell melanoma vaccine. *Clin. Cancer Res.* 2009; 15:7029–7035. [PubMed: 19903777]
54. Dols A, Meijer SL, Smith JW II, et al. Allogeneic Breast Cancer Cell Vaccines. *Clin. Br Can.* 2008; 3:S173–S180.

55. Dols A, Smith JW, Meijer SL, et al. Vaccination of women with metastatic breast cancer, using a costimulatory gene (CD80)-modified, HLA-A2-matched, allogeneic, breast cancer cell line: clinical and immunological results. *Hum. Gene Ther.* 2003; 14:1117–1123. [PubMed: 12885350]
56. Dols A, Meijer SL, Hu H-M, et al. Identification of tumor-specific antibodies in patients with breast cancer vaccinated with gene-modified allogeneic tumor cells. *J. Immunother.* 2003; 26:163–170. [PubMed: 12616108]
57. Zaharoff DA, Rogers CJ, Hance KW, et al. Chitosan solution enhances the immunoadjuvant properties of GM-CSF. *Vaccine.* 2007; 25:8673–8686. [PubMed: 18037196]
58. Golumbek PT, Azhari R, Jaffee EM, et al. Controlled release, biodegradable cytokine depots: a new approach in cancer vaccine design. *Cancer Research.* 1993; 53:5841–5844. [PubMed: 8261390]
59. Rüttinger D, van den Engel NK, Winter H, et al. Adjuvant therapeutic vaccination in patients with non-small cell lung cancer made lymphopenic and reconstituted with autologous PBMC: first clinical experience and evidence of an immune response. *J Transl Med.* 2007; 5:43. [PubMed: 17868452]
60. Serafini P, Carbley R, Noonan KA, et al. High-dose granulocyte-macrophage colony-stimulating factor-producing vaccines impair the immune response through the recruitment of myeloid suppressor cells. *Cancer Research.* 2004; 64:6337–6343. [PubMed: 15342423]
61. Jinushi M, Nakazaki Y, Dougan M, et al. MFG-E8-mediated uptake of apoptotic cells by APCs links the pro- and antiinflammatory activities of GM-CSF. *J. Clin. Invest.* 2007; 117:1902–1913. [PubMed: 17557120]
62. Jinushi M, Nakazaki Y, Carrasco DR, et al. Milk fat globule EGF-8 promotes melanoma progression through coordinated Akt and twist signaling in the tumor microenvironment. *Cancer Research.* 2008; 68:8889–8898. [PubMed: 18974133]
63. Ali OA, Emerich D, Dranoff G, et al. In Situ Regulation of DC Subsets and T Cells Mediates Tumor Regression in Mice. *Sci Transl Med.* 2009; 1:8ra19–8ra19.
64. Kruit W, Suciu S, Dreno B, et al. Immunization with recombinant MAGE-A3 protein combined with adjuvant systems AS15 or AS02B in patients with unresectable and progressive metastatic cutaneous melanoma: A randomized open-label phase II study of the EORTC melanoma group (16032-18031). *J Clin Oncol.* 2008; 27(suppl) Abstract: 9011.
65. Valmori D, Souleimanian NE, Tosello V, et al. Vaccination with NY-ESO-1 protein and CpG in Montanide induces integrated antibody/Th1 responses and CD8 T cells through cross-priming. *Proc. Natl. Acad. Sci. U.S.A.* 2007; 104:8947–8952. [PubMed: 17517626]
66. Disis ML, Wallace DR, Gooley TA, et al. Concurrent Trastuzumab and HER2/neu-Specific Vaccination in Patients With Metastatic Breast Cancer. *J Clin Oncol.* 2009; 27:4685–4692. [PubMed: 19720923]
67. Nesslinger NJ, Ng A, Tsang K-Y, et al. A viral vaccine encoding prostate-specific antigen induces antigen spreading to a common set of self-proteins in prostate cancer patients. *Clin. Cancer Res.* 2010; 16:4046–4056. [PubMed: 20562209]
68. Corbière V, Chapiro J, Stroobant V, et al. Antigen spreading contributes to MAGE vaccination-induced regression of melanoma metastases. *Cancer Research.* 2011; 71:1253–1262. [PubMed: 21216894]
69. Disis ML, Goodell V, Schiffman K, et al. Humoral epitope-spreading following immunization with a HER-2/neu peptide based vaccine in cancer patients. *J. Clin. Immunol.* 2004; 24:571–578. [PubMed: 15359116]
70. Knutson KL, Disis ML. Clonal diversity of the T-cell population responding to a dominant HLA-A2 epitope of HER-2/neu after active immunization in an ovarian cancer patient. *Hum. Immunol.* 2002; 63:547–557. [PubMed: 12072190]
71. Butterfield LH, Ribas A, Dissette VB, et al. Determinant spreading associated with clinical response in dendritic cell-based immunotherapy for malignant melanoma. *Clin. Cancer Res.* 2003; 9:998–1008. [PubMed: 12631598]
72. Brossart P, Wirths S, Stuhler G, et al. Induction of cytotoxic T-lymphocyte responses in vivo after vaccinations with peptide-pulsed dendritic cells. *Blood.* 2000; 96:3102–3108. [PubMed: 11049990]

73. Greten TF, Jaffee EM. Cancer vaccines. *J. Clin. Oncol.* 1999; 17:1047–1060. [PubMed: 10071300]
74. Wirth TC, Harty JT, Badovinac VP. Modulating numbers and phenotype of CD8+ T cells in secondary immune responses. *Eur. J. Immunol.* 2010; 40:1916–1926. [PubMed: 20411564]
75. McNally JM, Zarozinski CC, Lin MY, et al. Attrition of bystander CD8 T cells during virus-induced T-cell and interferon responses. *J. Virol.* 2001; 75:5965–5976. [PubMed: 11390598]
76. Hansen SG, Ford JC, Lewis MS, et al. Profound early control of highly pathogenic SIV by an effector memory T-cell vaccine. *Nature.* 2011; 473:523–527. [PubMed: 21562493]
77. Kantoff PW, Schuetz TJ, Blumenstein BA, et al. Overall survival analysis of a phase II randomized controlled trial of a Poxviral-based PSA-targeted immunotherapy in metastatic castration-resistant prostate cancer. *J Clin Oncol.* 2010; 28:1099–1105. [PubMed: 20100959]
78. Gulley JL, Arlen PM, Madan RA, et al. Immunologic and prognostic factors associated with overall survival employing a poxviral-based PSA vaccine in metastatic castrate-resistant prostate cancer. *Cancer Immunol. Immunother.* 2010; 59:663–674. [PubMed: 19890632]
79. Ribas A, Weber JS, Chmielowski B, et al. Intra-Lymph Node Prime-Boost Vaccination against Melan A and Tyrosinase for the Treatment of Metastatic Melanoma: Results of a Phase 1 Clinical Trial. *Clin. Cancer Res.* 2011; 17:2987–2996. [PubMed: 21385924]
80. Weber J, Boswell W, Smith J, et al. Phase 1 Trial of Intranodal Injection of a Melan-A/MART-1 DNA Plasmid Vaccine in Patients With Stage IV Melanoma. *J Immunother.* 2008; 31:215–223. [PubMed: 18481391]
81. Apetoh L, Ghiringhelli F, Tesniere A, et al. The interaction between HMGB1 and TLR4 dictates the outcome of anticancer chemotherapy and radiotherapy. *Immunol. Rev.* 2007; 220:47–59. [PubMed: 17979839]
82. Green DR, Ferguson T, Zitvogel L, et al. Immunogenic and tolerogenic cell death. *Nat Rev Immunol.* 2009; 9:353–363. [PubMed: 19365408]
83. Jinushi M, Vanneman M, Munshi NC, et al. MHC class I chain-related protein A antibodies and shedding are associated with the progression of multiple myeloma. *Proc. Natl. Acad. Sci. U.S.A.* 2008; 105:1285–1290. [PubMed: 18202175]
84. Raulet DH. Roles of the NKG2D immunoreceptor and its ligands. *Nat Rev Immunol.* 2003; 3:781–790. [PubMed: 14523385]
85. Bahjat KS, Korman AJ. Elevated soluble major histocompatibility complex class I chain-related protein A predicts nonresponsiveness to cytotoxic T lymphocyte antigen-4 blockade. *J. Immunother.* 2009; 32 (abstracts):1002.
86. Jinushi M, Hodi FS, Dranoff G. Therapy-induced antibodies to MHC class I chain-related protein A antagonize immune suppression and stimulate antitumor cytotoxicity. *Proc. Natl. Acad. Sci. U.S.A.* 2006; 103:9190–9195. [PubMed: 16754847]
87. Gattinoni L, Finkelstein SE, Klebanoff CA, et al. Removal of homeostatic cytokine sinks by lymphodepletion enhances the efficacy of adoptively transferred tumor-specific CD8+ T cells. *J. Exp. Med.* 2005; 202:907–912. [PubMed: 16203864]
88. Jaffee EM, Hruban RH, Biedrzycki B, et al. Novel allogeneic granulocyte-macrophage colony-stimulating factor-secreting tumor vaccine for pancreatic cancer: a phase I trial of safety and immune activation. *J. Clin. Oncol.* 2001; 19:145–156. [PubMed: 11134207]
89. Lutz E, Yeo CJ, Lillemoe KD, et al. A lethally irradiated allogeneic granulocyte-macrophage colony stimulating factor-secreting tumor vaccine for pancreatic adenocarcinoma. A Phase II trial of safety, efficacy, and immune activation. *Ann. Surg.* 2011; 253:328–335.
90. Laheru D, Lutz E, Burke J, et al. Allogeneic granulocyte macrophage colony-stimulating factor-secreting tumor immunotherapy alone or in sequence with cyclophosphamide for metastatic pancreatic cancer: a pilot study of safety, feasibility, and immune activation. *Clin. Cancer Res.* 2008; 14:1455–1463. [PubMed: 18316569]
91. Smith BD, Kasamon YL, Kowalski J, et al. K562/GM-CSF immunotherapy reduces tumor burden in chronic myeloid leukemia patients with residual disease on imatinib mesylate. *Clin. Cancer Res.* 2010; 16:338–347. [PubMed: 20048335]
92. Borrello IM, Levitsky HI, Stock W, et al. Granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting cellular immunotherapy in combination with autologous stem cell

- transplantation (ASCT) as postremission therapy for acute myeloid leukemia (AML). *Blood*. 2009; 114:1736–1745. [PubMed: 19556425]
93. Emens LA, Asquith JM, Leatherman JM, et al. Timed sequential treatment with cyclophosphamide, doxorubicin, and an allogeneic granulocyte-macrophage colony-stimulating factor-secreting breast tumor vaccine: a chemotherapy dose-ranging factorial study of safety and immune activation. *J Clin Oncol*. 2009; 27:5911–5918. [PubMed: 19805669]
 94. MacLean GD, Miles DW, Rubens RD, et al. Enhancing the effect of THERATOPE STn-KLH cancer vaccine in patients with metastatic breast cancer by pretreatment with low-dose intravenous cyclophosphamide. *J Immunother Emphasis Tumor Immunol*. 1996; 19:309–316. [PubMed: 8877724]
 95. Gulley JL, Arlen PM, Bastian A, et al. Combining a recombinant cancer vaccine with standard definitive radiotherapy in patients with localized prostate cancer. *Clin. Cancer Res*. 2005; 11:3353–3362. [PubMed: 15867235]
 96. Zou W. Regulatory T cells, tumor immunity and immunotherapy. *Nat Rev Immunol*. 2006; 6:295–307. [PubMed: 16557261]
 97. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med*. 2004; 10:942–949. [PubMed: 15322536]
 98. Gnjjatic S, Altorki NK, Tang DN, et al. NY-ESO-1 DNA vaccine induces T-cell responses that are suppressed by regulatory T cells. *Clin. Cancer Res*. 2009; 15:2130–2139. [PubMed: 19276258]
 99. [Accessed: June 2011] www.clinicaltrials.gov
 100. Kim YH, Duvic M, Obiz E, et al. Clinical efficacy of zanolimumab (HuMax-CD4): two phase 2 studies in refractory cutaneous T-cell lymphoma. *Blood*. 2007; 109:4655–4662. [PubMed: 17311990]
 101. Janssen EM, Droin NM, Lemmens EE, et al. CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. *Nature*. 2005; 434:88–93. [PubMed: 15744305]
 102. Sun JC, Bevan MJ. Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science*. 2003; 300:339–342. [PubMed: 12690202]
 103. Rech AJ, Vonderheide RH. Clinical use of anti-CD25 antibody daclizumab to enhance immune responses to tumor antigen vaccination by targeting regulatory T cells. *Ann. N. Y. Acad. Sci*. 2009; 1174:99–106. [PubMed: 19769742]
 104. Dannull J, Su Z, Rizzieri D, et al. Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. *J. Clin. Invest*. 2005; 115:3623–3633. [PubMed: 16308572]
 105. Barnett B, Kryczek I, Cheng P, et al. Regulatory T cells in ovarian cancer: biology and therapeutic potential. *Am. J. Reprod. Immunol*. 2005; 54:369–377. [PubMed: 16305662]
 106. Foss FM, Waldmann TA. Interleukin-2 receptor-directed therapies for cutaneous lymphomas. *Hematol. Oncol. Clin. North Am*. 2003; 17:1449–1458. [PubMed: 14710895]
 107. Jacobs JM, Punt CJA, Lesterhuis WJ, et al. Dendritic cell vaccination in combination with anti-CD25 monoclonal antibody treatment: a phase I/II study in metastatic melanoma patients. *Clin. Cancer Res*. 2010; 16:5067–5078. [PubMed: 20736326]
 108. de Vries IJM, Castelli C, Huygens C, et al. Frequency of circulating Tregs with demethylated FOXP3 intron 1 in melanoma patients receiving tumor vaccines and potentially Treg-depleting agents. *Clin. Cancer Res*. 2011; 17:841–848. [PubMed: 21177412]
 109. Powell DJ, Attia P, Ghetie V, et al. Partial reduction of human FOXP3+ CD4 T cells in vivo after CD25-directed recombinant immunotoxin administration. *J. Immunother*. 2008; 31:189–198. [PubMed: 18481388]
 110. Wuest SC, Edwan JH, Martin J, et al. A role for interleukin-2 trans-presentation in dendritic cell-mediated T cell activation in humans, as revealed by daclizumab therapy. *Nat Med*. 2011; 17:604–609. [PubMed: 21532597]
 111. Triplett TA, Weinberg A. Evaluation of a novel human CD4+CD25DIMFOXP3-T-cell population in cancer patients undergoing interleukin-2 treatment. *J. Immunother*. 2009; 32 (abstract) 963–964.

112. Furtado GC, Curotto de Lafaille MA, Kutchukhidze N, et al. Interleukin 2 signaling is required for CD4(+) regulatory T cell function. *J. Exp. Med.* 2002; 196:851–857. [PubMed: 12235217]
113. Sharfe N, Dadi HK, Shahar M, et al. Human immune disorder arising from mutation of the alpha chain of the interleukin-2 receptor. *Proc. Natl. Acad. Sci. U.S.A.* 1997; 94:3168–3171. [PubMed: 9096364]
114. Mantovani A, Sozzani S, Locati M, et al. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol.* 2002; 23:549–555. [PubMed: 12401408]
115. Suzuki E, Kim S, Cheung H-K, et al. A novel small-molecule inhibitor of transforming growth factor beta type I receptor kinase (SM16) inhibits murine mesothelioma tumor growth in vivo and prevents tumor recurrence after surgical resection. *Cancer Research.* 2007; 67:2351–2359. [PubMed: 17332368]
116. Rausch MP, Hahn T, Ramanathapuram L, et al. An orally active small molecule TGF-beta receptor I antagonist inhibits the growth of metastatic murine breast cancer. *Anticancer Res.* 2009; 29:2099–2109. [PubMed: 19528470]
117. Youn J-I, Nagaraj S, Collazo M, et al. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. *J Immunol.* 2008; 181:5791–5802. [PubMed: 18832739]
118. Kusmartsev S, Su Z, Heiser A, et al. Reversal of myeloid cell-mediated immunosuppression in patients with metastatic renal cell carcinoma. *Clin. Cancer Res.* 2008; 14:8270–8278. [PubMed: 19088044]
119. Kusmartsev S, Cheng F, Yu B, et al. All-trans-retinoic acid eliminates immature myeloid cells from tumor-bearing mice and improves the effect of vaccination. *Cancer Research.* 2003; 63:4441–4449. [PubMed: 12907617]
120. Serafini P, Meckel K, Kelso M, et al. Phosphodiesterase-5 inhibition augments endogenous antitumor immunity by reducing myeloid-derived suppressor cell function. *J. Exp. Med.* 2006; 203:2691–2702. [PubMed: 17101732]
121. Olkhanud PB, Damdinsuren B, Bodogai M, et al. Tumor-Evoked Regulatory B Cells Promote Breast Cancer Metastasis by Converting Resting CD4+ T Cells to T-Regulatory Cells. *Cancer Research.* 2011; 71:3505–3515. [PubMed: 21444674]
122. Wijesuriya R, Maruo S, Zou JP, et al. B cell-mediated down-regulation of IFN-gamma and IL-12 production induced during anti-tumor immune responses in the tumor-bearing state. *Int. Immunol.* 1998; 10:1057–1065. [PubMed: 9723691]
123. Bouaziz J-D, Yanaba K, Venturi GM, et al. Therapeutic B cell depletion impairs adaptive and autoreactive CD4+ T cell activation in mice. *Proc. Natl. Acad. Sci. U.S.A.* 2007; 104:20878–20883. [PubMed: 18093919]
124. Tadmor T, Zhang Y, Cho H-M, et al. The absence of B lymphocytes reduces the number and function of T-regulatory cells and enhances the anti-tumor response in a murine tumor model. *Cancer Immunol. Immunother.* 2011; 60:609–619. [PubMed: 21253724]
125. Gilbert KM, Weigle WO. Tolerogenicity of resting and activated B cells. *J. Exp. Med.* 1994; 179:249–258. [PubMed: 7505799]
126. Schwartzenruber DJ, Lawson DH, Richards JM, et al. gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma. *N. Engl. J. Med.* 2011; 364:2119–2127. [PubMed: 21631324]
127. Surh CD, Sprent J. Homeostasis of Naive and Memory T Cells. *Immunity.* 2008; 29:848–862. [PubMed: 19100699]
128. Pellegrini M, Calzascia T, Toe JG, et al. IL-7 Engages Multiple Mechanisms to Overcome Chronic Viral Infection and Limit Organ Pathology. *Cell.* 2011; 144:601–613. [PubMed: 21295337]
129. Wang L-X, Li Y, Yang G, et al. CD122+CD8+ Treg suppress vaccine-induced antitumor immune responses in lymphodepleted mice. *Eur. J. Immunol.* 2010; 40:1375–1385. [PubMed: 20186876]
130. Rosenberg SA, Sportés C, Ahmadzadeh M, et al. IL-7 administration to humans leads to expansion of CD8+ and CD4+ cells but a relative decrease of CD4+ T-regulatory cells. *J Immunother.* 2006; 29:313–319. [PubMed: 16699374]

131. Sportés C, Babb RR, Krumlauf MC, et al. Phase I study of recombinant human interleukin-7 administration in subjects with refractory malignancy. *Clin. Cancer Res.* 2010; 16:727–735. [PubMed: 20068111]
132. Lugli E, Goldman CK, Perera LP, et al. Transient and persistent effects of IL-15 on lymphocyte homeostasis in nonhuman primates. *Blood.* 2010; 116:3238–3248. [PubMed: 20631381]
133. Waldmann TA. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. *Nat Rev Immunol.* 2006; 6:595–601. [PubMed: 16868550]
134. Klebanoff CA, Finkelstein SE, Surman DR, et al. IL-15 enhances the in vivo antitumor activity of tumor-reactive CD8+ T cells. *Proc. Natl. Acad. Sci.* 2004; 101:1969–1974. [PubMed: 14762166]
135. Roberti MP, Barrio MM, Bravo AI, et al. IL-15 and IL-2 increase Cetuximab-mediated cellular cytotoxicity against triple negative breast cancer cell lines expressing EGFR. *Breast Cancer Res Treat.* 2011 Epub.
136. Cooke M, Ohlén C, Greenberg P. Interleukin-15 rescues tolerant CD8+ T cells for use in adoptive immunotherapy of established tumors. *Nature.* 2006; 12:335–341.
137. Dubois S, Patel H, Zhang M, et al. Preassociation of IL-15 with IL-15R α -IgG1-Fc Enhances Its Activity on Proliferation of NK and CD8+/CD44^{high} T Cells and Its Antitumor Action. *J Immunol.* 2008; 180:2099–2106. [PubMed: 18250415]
138. Hinrichs CS, Spolski R, Paulos CM, et al. IL-2 and IL-21 confer opposing differentiation programs to CD8+ T cells for adoptive immunotherapy. *Blood.* 2008; 111:5326–5333. [PubMed: 18276844]
139. Li Y, Yee C. IL-21 mediated Foxp3 suppression leads to enhanced generation of antigen-specific CD8+ cytotoxic T lymphocytes. *Blood.* 2007; 111:229–235. [PubMed: 17921346]
140. Peluso I, Fantini MC, Fina D, et al. IL-21 counteracts the regulatory T cell-mediated suppression of human CD4+ T lymphocytes. *J. Immunol.* 2007; 178:732–739. [PubMed: 17202333]
141. Li Y, Bleakley M, Yee C. IL-21 influences the frequency, phenotype, and affinity of the antigen-specific CD8 T cell response. *J. Immunol.* 2005; 175:2261–2269. [PubMed: 16081794]
142. Davis ID, Brady B, Kefford RF, et al. Clinical and biological efficacy of recombinant human interleukin-21 in patients with stage IV malignant melanoma without prior treatment: a phase IIa trial. *Clin. Cancer Res.* 2009; 15:2123–2129. [PubMed: 19276257]
143. Huang AY, Golumbek P, Ahmadzadeh M, et al. Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. *Science.* 1994; 264:961–965. [PubMed: 7513904]
144. Li Y, Wang LX, Pang P, et al. Cross-presentation of tumor associated antigens through tumor-derived autophagosomes. *Autophagy.* 2009; 5:576–577. [PubMed: 19333005]
145. Yewdell JW, Nicchitta CV. The DRiP hypothesis decennial: support, controversy, refinement and extension. *Trends Immunol.* 2006; 27:368–373. [PubMed: 16815756]
146. Li Y, Wang LX, Yang G, et al. Efficient Cross-presentation Depends on Autophagy in Tumor Cells. *Cancer Research.* 2008; 68:6889–6895. [PubMed: 18757401]
147. Twitty C, Jensen SM, Hu H-M, et al. Tumor-Derived Autophagosome Vaccine: Induction of Cross-Protective Immune Responses Against Short-Lived Proteins Through a P62-Dependent Mechanism. *Clin Cancer Res.* 2011 in press.
148. Robbins PF, Dudley ME, Wunderlich J, et al. Cutting edge: persistence of transferred lymphocyte clonotypes correlates with cancer regression in patients receiving cell transfer therapy. *J. Immunol.* 2004; 173:7125–7130. [PubMed: 15585832]
149. Kantoff PW, Higano CS, Shore ND, et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N. Engl. J. Med.* 2010; 363:411–422. [PubMed: 20818862]
150. Lee P, Wang F, Kuniyoshi J, et al. Effects of interleukin-12 on the immune response to a multipptide vaccine for resected metastatic melanoma. *J. Clin. Oncol.* 2001; 19:3836–3847. [PubMed: 11559721]
151. Atanackovic D, Altorki NK, Cao Y, et al. Booster vaccination of cancer patients with MAGE-A3 protein reveals long-term immunological memory or tolerance depending on priming. *Proc. Natl. Acad. Sci. U.S.A.* 2008; 105:1650–1655. [PubMed: 18216244]
152. Atanackovic D, Altorki NK, Stockert E, et al. Vaccine-induced CD4+ T cell responses to MAGE-3 protein in lung cancer patients. *J. Immunol.* 2004; 172:3289–3296. [PubMed: 14978137]

153. van Baren N, Bonnet M-C, Dréno B, et al. Tumoral and immunologic response after vaccination of melanoma patients with an ALVAC virus encoding MAGE antigens recognized by T cells. *J. Clin. Oncol.* 2005; 23:9008–9021. [PubMed: 16061912]
154. Slingluff CL, Petroni GR, Yamshchikov GV, et al. Clinical and immunologic results of a randomized phase II trial of vaccination using four melanoma peptides either administered in granulocyte-macrophage colony-stimulating factor in adjuvant or pulsed on dendritic cells. *J. Clin. Oncol.* 2003; 21:4016–4026. [PubMed: 14581425]
155. Butterfield LH, Palucka AK, Britten CM, et al. Recommendations from the iSBTc-SITC/FDA/NCI Workshop on Immunotherapy Biomarkers. *Clin. Cancer Res.* 2011; 17:3064–3076. [PubMed: 21558394]
156. Galon J, Costes A, Sanchez-Cabo F, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science.* 2006; 313:1960–1964. [PubMed: 17008531]
157. Tosolini M, Kirilovsky A, Mlecnik B, et al. Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, th2, treg, th17) in patients with colorectal cancer. *Cancer Research.* 2011; 71:1263–1271. [PubMed: 21303976]
158. Milne K, Köbel M, Kalloger SE, et al. Systematic Analysis of Immune Infiltrates in High-Grade Serous Ovarian Cancer Reveals CD20, FoxP3 and TIA-1 as Positive Prognostic Factors. *PLoS ONE.* 2009; 4:e6412. [PubMed: 19641607]
159. Harlin H, Meng Y, Peterson AC, et al. Chemokine expression in melanoma metastases associated with CD8+ T-cell recruitment. *Cancer Research.* 2009; 69:3077–3085. [PubMed: 19293190]
160. Vansteenkiste J, Zielinski M, Dahabreh J, et al. Association of gene expression signature and clinical efficacy of MAGE-A3 antigen-specific cancer immunotherapeutic (ASCI) as adjuvant therapy in resected stage IB/II non-small cell lung cancer (NSCLC). *J Clin Oncol.* 2008; (suppl) Abstract: 7501.
161. Fox BA, Schendel DJ, Butterfield LH, Aamdal S, Allison JP, Ascierto PA. Defining the critical hurdles in cancer immunotherapy. *J Transl Med.* 2011 in press.
162. Cheever MA. Twelve immunotherapy drugs that could cure cancers. *Immunol. Rev.* 2008; 222:357–368. [PubMed: 18364014]
163. Cheever MA, Allison JP, Ferris AS, et al. The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. *Clin. Cancer Res.* 2009; 15:5323–5337. [PubMed: 19723653]
164. Cheever MA, Schlom J, Weiner LM, et al. Translational Research Working Group developmental pathway for immune response modifiers. *Clin. Cancer Res.* 2008; 14:5692–5699. [PubMed: 18794077]
165. Puri S, Thompson JA, Siebert JC, et al. Exploiting the Humoral Immune Response to Identify Tumor/Tumor-Associated Antigens Recognized after Combination Immunotherapy in Men With Hormone-refractory Prostate Cancer. *J. Immunother.* 2009 (abstract):975.
166. Salgaller ML, Marincola FM, Cormier JN, et al. Immunization against epitopes in the human melanoma antigen gp100 following patient immunization with synthetic peptides. *Cancer Research.* 1996; 56:4749–4757. [PubMed: 8840994]
167. Kammula US, Marincola FM, Rosenberg SA. Real-time quantitative polymerase chain reaction assessment of immune reactivity in melanoma patients after tumor peptide vaccination. *J. Natl. Cancer Inst.* 2000; 92:1336–1344. [PubMed: 10944556]
168. Sosman JA, Carrillo C, Urban WJ, et al. Three Phase II Cytokine Working Group Trials of gp100 (210M) Peptide Plus High-Dose Interleukin-2 in Patients With HLA-A2-Positive Advanced Melanoma. *J Clin Oncol.* 2008; 26:2292–2298. [PubMed: 18467720]
169. Adams S, O'Neill DW, Nonaka D, et al. Immunization of malignant melanoma patients with full-length NY-ESO-1 protein using TLR7 agonist imiquimod as vaccine adjuvant. *J. Immunol.* 2008; 181:776–784. [PubMed: 18566444]
170. Bioley G, Guillaume P, Luescher I, et al. Vaccination with a recombinant protein encoding the tumor-specific antigen NY-ESO-1 elicits an A2/157-165-specific CTL repertoire structurally distinct and of reduced tumor reactivity than that elicited by spontaneous immune responses to NY-ESO-1-expressing Tumors. *J. Immunother.* 2009; 32:161–168. [PubMed: 19238015]

171. Rosenberg SA, Yang JC, Schwartzentruber DJ, et al. Recombinant fowlpox viruses encoding the anchor-modified gp100 melanoma antigen can generate antitumor immune responses in patients with metastatic melanoma. *Clin. Cancer Res.* 2003; 9:2973–2980. [PubMed: 12912944]

Figure 1. Proposed model for how immune signature can be used to personalize vaccine strategies and improve patient outcomes

A. An active immune signature identified by phenotypic or genetic analysis of immune components within the tumor signals pre-existing immune response that is easier to augment by booster vaccines. This is the cohort of patients that respond to effective boosting vaccines. B. Tumors that lack an immune signature contain few immune cells, signaling the lack of an anti-tumor immune response. Vaccine strategies need to stress both priming of a *de novo* anti-tumor immune response as well as boosting of the immune response in order to be effective. C. Immunosuppressive gene signature including Tregs, MDSC, Bregs and/or tolerogenic cytokines will block the immune response to vaccination and may have eliminated tumor-specific T cells. Vaccine strategies need to start with elimination of the suppressive environment/cells and subsequently prime and boost the anti-tumor immune response in order to develop a therapeutic immune response. All three scenarios are expected to benefit from agents that induce immunogenic death.

Table 1
Summary of selected studies with kinetic analysis of the T cell response data for patients receiving multiple vaccinations

Vaccine Type	Tumor	Antigen	Adjuvant	Vaccine #	Early ^a FreqFunc	Late ^b FreqFunc	Assay ^c	Summary of immune response	Ref
Peptide	Melanoma	gp100 HLA I	Montanide	2-7	++	+	Tet CRA CTL	79.6% of patients in 2 trials had peptide-specific IFN γ production. 100% of patients tested had gp100-specific CD8 T cells (n=7). Quantity of IFN γ secreted by PBMCs increased from 2 to 4 vaccinations.	29, 31, 166, 167
					++	+			
	Melanoma	gp100(209-2M) HLA I	Montanide	2-3 or 7-11	++	+	IVS Tet IVS MFCS IVS ICS	Increased peptide-specific effector and memory T cells in sentinel LN and blood with 2-3 vaccinations. No further increase in peptide specific cells with booster vaccination	32
					++	+			
	Melanoma	Separate gp100(209-2M) Tyrosinase(370D) HLA I Combined gp100(209-2M) Tyrosinase(370D) HLA I	IFA	10, 4 or 12	++	+++	Tet IVS ELISPOT CRA	4-21% of patients had tyrosinase-specific T cells and 70-72% had gp100-specific T cells. 17% of patients had >10% tetramer-specific CD8 T cells. 52.6-56% had gp100-specific IFN γ T cells. 54% of patients had tyrosinase-specific T cells and 20% had gp100-specific T cells. 4.2-13% gp100-specific IFN γ CD8 T cells.	33, 34
					++	++			
	Melanoma	gp100(209-2M) MART-1 HLA I gp100:44-59 HLA II gp100(209-2M) MART-1 HLA I	IFA	2-4	+	+	IVS ELISPOT CRA	50% of patients generated de novo gp100-specific cells. 70% of patients had gp100-specific cells after 4 vaccines. 95% of patients generated de novo gp100-specific cells.	36
					++	++			
	Melanoma	12 HLA I melanoma and tetanus HLA II 12 HLA I and 6 HLA II melanoma	Montanide and/or CY	6	ND	++	ELISPOT	78% of patients had a CD8 T cell response and 93% of patients had a CD4 T cell response. 19% of patients had a CD8 T cell response and 48% of patients had a CD4 T cell response.	37
					ND	+			
	Melanoma	gp100(209-2M) HLA I	Montanide IL-2	4	-	ND	Tet MFCS	No immune correlates were observed following vaccination when comparing clinical responders to non-responders.	168
					+	++			
	Melanoma	gp100(209-2M) MART-1 Tyrosinase(370D) HLA I (HLA-A2 epitopes)	Montanide and/or GM-CSF IL-2 IFN α 2b	3-6	+	++	ELISPOT	21.2% of patients have an IFN γ response to any peptide after 3 vaccines. 37% of patients have an IFN γ response to any peptide after 6 vaccines. Increase in immune response correlates with clinical outcome (21.3 vs 13.4 months, p<0.046).	46
					+	++			

Vaccine Type	Tumor	Antigen	Adjuvant	Vaccine #	Early ^a FreqFunc	Late ^b FreqFunc	Assay ^c	Summary of immune response	Ref
	Melanoma	gp100(209-2M) Tyrosinase(370D) HLA I	Montanide IL-12	2-8	+	++	Tet CRA	Immune response not increased with either cytokine. 88% of patients had gp100-specific T cells. 86.8% of patients had vaccine specific IFN γ production.	150
	Melanoma	MelanA Tyrosinase HLA I	Plasmid	6-12	ND	++	Tet ELISPOT	50% of evaluable patients made an anti-vaccine immune response. 29.9% of patients had tyrosinase-specific T cells and 39.6% had MelanA specific CD8 T cell response.	79
	Breast Ovarian	HER-2/neu HLA II (HLA-A2 epitopes) HER-2/neu ICD HLA II HER-2/neu ECD HLA II	GM-CSF	2-6	++	+	ELISPOT IVS CTL LDSA	92% of evaluable patients developed a HER-2/neu-specific response. Detailed kinetic analysis of individual patients showed HER-2/neu-specific T cell responses went down with more vaccinations. 50% of HLA-A2 ⁺ patients had HER-2/neu-specific IFN γ ⁺ CD8 T cells. Responding patients developed intermolecular epitope-spreading. Peptide-specific T cells killed tumor targets <i>ex vivo</i> even at late time points (>17 months). Great deal of variation between patients.	40, 44
Long peptide	Cervical	HPV E6/E7 both peptides / one site	Montanide	2-4	++	+	ELISPOT ICS	87.5% of patients had an E6-specific CD8 T cell response after 2 vaccines, but this diminished after 4 vaccines for patients with peptides combined (one site). No decrease when peptides separated at 2 sites. HPV16-specific IFN γ ⁺ CD4 T cells were increased after 2, but not after 4 vaccines for one CR patient.	38
		HPV E6/E7 separate sites		Montanide	2-4	++			++
Protein	Melanoma	NY-ESO-1	Imiquimod	4	+	ND	IVS ELISPOT IVS ICS	30% of patients had increase in ESO-specific CD8 T cells. Of responders tetramer positive CD8 T cells were also IFN γ ⁺ . 75% of evaluable patients had IFN γ ⁺ CD4 T cells.	169, 170
	Melanoma Breast Sarcoma Ovarian	NY-ESO-1	Montanide CpG	2-4	ND	+	IVS ICS	94.4% of patients had IFN γ ⁺ CD4 T cells and 50% had IFN γ ⁺ CD8 T cells. Kinetic analysis showed a trend that patients had more IFN γ ⁺ CD4 and CD8 T cells after 4 vaccines than after two.	65
	Breast Ovarian	HER-2/neu	GM-CSF	2-6	++	++	LDSA	89% of patients had HER-2/neu-specific T cell immunity. HER-2/neu immunity developed earlier using high-doses of vaccine. 50% of patients retained specific T cells 9-12 months after immunization.	41

Vaccine Type	Tumor	Antigen	Adjuvant	Vaccine #	Early ^a FreqFunc	Late ^b FreqFunc	Assay ^c	Summary of immune response	Ref
	NSCLC	MAGE-A3	No adjuvant or MPL and Q521	4-8	+	++	IVS Tet IVS ELISPOT	50% of patients receiving 4 vaccines plus adjuvant developed IFN γ producing CD4 T cells. 71.4% of patients receiving 8 vaccines plus adjuvant developed IFN γ producing CD4 T cells.	151, 152
Virus	Melanoma	gp100	No adjuvant	2	++	ND	CRA	54% of patients had peptide-specific IFN γ -production.	171
	Prostate	PSA (PROSTVAC)	No adjuvant or GM-CSF	1 prime + monthly boost	++	+	ELISPOT IVS SFA	44.8% had IFN γ response to PSA. Non-responding patients had increased suppressive function of CD4 ⁺ CD25 ⁺ T cells after 3 vaccinations, measured <i>in vitro</i> .	78
	Melanoma NSCLC H&N	MAGE-A1/3 (ALVAC)	None	4 viral + 3-15 peptides	+	++	IVS Tet IVS ELISPOT	50% of patients given 7-19 vaccinations had a CD8 T cell response. 22.2% of patients given booster vaccination had MAGE-A3-specific CD8 T cells. One PR patient had MAGE specific IFN γ ⁺ cells and antigen spreading.	68, 153
Whole tumor	Melanoma	Autologous tumor	GM-CSF	3-12	+	+	IHC CRA CTL	68.7% of patients had increased T cell infiltration at metastatic tumor sites. Tumor-specific type 2 cytokines were produced and concentration of cytokines increased with more vaccinations.	47, 48
	Breast	Allogeneic MDA-MB-231	GM-CSF or BCG	2-12	+	-	ICS CRA	44.4% of evaluable patients developed a CD8 T cell response to tumor cell lines. One patient with CR had >5% vaccine-specific T cells early and maintained vaccine-specific response >3 years.	54, 55

^aEarly: 1-3 vaccinations or time period after primary vaccine administration

^bLate: 4 vaccinations or 6 months following vaccination.

^cAssays performed following an *in vitro* stimulation (IVS) are identified by IVS prior to assay design.

Abbreviations: ICS – intracellular cytokine staining, Tet – tetramer, CRA – cytokine release assay, MFCS- multi-color flow cytometry, SFA – suppressive function assay, LDSEA – limiting dilution stimulation assay, CTL – cytotoxic T lymphocyte assay, IFA – incomplete Freund's adjuvant, CY – cyclophosphamide, NSCLC – non-small cell lung cancer, H&N – head and neck, ICD – intracellular domain, ECD – extracellular domain, MPL – monophosphoryl lipid, BCG – bacilli calmette-guerin

- no measurable response

^dEarly: increase from pre-, Late: decrease from early

⁺⁺Early: large increase from pre-, Late: same as early or increased from + early

+++Late: increase from early

ND: not determined