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Analysis of Vaccine-Induced T Cells in Humans with Cancer

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Abstract

Over the past several years, progress in the field of tumor immunology has lead to advances in active immunotherapy and vaccination as a means of eliciting tumor-specific immune responses to mediate tumor regression and clearance. Developing vaccines targeted against cancer became an important focus as a therapy following the success of viral vaccines in preventing infection and disease. In humans with cancer, similar to viral infections, the host immune system is capable of recognizing antigens expressed on tumor cells. This similarity allows the immunological framework of the viral vaccine to be adapted to the cancer setting in hopes of enhancing human T-cell reactivity against tumor.¹ It is generally believed that a requirement for tumor destruction to occur is the induction of sufficient levels of immune cells with high avidity for recognition of tumor antigens. Moreover, the cells must be targeted to the tumor site and be capable of infiltrating tumor stroma.² Several tumor-associated antigens (TAA) have been identified in the melanoma model which has allowed for immunization trials to evaluate therapeutic potential of tumor-specific T-cell induction. Some clinical trials reported limited success of T-cell mediated tumor rejection, reporting partial or complete regression in 10 to 30% of patients.³ Although tumor regression was not observed following active immunization in vivo, ex vivo assays evaluating TAA-specific T cells demonstrated tumor recognition and subsequent T-cell activation suggesting that tumor-specific T-cell induction indeed occurs but alone is not adequate to induce tumor regression.¹ Recently, the usefulness and success of active-specific immunization (ASI) against TAAs as a means of eliciting a tumor-specific immune response leading to tumor regression and clearance has been a topic of debate and discussion.

Argument against Potential of Tumor Vaccination

Those unconvinced of the potential usefulness of cancer vaccines as a therapy argue that the clinical endpoint of ASI is tumor destruction and clinical trials have yet to successfully correlate ASI with clinical regression. Instead, other therapeutic methods should be investigated such as adoptive immunotherapy. In the viral model, vaccination shows no benefit to the host when administered during acute viral infection and Rosenberg et al⁴ suggest that a similar phenomenon may be occurring during ASI against cancer and that

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cancer vaccines may be more useful if used as a preventative. In animal models, antitumor vaccinations were administered both in prophylactic and therapeutic settings. Prophylactic vaccinations against viral diseases and to prevent virally induced tumors were effective when synthetic peptide was used. However, nonvirally induced tumors did not respond to prophylactic vaccination, though antitumor efficacy was present. As a therapy, vaccination was effective in only limited number animal models.

Rather than measuring success based on immunologic data such as circulating TAA-specific T-lymphocytes, presence of tumor infiltrating lymphocytes (TILs) and histology, it has been suggested that success should be based on clinical regression following the Response Evaluation Criteria for Solid Tumors (RECIST) guidelines which require a 30% reduction in the sum of maximum lesion diameter and no novel or progressing lesions.⁵⁻⁷ However, the more conventional criteria to characterize a clinical response are a “50% reduction in the sum of the products of the perpendicular diameters of all lesions without 25% growth of any lesion or the appearance of new lesions”. Using this more common approach, Rosenberg et al⁴ reported an objective response rate of only 2.6% in 440 patients following vaccine administration to patients with various types of metastatic cancer including melanoma, renal cell, ovarian, colorectal and breast, a rate which they found to be comparable to other vaccine trials. Patients were administered various vaccines such as peptide, viral vectors and naked DNA encoding tumor antigen. Although T cells activated against specific TAAs can be successfully generated in vivo, the lack of correlation to clinical response still exists. However, the report was biased by the aggregation of heterogeneous protocols into a single analysis and by the lack of mechanistic interpretation of the reasons for the lack of correlation between frequency of TAA-specific T cells and tumor regression.⁸

Various obstacles exist in the cancer ASI setting. One issue is that although T-lymphocytes capable of recognizing TAAs are generated, antigen recognition is not enough to mediate regression and T-cell mediated rejection of vascularized tumors.³ In addition to recognizing TAAs, T cells must also be capable of localizing and surviving in target tissue. Another concern is that the number of circulating T cells may be inadequate to mount a clinical response. In adoptive therapy studies, it has been reported that between 5 and 75% of antitumor T cells are necessary to achieve somewhat successful clinical effectiveness.⁹ Other important elements that should be addressed are the inability of tumor to activate quiescent or precursor lymphocytes, tolerance mechanisms including anergy,³ suppressor influences by tumor or immune system^{10,11} and tumor escape mechanisms.

Argument for Potential of Tumor Vaccination

Expectations from immunizations against cancer are similar to those from immunizations against pathogens. In the pathogenic immunization pathway an inflammatory response at the injection site activates monocytes making them capable of antigen uptake. The immunogen or adjuvant can cause monocyte maturation into professional antigen presenting cells (APCs) which migrate to loco regional lymph nodes where they interact with naive and memory T cells, including those that can recognize the antigen and initiate the afferent loop of immunization. Differences between anti-cancer and anti-infectious ASI occur in the efferent arm. In a pathogenic model, after T cells are primed in the lymph nodes, they

migrate back to the site of infection which is usually associated with inflammation allowing the T cells to become activated and to perform their cytolytic function. However, because tissue damage in cancer is typically not as extensive as in a pathogenic model, the tumor microenvironment is less conducive to producing inflammatory responses capable of stimulating tumor-specific T cells to perform effector functions. The afferent arm of immunization however, is functioning properly as demonstrated by identification of circulating antigen-specific T cells suggesting that the problem lies in effector function performance.¹

Because T-cell induction does not equal clinical regression, certain aspects should be investigated such as effector function adequacy, frequency of immunogen-specific T cells, T-cell localization and function at tumor site, secondary stimuli and tumor escape mechanisms. The fact that tumor and tumor-specific circulating or intra-tumoral cytotoxic T-lymphocytes (CTLs) can coexist in the host suggests that there may be an issue with the adequacy of T-lymphocyte effector function. Following immunization, TAA-specific T cells typically express T-cell activation markers and secrete interferon- γ (IFN- γ) when stimulated ex vivo by cognate tumor. However, they do not express perforin and other effector molecules and are small in size which is similar to that of resting T cells.^{12,13} In addition, it is possible that TAA-specific T cells are not produced at a high enough frequency to induce tumor regression since there is evidence that in the virus model T-cell frequency directly correlates with disease clearance or resurgence. Tissue sensitivity to CTLs may also be variable depending on the tumor, which may determine the frequency necessary for regression. Mouse studies have shown that immune response intensity directly correlates with tumor regression¹⁴ and that T-cell frequency directly correlates with number of immunizations administered. It has been reported that in order to reach a T-cell frequency comparable to that of acute infection, between 16 and 24 rounds of immunization might be necessary¹⁵ and in most cancer vaccine trials only a few rounds of immunization are administered, which may limit the ability or success of the vaccine. Longer immunization schedules could be beneficial, albeit in a population not requiring urgent palliative or therapeutic intervention. Moreover, although T cells seem to have the ability to reach the tumor site, recognize antigen and produce IFN- γ , they are not able to expand, nor limit tumor growth which is similar to the immune response in chronic viral infection in which CTLs are circulating but not eliminating virus.¹⁶ It is possible that secondary stimuli such as interleukin-2 (IL-2) may be required in order to activate CTL effector function. Although tumor escape mechanisms offer an attractive explanation for the lack of correlation between TAA-induced T cells and clinical regression, we are inclined to believe that this is probably not the case and that it is instead a result of T cells that are not adequately stimulated for killing.

Although the RECIST guidelines are useful to determine tumor shrinkage, this may not be the best suited method to measure success in the cancer vaccination setting. In a literature review of clinical ASI studies Mocellin et al⁸ report a response rate of tumor shrinkage in 10% of subjects, which would be even higher under RECIST guidelines however, this number does not correlate with clinical regression.¹⁷ This demonstrates that contrasting “tumor response” from RECIST with “patient response” from increased survival following immunization may be more useful and that solely using the same RECIST criteria regardless of therapy, type of cancer and stage of disease may be dangerous. In clinical cancer vaccine

trials, few have demonstrated robust responses satisfying RECIST guidelines, however prolonged survival has been observed as a measurable endpoint.¹⁸

Methods for Immune Monitoring following Active-Specific Immunization

Systemic Response

Developing a standardized method for immune monitoring of vaccine induced immune responses is of great importance for the development and evaluation of cancer vaccines. Standardization would allow vaccine study comparison between institutions however standardizing parameters and laboratory techniques among the large variety of cellular and molecular assays that are used to detect responses to vaccination is quite difficult.¹⁹ A vast array of techniques and assays are regularly employed to monitor the systemic immune response following ASI. Furthermore, it should be emphasized that the human biology is the independent variable and relevant clinical parameters should be easily reproducible independent of the assays if they are truly associated with a particular determinism. Thus, excessive emphasis on assay validation and cross-validation may be unwarranted when the biology evaluated is not clearly relevant to disease outcome or the phenomenon studied is poorly linked to a clinical parameter.

Limiting Dilution Assays (LDA)

Two types of limiting dilution assays (LDAs) are typically used to measure systemic activation of circulating T cells, one measures antigen-specific T-cell proliferation and the other T-cell ability to lyse labeled tumor cells. Antigen-induced clonal expansion is detected via radiolabel incorporation into DNA to measure expansion of CTL and helper T cells. Briefly, cells are incubated for approximately 5 days in the presence of soluble antigen and ³H-thymidine is added for several hours. DNA synthesis, which is the first response of cells to the mitogenic potential of the antigen, is determined by measuring radioisotope incorporation associated with the cells. As a positive control, lectin PHA can be added as a nonspecific T-cell activator. This assay is extensively used because the desired outcome of any vaccination protocol is the expansion of an antigen-specific T-cell population.²⁰ The other assay measures the lytic ability of CTLs and helper T cells that are measured through radio-labeled tumor cells. This assay is important in immunization immunology because it is assumed that the ability to lyse and kill tumor targets in vitro is similar to CTL killing ability in vivo, however this has been difficult to prove. Tumor lysis can occur via two methods (1) CTL release of lytic granules containing perforin and granzymes causing pore formation in target membrane followed by lysis and (2) through the Fas-Fas ligand apoptotic pathway. The chromium release assay (CRA) elucidates CTL function by measuring the amount of ⁵¹Chromium (Cr) released following lysis of labeled cells. Tumor target cells are labeled with ⁵¹Cr and mixed with T-lymphocytes. Target cells spontaneously release ⁵¹Cr slowly, so rapid ⁵¹Cr release demonstrates target cell lysis. In a similar colorimetric assay, MTT tetrazolium salt is hydrolyzed by viable cells to form a blue crystal measurable in a microtiter plate reader. Additionally, fluorimetric methods including MUH and AlamarBlue give more sensitive results when compared to the CRA method. Fluorimetric assays are attractive because they avoid radioisotope use, however they do have longer assay times and require the purchase of a microplate reader.²⁰ From a clinical standpoint, CRA has often

been used to monitor clinical immunization trials and to determine immunogenicity of tumor-related proteins however no correlation has been documented between antigen-specific proliferation and clinical outcome. In theory, vaccine-naïve patients with cancer or volunteers without cancer should not have detectable TAA-specific immunity. However, it has been reported in some cases that melanoma patients, as well as volunteers without cancer do have immune responses to melanoma differentiation antigens, making evaluation of the prevalence of immunity in the naive population important.^{21,22}

Enzyme-Linked Immunospot Assays (ELISPOT)

The enzyme-linked immunospot assay (ELISPOT) is based on the ELISA principles and was originally established to detect antibody-secreting cells and was later adapted to detect antigen-specific T cells and T-cell frequency. Practically speaking, a 96-well nitrocellulose-bottomed microtiter plate is coated with an antibody that traps a specific cytokine. Peripheral blood mononuclear cells (PBMCs), isolated CD8⁺ or CD4⁺ lymphocytes are incubated in the wells in the presence of an antigen for 6 to 48 hours. If cells respond to antigen, they will release said cytokine which is then bound by the antibody in close proximity to T cell in the well. Cells are then washed from the wells to visualize cytokine release by T-lymphocytes by an enzyme-labeled detection antibody and its chromogenic substrate that attaches to the well surface. The final product consists of colored spots in the wells; each spot corresponds to one cell secreting the candidate cytokine. IFN- γ production is often used as a read out for T-cell activity because it is not typically spontaneously secreted in unstimulated T cells that occur with other cytokines, such as TNF- α in a small fraction of cells. Various studies have used ELISPOT to measure tumor-reactive T-lymphocytes in peripheral blood of patients with tumors and data suggest that the assay is capable of detecting low frequency T-cell responses.²⁰ One advantage of using ELISPOT over assays such as LDAs is that it does not rely on cell proliferation which better reflects individual IFN- γ producing cells and functional state in vivo.¹⁹ Clinically, this method of immune monitoring has been useful in vaccination trials, most of which measured responses against peptides, melanoma cells, or idiotype protein in patients with myeloma.

Cytokine Flow Cytometry (CFC)

Cytokine flow cytometry (CFC) can be used to detect stimulated CD4⁺ and CD8⁺ T-lymphocytes at a low frequency following ex vivo stimulation with antigen by measuring intracellular IFN- γ as a surrogate of T-cell activation. CFC can be successfully performed with mononuclear cells obtained from PBMC,²³ whole blood,²⁴ lymph nodes or other biologic fluids.² Briefly, mononuclear cells are incubated for a total of 6 hours in presence of stimulating antigen to allow generation of high cytokine levels and for optimal cytokine staining. After 1 to 2 hours of stimulation, a cytokine secretion inhibitor such as brefeldin A is added to the culture. The cells are then fixed at 6 hours post stimulation, permeabilized and stained with monoclonal antibodies that recognize both surface and intracellular proteins to be characterized via flow cytometric analysis. Due to the short stimulation time, problems associated with increased culture times such as apoptosis and proliferation need not be addressed. As we have previously shown^{25–27} unstimulated lymphocytes and lymphocytes stimulated with irrelevant peptide do not exhibit cytokine secretion and thus background noise is rare. Moreover, super-antigens such as staphylococcal enterotoxin B can effectively

stimulate a large proportion of lymphocytes as a positive control.²⁶ In studies investigating T-cell activation in response to stimulation with CMV and EBV viral epitopes, CFC was reliable in producing measureable amounts of IFN- γ .^{25,26} Because tumor-specific T cells often produce less IFN- γ than virally stimulated T cells, they may be theoretically more difficult to detect using this method.^{20,28} However, studies have demonstrated that CFC is in fact sensitive enough to detect immune responses to tumor antigens in spite of the fact that IFN- γ frequencies are lower than when compared to infection.²⁹ Others report the need for in vitro sensitization for frozen and thawed samples, rather than direct ex vivo testing.³⁰ In addition to immune monitoring, CFC may also be a powerful tool in vaccine development by identifying novel TAA capable of eliciting immune responses.

Tetramer Analysis with Soluble Major Histocompatibility Complex (MHC)/Peptide Complexes

Soluble MHC/peptide tetramers can be produced that are conjugated to a fluorochrome and stably bind to a specific T-cell receptor (TCR). Tetramers can be generated for MHC class I CD8+ T-cell screening as well as for MHC class II CD4+ T-cell screening. Fluorescent MHC/peptide tetramers when incubated with a heterogeneous population of T cells will bind those T cells expressing MHC/peptide-specific TCRs which can then be detected by flow cytometric assays. This tool is useful in identification of antigen-specific CD8+ and CD4+ T cells in a polyclonal T-cell population and to generate information on T-cell functionality when combined with additional assays.^{19,20} Methodology for tetramer generation has been established and described.^{31–33} Tetramer analysis following vaccination has numerous advantages over some other methods. Tetramers allow cell enumeration without employing indirect functional assays in vitro and also allows cell sorting to isolate antigen-specific T cells which provides a source for TCR analysis and for cells targeted to adoptive transfer therapy. In addition, cellular phenotype can be obtained by using markers for activation status, costimulatory receptors, homing and others, while simultaneously staining for intracellular proteins to study T-cell stages of those responding to vaccination.¹⁹ Although tetramers are a powerful tool, they do have some limitations such as that MHC/peptide tetramers bind.

TCR with minimal avidity, which may allow some T cells of functional and clinical importance to be overlooked. In addition, some clinically important epitopes bind MHC with low affinity eliminating the possibility to produce an effective tetramer complex.

Quantitative Reverse Transcription-Polymerase Chain Reaction

Originally, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was developed to measure viral loads in patients and for monitoring viral infection following transplantation. Investigators at the National Cancer Institute Surgery Branch adapted this procedure for use in evaluating T-cell activation by measuring IFN- γ transcript levels in melanoma patients following ASI with peptide. Kammula et al.^{34,35} compared IFN- γ transcript levels in PBMC pre and postvaccination as well as in PBMC stimulated ex vivo with the relevant epitope. Data from this study found that the qRT-PCR results correlated well with results from HLA/peptide tetrameric complexes and intracellular CFC. This assay allows the investigation of gene expression of an unlimited number of genes from small

samples, such as FNAs, from a likewise minimal amount of candidate RNA. Because this is a sequence-based method, it also allows for investigation of any gene with a known sequence. Additionally, after cDNA is generated from the RNA of clinical samples, it can be stored safely for a long time for future analyses. This method also provides flexibility in that it can be applied to T-cell reactivity to whole proteins, protein mixtures, whole tumor cells without first determining relevant peptides/HLA restrictions.²⁰

Transcriptional Analysis of Circulating T Cells Following Active-Specific Immunization

In a transgenic mouse model Kaech et al¹² characterized time-dependent phenotypes of CD8⁺ T cells following acute exposure to antigen. One week following antigen exposure, expanding CD8⁺ T cells were found to be cytotoxic when tested ex vivo and had a transcriptional profile rich in effector function. In the following two weeks during the contraction phase, a memory phenotype was observed that responded to cognate stimulus measured by IFN- γ secretion, but was not capable of cytolytic activity and other effector functions. These findings correlate well with the TAA-specific immunization model in which a time-limited course of TAA-exposure is followed by a rest period of a few weeks. In this model, immunization induced T cells retain the effector phenotype (CD27, CCR7CD45RA^{high}) and IFN- γ responsiveness, however they cannot exert effector functions.^{1,36} Monsurro et al¹³ described this 'quiescent' phenotype through transcriptional profiling of an immunization-induced T-cell population not capable of exerting ex vivo cytotoxicity that was found to lack gene expression associated with T-cell activation, proliferation and effector function. This study was important because it demonstrated the significance of evaluating the functional status of vaccine-induced T cells at the global level pointing out that circulating T cells induced by vaccines do not have a phenotype of true effector cells and this finding may provide the most likely explanation for the lack of correlation between TAA-specific T-cell frequency in the blood and tumor regression.¹ Importantly, this study also demonstrated that transcriptional analysis of rare sub-populations of T cells can be performed using sorting procedures; further improvement in sorting technologies using high-speed FACS sorters has increased the yield and purity of such subsets allowing sophisticated distinctions among various circulating lymphocytes and subtleties about their interactions.^{27,37}

Tumor-Site Response, Tumor Microenvironment

The Immune Surveillance Hypothesis

Immune surveillance, as described by Wang et al is a hypothesis that may be useful in understanding spontaneous cancer rejection.³⁸ Although no direct way exists to test this hypothesis, it has been suggested that the immune system is in a constant battle in surveillance against neoplastic development.³⁹ Examples of spontaneous rejection that drove the hypothesis include Rosenberg's patient with gastric cancer who was found to be disease free years later⁴⁰ and studies that demonstrated a reduction in size or complete regression of renal cancer pulmonary metastases following primary tumor removal.^{41–43} In these instances, it is suggested that perhaps through immune surveillance, the host responded to a systemic presence of cancer. These occurrences support experimental evidence that demonstrates an increased prevalence of cancer in mice that lack immune

effector mechanisms such as IFN- γ production or are deficient in T-cell function.⁴⁴ Remarkable human examples occur within Epstein-Barr Virus (EBV)-induced lymphomas that are often observed during immunosuppression. These lymphomas are readily reversed both when immunosuppression is discontinued and when EBV-specific CTL are adoptively transferred to the patient.³⁸ Thus, there is sufficient evidence that innate and adaptive immune responses play a role in the modulation of the growth of at least a subset of cancers.

Transcriptional Profiling Immune Responses against Tumors

Although many studies have focused on measuring immune responsiveness by way of circulating peripheral T cells, it is also important to study the immune responses occurring in the target organ and in the tumor microenvironment. Studying the tumor microenvironment is relevant as it has been shown that cancer cells can significantly affect the surrounding environment,⁴⁵ some cancers are more sensitive to immune responses than others,^{46–48} immune responses for particular cancers may be predetermined⁴⁹ and it may provide insight into the effects of IL-2 on microenvironment.^{2,16,50–53} For example, the importance of tumor microenvironment has been observed during the evaluation of tumor-free peritoneum in patients with epithelial ovarian cancer. Alterations in surrounding tissue were observed that seemed to be a result of soluble factor secretion from tumor deposits that activated cell-cell interactions and adhesions as well as extra-cellular matrix modulation and growth.⁴⁸ Use of transcriptional profiling techniques on tumors as well on as surrounding tissues may be important in providing insight into the intricacies of tumor growth and persistence.

Use of transcriptional analysis at the level of tumor microenvironment has proven to be valuable. Initially, from transcriptional analysis of frozen tissue and cell lines, melanomas were thought to segregate into two molecular subclasses.⁵⁴ However, additional transcript analysis of melanoma lesions sampled by repeated FNAs have shown that what were thought to be subclasses are probably two phases of an evolving process that eventually leads to loss of gene expression associated with melanoma ontogeny.⁴⁹ As such, transcriptional analysis is beneficial in evaluating effects of ontogeny of molecular sub-classifications. This approach was used to compare profiles of normal kidney samples and primary cancers of varying histology to renal cell cancer (RCC) profiles and confirmed that the molecular basis of the subclasses correlates to the level of differentiation of individual cancers. When the genes that were co-expressed by normal kidney tissue were removed from analysis, RCC displayed the same profile as other cancers, demonstrating similarities in oncogenic processes.⁴⁸ In fact, use of transcriptional profiling allowed Wang et al⁴⁶ to elucidate molecular signatures for melanoma in which most melanoma-restricted immune-associated genes cluster tightly together including those genes associated with natural killer (NK) cell and activated CD8+ T-cell function. Moreover, a large cluster of genes is shared between melanoma and RCC; although the significance of these similarities should be investigated further.

IL-2 has been credited with inducing regression of both melanoma and metastatic RCC and is also thought to play a role in immune-mediated cancer regression. Because of this capability, Panelli et al investigated the transcriptional profile of FNA obtained from melanoma metastases before and during IL-2 therapy. In this study, it was observed that IL-2

does not cause migration, activation, or proliferation of T cells at tumor site, it does however induce a cytokine storm that is surged by monocytes and NK cells, mimicking acute inflammation. Monocytes and NK cells contribute to immune response by destroying cancer cells and taking up shed TAAs that are then presented to adaptive immune cells.⁵⁰ The transcriptional analysis of a lesion responding to IL-2 demonstrated gene activation that overlapped with those genes identified in the profile of TAA-specific T-cell activation in vitro.¹³ It is likely that IL-2 does not directly alter the tumor microenvironment, but that alterations are dependent on the downstream production of immune modulators by IL-2 stimulated cells, which then affect the microenvironment.^{51,55} In order to investigate the potential effect of the cytokine storm on intra-tumoral mononuclear phagocytes Wang et al³⁶ analyzed the profile of target cells following stimulation with panel of cytokines and identified two major cytokine classes capable of inducing classical and alternative mononuclear phagocyte activation. In summary, the study of circulating T-cell responses needs to be complemented by the study of functional signatures within the tumor microenvironment at time points relevant to their function. In particular, the dynamic phase of the immune response in which a switch is observed between a chronic inflammatory process conducive to cancer growth onto an acute one leading to cancer destruction needs to be studied by comparing circulating and peripheral immune responses.² Following this strategy we have recently proposed a model representative of this dynamic phase of the immune response which is relevant not only to tumor rejection but, more generally, to immune-mediated tissue-destruction in the context of allograft rejection, pathogen clearance and autoimmunity; we called this model “the immunologic constant of rejection”¹⁶ (see also next section). The immunologic constant of rejection predicts that tissue-specific destruction is mediated to activation of Type II interferon signatures inclusive of CXCL-9 to -11 and CCL5 chemokines, activation of cytotoxic T cells and Natural Killer cells with their localization, expansion and activation at the tumor site leading to high levels of expression of immune effector genes such as granzyme A and B, Perforin and FAS. Thus, immune responses switch during immune rejection from a quiescent circulating phenotype onto an activated effector natural-killer cell type within the target organ.^{1,16}

Conclusion

Although investigation into T-cell responses to cancer vaccines is far from complete, great progress has been made to begin understanding the complex interactions leading to cancer regression. Although vaccination against TAAs increases antigen-specific T cells in peripheral blood, this increase is not correlated with clinical regression, which sparked investigation into T-cell function following immunization. One explanation that may describe this phenomenon is immunoediting as a means of tumor escape from immune recognition.^{56–58} Alternatively, we and others have hypothesized that although tumor-specific T cells are induced by vaccination, their function is inadequate for tumor regression.^{59–61} This lack of function may result from various problems during tumor-host interactions including inadequate T-cell receptor (TCR) engagement with epitope, insufficient host costimulation, lack of T-cell localization to target tissue and the complexity of tumor-host interactions in the tumor environment resulting from varying tumor phenotypes and the immune mediators secreted into the microenvironment.⁶⁰ In order to

determine functional and genetic differences, Monsurro et al¹³ compared an antigen-specific subset of T-lymphocytes to properly functioning T-lymphocytes. The immunization-induced subset of cells was described as having a quiescent effector phenotype lacking proliferative and cytotoxic capabilities *ex vivo*. This phenotype was also characterized by down-regulation of genes important in T-cell activation, proliferation and effector function. In one clinical study, patients with Stage I-III melanoma were vaccinated with a modified gp100 peptide. Although in the majority of patients vaccinated induced high avidity, tumor-specific T cells, they were still found to be of low function in tumor lysis assays.⁶² Moreover, Chen et al⁶³ developed a high throughput array method using peptide/MHC complexes with antibodies against secreted factors to capture T-lymphocyte secreted cytokines. This methodology is useful for characterization of antigen-specific CD8+ T-lymphocyte functionality in clinical samples following vaccination and may be useful in correlating lymphocyte function to clinical outcome. The clinical samples from ten melanoma patients vaccinated with a gp100 peptide evaluated in this study displayed distinct differences in cytokine secretion profiles both in patient-specific and antigen-specific CD8+ lymphocytes, demonstrating the variability in T-cell function following vaccination.

Another point that has been elucidated in recent years is that TAA-specific T cells must not only be induced following immunization, they must be active and functioning at the tumor site. We propose that T-cell function and ultimately tissue destruction in cancer may occur through a route similar to other pathological processes such as infection, allograft rejection and autoimmunity and suggest that an “immunological constant of rejection” may exist as the common mechanism for these disease processes. Transcriptional profiling studies revealed that this immunological constant includes activation of interferon stimulated genes (ISGs) and immune effector functions (IEFs).¹⁶ Sarwal et al⁶⁴ studied the basis of acute rejection in kidney allografts and identified ISGs, granzymes, B and T-cell signature. Similarly, studies regarding immune-mediated melanoma metastasis rejection during IL-2 therapy demonstrated activation of ISGs, granzymes as well as transcripts for activated CTL and NK cells.^{49,50,52} Based on these results, we hypothesized that the last step in the pathway leading to cancer rejection is broad activation of cytotoxic mechanisms by innate or adaptive immune cells. This hypothesis was studied in the Imiquimod-mediated rejection of basal cell carcinoma (BCC), in which rejection was associated with expression of ISGs, IEFs, IFN- α , IFN- γ and infiltration of CTL and NK cells, with a complete lack of B-cell involvement.⁵² These studies, among others, have demonstrated the association of Type I pro-inflammatory modulators, especially IFN- α and IFN- γ with tissue-specific destruction. Because this activation is present in many chronic inflammatory conditions, it alone is unlikely to be adequate for tumor or tissue rejection, but in combination with additional immune responders may recruit CTLs and initiate a cascade leading to rejection.¹⁶

References

1. Monsurro V, Wang E, Panelli MC, et al. Active-specific immunization against melanoma: is the problem at the receiving end? *Sem Cancer Biol.* 2003; 13:473–80.
2. Mantovani A, Romero P, Palucka AK, et al. Tumor immunity: effector response to tumor and the influence of the microenvironment. *Lancet.* 2008; 371(9614):771–83. [PubMed: 18275997]

3. Parmiani G, Castelli C, Dalerba P, et al. Cancer immunotherapy with peptide-based vaccines: what have we achieved? Where are we going? *J Natl Cancer Inst.* 2002; 94(11):805–18. [PubMed: 12048268]
4. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. *Nat Med.* 2004; 10(9):909–15. [PubMed: 15340416]
5. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst.* 2000; 92(3): 205–16.
6. Therasse P, Eisenhauer EA, Verweij J. RECIST revisited: a review of validation studies on tumour assessment. *Eur J Cancer.* 2006; 42(8):1031–9. [PubMed: 16616487]
7. Therasse P, Eisenhauer EA, Buyse M. Update in methodology and conduct of cancer clinical trials. *Eur J Cancer.* 2006; 42(10):1322–30. [PubMed: 16737813]
8. Mocellin S, Mandruzzato S, Bronte V, et al. Correspondence 1: Cancer vaccines: pessimism in check. *Nat Med.* 2004; 10(12):1278–9. [PubMed: 15580242]
9. Dudley ME, Wunderlich JR, Robbins PF, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science.* 2002; 298(5594):850–4. [PubMed: 12242449]
10. Marincola FM, Jaffe EM, Hicklin DJ, et al. Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. *Adv Immunol.* 2000; 74:181–273. [PubMed: 10605607]
11. Speiser DE, Miranda R, Zakarian A, et al. Self antigens expressed by solid tumors Do not efficiently stimulate naive or activated T-cells: implications for immunotherapy. *J Exp Med.* 1997; 186(5):645–53. [PubMed: 9271580]
12. Kaech SM, Hemby S, Kersh E, et al. Molecular and functional profiling of memory CD8 T-cell differentiation. *Cell.* 2002; 111:837–51. [PubMed: 12526810]
13. Monsurro V, Wang E, Yamano Y, et al. Quiescent phenotype of tumor-specific CD8₊ T-cells following immunization. *Blood.* 2004; 104(7):1970–8. [PubMed: 15187028]
14. Perez-Diez A, Spiess PJ, Restifo NP, et al. Intensity of the vaccine-elicited immune response determines tumor clearance. *J Immunol.* 2002; 168:338–47. [PubMed: 11751979]
15. Monsurro V, Nielsen M-B, Perez-Diez A, et al. Kinetics of TCR use in response to repeated epitope-specific immunization. *J Immunol.* 2001; 166:5817–25. [PubMed: 11313426]
16. Wang E, Worschech A, Marincola FM. The immunologic constant of rejection. *Trends Immunol.* 2008; 29(6):256–62. [PubMed: 18457994]
17. Mocellin S, Mandruzzato S, Bronte V, et al. Part I: Vaccines for solid tumours. *Lancet Oncol.* 2004; 5(11):681–9. [PubMed: 15522656]
18. Schlom J, Arlen PM, Gulley JL. Cancer vaccines: moving beyond current paradigms. *Clin Cancer Res.* 2007; 13(13):3776–82. [PubMed: 17606707]
19. Speiser DE, Pittet MJ, Rimoldi D, et al. Evaluation of melanoma vaccines with molecularly defined antigens by ex vivo monitoring of tumor specific T-cells. *Semin Cancer Biol.* 2003; 13(6): 461–72.
20. Keilholz U, Weber J, Finke J, et al. Immunologic monitoring of cancer vaccine therapy: results of a Workshop sponsored by the Society of Biological Therapy. *J Immunother.* 2002; 25(2):97–138. [PubMed: 12074049]
21. Marincola FM, Rivoltini L, Salgaller ML, et al. Differential anti-MART-1/MelanA CTL activity in peripheral blood of HLA-A2 melanoma patients in comparison to healthy donors: evidence for in vivo priming by tumor cells. *J Immunother.* 1996; 19(4):266–77.
22. D'Souza S, Rimoldi D, Lienard D, et al. Circulating Melan-A/Mart-1 specific cytolytic T-lymphocyte precursors in HLA-A2₊ melanoma patients have a memory phenotype. *Int J Cancer.* 1998; 78(6):699–706. [PubMed: 9833762]
23. Picker LJ, Singh MK, Zdraveski Z, et al. Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T-cells by flow cytometry. *Blood.* 1995; 86(4):1408–19. [PubMed: 7632949]

24. Suni MA, Picker LJ, Maino VC. Detection of antigen-specific T-cell cytokine expression in whole blood by flow cytometry. *J Immunol Methods*. 1998; 212(1):89–98. [PubMed: 9671156]
25. Provenzano M, Selleri S, Jin P, et al. Comprehensive epitope mapping of the Epstein-Barr virus latent membrane protein-2 in normal, non tumor-bearing individuals. *Cancer Immunol Immunother*. 2007 Jul; 56(7):1047–63. [PubMed: 17124584]
26. Slezak SL, Bettinotti M, Selleri S, et al. CMV pp65 and IE-1 T-cell epitopes recognized by healthy subjects. *J Transl Med*. 2007; 5:17. [PubMed: 17391521]
27. Selleri S, Deola S, Pos Z, et al. GM-CSF/IL-3/IL-5 receptor common B chain (CD131) as a biomarker of antigen-stimulated CD8₊ T-cells. *J Transl Med*. 2008 in press.
28. Keilholz U, Martus P, Scheibenbogen C. Immune monitoring of T-cell responses in cancer vaccine development. *Clin Cancer Res*. 2006; 12(7 Pt 2):2346s–52s. [PubMed: 16609057]
29. Nielsen M-B, Monsurro V, Miguelse S, et al. Status of activation of circulating vaccine-elicited CD8₊ T-cells. *J Immunol*. 2000; 165(4):2287–96. [PubMed: 10925318]
30. Bercovici N, Haicheur N, Massicard S, et al. Analysis and characterization of antitumor T-cell response after administration of dendritic cells loaded with allogeneic tumor lysate to metastatic melanoma patients. *J Immunother*. 2008; 31(1):101–12. [PubMed: 18157017]
31. Altman JD, Moss PA, Goulder PR, et al. Phenotypic analysis of antigen-specific T-lymphocytes [published erratum appears in *Science* 1998; 280(5371):1821]. *Science*. 1996; 274(5284):94–6. [PubMed: 8810254]
32. Lee K-H, Wang E, Nielsen M-B, 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; 163:6292–300. [PubMed: 10570323]
33. Bettinotti MP, Panelli MC, Ruppe E, et al. Clinical and immunological evaluation of patients with metastatic melanoma undergoing immunization with the HLA-C2*0702 associated epitope MAGE-A12:170–178. *Int J Cancer*. 2003; 105:210–6. [PubMed: 12673681]
34. Kammula US, Lee K-H, Riker A, et al. Functional analysis of antigen-specific T-lymphocytes by serial measurement of gene expression in peripheral blood mononuclear cells and tumor specimens. *J Immunol*. 1999; 163:6867–79. [PubMed: 10586088]
35. 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(16):1336–44. [PubMed: 10944556]
36. Wang E, Panelli MC, Marincola FM. Gene profiling of immune responses against tumors. *Curr Opin Immunol*. 2005; 17(4):423–7. [PubMed: 15950448]
37. Deola S, Panelli MC, Maric D, et al. “Helper” B-cells promote cytotoxic T-cell survival and proliferation independently of antigen presentation through CD27-CD70 interactions. *J Immunol*. 2008; 130(3):1362–72.
38. Wang E, Selleri S, Sabatino M, et al. Spontaneous and tumor-induced cancer rejection in humans. *Exp Opin Biol Ther*. 2008; 8(3):337–49.
39. Burnet FM. The concept of immunological surveillance. *Prog Exp Tumor Res*. 1970; 13:1–27. [PubMed: 4921480]
40. Barry JM, Rosenberg SA. The transformed cell: unlocking the mysteries of cancer. 1st. New York, NY: G.P. Putnam's Sons; 1992.
41. Davis SD, Koizumi JH, Pitts WR. Spontaneous regression of pulmonary metastases from renal cell carcinoma. *Urology*. 1989; 33(2):141–4. [PubMed: 2916288]
42. Lokich J. Spontaneous regression of metastatic renal cancer. Case report and literature review. *Am J Clin Oncol*. 1997; 20(4):416–8. [PubMed: 9256902]
43. Sanchez-Ortiz RF, Tannir N, Ahrar K, et al. Spontaneous regression of pulmonary metastases from renal cell carcinoma after radio frequency ablation of primary tumor: an in situ tumor vaccine? *J Urol*. 2003; 170(1):178–9. [PubMed: 12796677]
44. Shankaran V, Ikeda H, Bruce AT, et al. IFN- γ and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature*. 2001; 410:1107–11. [PubMed: 11323675]
45. Wang E, Ngalame Y, Panelli MC, et al. Peritoneal and sub-peritoneal stroma may facilitate regional spread of ovarian cancer. *Clin Cancer Res*. 2005; 11(1):113–22. [PubMed: 15671535]

46. Wang E, Panelli MC, Zavaglia K, et al. Melanoma-restricted genes. *J Transl Med.* 2004; 2:34. [PubMed: 15488140]
47. Mandruzzato S, Callegaro A, Turcatel G, et al. A gene expression signature associated with survival in metastatic melanoma. *J Transl Med.* 2006; 4:50. [PubMed: 17129373]
48. Wang E, Lichtenfels R, Bukur J, et al. Ontogeny and oncogenesis balance the transcriptional profile of renal cell cancer. *Cancer Res.* 2004; 64(20):7279–87. [PubMed: 15492247]
49. Wang E, Miller LD, Ohnmacht GA, et al. Prospective molecular profiling of subcutaneous melanoma metastases suggests classifiers of immune responsiveness. *Cancer Res.* 2002; 62:3581–6. [PubMed: 12097256]
50. Panelli MC, Wang E, Phan G, et al. Gene-expression profiling of the response of peripheral blood mononuclear cells and melanoma metastases to systemic IL-2 administration. *Genome Biol.* 2002; 3(7) RESEARCH0035.
51. Panelli MC, Martin B, Nagorsen D, et al. A genomic and proteomic-based hypothesis on the eclectic effects of systemic interleukin-2 administration in the context of melanoma-specific immunization. *Cells Tissues Organs.* 2003; 177(3):124–31.
52. Panelli MC, Stashower M, Slade HB, et al. Sequential gene profiling of basal cell carcinomas treated with Imiquimod in a placebo-controlled study defines the requirements for tissue rejection. *Genome Biol.* 2006; 8(1):R8.
53. Wang E, Marincola FM. Bottom up: a modular view of immunology. *Immunity.* 2008 in press.
54. Bittner M, Meltzer P, Chen Y, et al. Molecular classification of cutaneous malignant melanoma by gene expression: shifting from a continuous spectrum to distinct biologic entities. *Nature.* 2000; 406:536–840. [PubMed: 10952317]
55. Panelli MC, White RL Jr, Foster M, et al. Forecasting the cytokine storm following systemic interleukin-2 administration. *J Transl Med.* 2004; 2:17. [PubMed: 15175100]
56. Dunn GP, Bruce AT, Ikeda H, et al. Cancer immunoediting: from immunosurveillance to tumor escape. *Nature Immunol.* 2002; 3(11):991–8. [PubMed: 12407406]
57. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. *Annu Rev Immunol.* 2004; 22:329–60. [PubMed: 15032581]
58. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity.* 2004; 21(2):137–48. [PubMed: 15308095]
59. Khong HT, Restifo NP. Natural selection of tumor variants in the generation of “tumor escape” phenotypes. *Nature Immunol.* 2002; 3(11):999–1005. [PubMed: 12407407]
60. Marincola FM, Wang E, Herlyn M, et al. Tumors as elusive targets of T-cell-based active immunotherapy. *Trends Immunol.* 2003; 24(6):335–42. [PubMed: 12810110]
61. Marincola FM. A balanced review of the status of T-cell-based therapy against cancer. *J Transl Med.* 2005; 3(1):16. [PubMed: 15831096]
62. Meijer SL, Dols A, Jensen SM, et al. Induction of circulating tumor-reactive CD8₊ T-cells after vaccination of melanoma patients with the gp100 209-2M peptide. *J Immunother.* 2007; 30(5): 533–43. [PubMed: 17589294]
63. Chen DS, Soen Y, Stuge TB, et al. Marked differences in human melanoma antigen-specific T-cell responsiveness after vaccination using a functional microarray. *PLoS Med.* 2005; 2(10):e265. [PubMed: 16162034]
64. Sarwal M, Chua MS, Kambham N, et al. Molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling. *N Engl J Med.* 2003; 349(2):125–38. [PubMed: 12853585]