GUEST COMMENTARY

Monitoring Specific T-Cell Responses to Melanoma Vaccines: ELISPOT, Tetramers, and Beyond

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The molecular identification of antigens expressed preferentially in tumor cells has enabled the development of antigen-specific vaccines against human cancer. The majority of human tumor antigens characterized to date have been isolated from melanoma cells based on class I major histocompatibility complex (MHC)-restricted recognition by CD8+ T cells (3, 16). However, tumor antigen peptides presented by class II MHC molecules recognized by CD4+ T cells also have recently been described (18). The availability of defined antigens has motivated clinical trials utilizing epitope-based rather than whole tumor cell-based vaccination strategies. Various vaccine approaches have been explored in murine preclinical models, and many of these have already entered human clinical trials. These include tumor antigen peptides mixed in saline or in lipid-containing adjuvants, peptides combined with immunomodulatory cytokines, peptide-loaded antigen-presenting cells (APCs), recombinant viruses, recombinant bacteria, and naked recombinant plasmid DNA (reviewed in reference 8). Inasmuch as the optimal vaccination strategy for inducing specific CD8+ cytotoxic T-lymphocyte (CTL) differentiation in vivo is not known, exploration of these myriad strategies for use in cancer patients is warranted.

Clinical testing of new therapies in cancer patients has traditionally involved phase I studies to determine the maximally tolerated dose of the new agent based on acceptable toxicity, followed by disease-specific phase II studies to ascertain the clinical response rate. However, with few exceptions, the clinical activity of new agents is modest, particularly in the heavily pretreated patient populations referred to academic medical centers. The potential reasons why some patients respond clinically to a new agent and others do not are explored infrequently, so that it often is not understood whether the desired biologic effect of the therapeutic intervention has been achieved. In addition, it is assumed that the maximally tolerated dose of an agent based on acceptable toxicity is also the optimal dose for the expected biologic activity, which may not be the case. Therefore, dose escalation in phase I studies until clinically significant toxicity is observed may not be appropriate for determining the recommended phase II dose for new biologic agents. Because of these ambiguities, there is growing interest in clinical trial design aimed toward measuring scientific intermediate end points to determine whether the anticipated biologic effect of the intervention has been achieved, irrespective of clinical efficacy. Synergistic activity with other therapies could then be investigated in subsequent studies using a confirmed biologically active dose and schedule. Clinical trials exploring active-specific cancer immunotherapy have led the way in the measurement of biologic intermediate end points.

Immunologic monitoring in cancer vaccine clinical trials is a first essential step toward understanding the mechanisms that govern clinical efficacy versus lack of response. As most defined melanoma antigen peptides are presented by class I MHC molecules, a measure of CD8+-T-lymphocyte activation is generally assessed. Induction of new T-cell responses in cancer patients is complicated by the generalized and possibly tumor-specific immunosuppressed state that has been observed in the setting of established malignancy (14). Thus, immunization to generate new tumor antigen-specific T cells might not be achievable in every patient. If T-cell priming is successfully induced, additional hurdles may lie in attaining expansion to sufficient numbers, long-term survival in the circulation, trafficking into tumor sites, and survival in the hostile tumor microenvironment, as well as in tumor cell escape from immunity-mediated destruction. Each of these potential defects that may mediate resistance to a T-cell-based cancer vaccine also is measurable and, if commonly observed, could potentially be overcome through additional therapeutic interventions.

TECHNIQUES FOR MONITORING CD8+-T-CELL RESPONSES

There are at least six in vitro assays and two in vivo assays that have been investigated to various degrees as potential measures of the presence of antigen-specific CD8+ T cells (Table 1). Although discussed here in the context of melanoma vaccines, these same techniques can be applied toward monitoring patients with other cancers, infectious diseases, or autoimmunity, provided that relevant antigens have been identified. Assays that are performed in vitro include limiting-dilution analysis (LDA) of cytolytic activity; antigen-specific proliferation, cytokine production, or cytolytic activity by bulk T-cell cultures; ELISPOT analysis of antigen-specific cytokine release; or flow cytometric staining with soluble peptide-MHC multimers, with or without cell sorting and functional analysis of positive cells. Tests performed in vivo include measuring delayed-type hypersensitivity (DTH) or examining the lymphocytes infiltrating vaccine sites (vaccine-induced lymphocytes). Each of these techniques offers potential advantages and disadvantages, and some have been utilized in cancer vaccine clinical trials already. Although the most appropriate T-cell assay for predicting the clinical efficacy of a cancer vaccine strategy is not yet defined, there is excitement in the field regarding the utilization of peptide-MHC multimers to detect
the presence of specific T cells in conjunction with functional assays to characterize those cells. A brief overview of these various immunologic techniques follows.

The LDA assay for CTL activity involves establishing cultures of T cells at various cell concentrations, along with a source of antigen, a syngeneic stimulator cell, and interleukin-2 (IL-2) (6). T cells can be unfraccionated or purified CD8 T cells; the stimulator cell may be a specific autologous tumor cell line or an autologous APC population loaded with the antigen of interest. Following a culture period for cell expansion, lysis is measured using a chromium release assay and specific versus control targets. By comparing the fractions of cultures giving rise to lytic activity at each cell dilution, a frequency of antigen-specific cells is obtained. The advantage of this assay is that it provides information regarding both quantity and quality, that is, the frequency of specific cells and a function of those cells, i.e., cytolytic activity. The disadvantages are that it is very cumbersome, involving thousands of microwell cultures, and that it depends on T-cell proliferation in vitro. Expansion of T cells in vitro with IL-2 may alter the functional phenotype of the cells, yielding results that may not accurately reflect the immune status of the subject in vivo. This caveat applies to several other assays described below.

In a second type of assay, bulk cultures of T cells isolated from the peripheral blood can be stimulated in vitro for a brief culture period to expand antigen-specific T cells of interest, followed by one of three functional analyses: antigen-specific proliferation, cytokine production, or cytolyis. The starting T cells again can be unfraccionated or purified CD8 T cells, and the stimulator cell population can be an autologous tumor cell line or peptide-loaded autologous APCs. Some investigators have utilized whole peripheral blood mononuclear cells mixed with a specific antigenic peptide plus IL-2, without purifying the stimulator and responder cell populations (4). This technique is much more rapid and straightforward than LDA and offers the possibility of performing three or more functional assays with the T cells expanded in the bulk culture. The disadvantages are that information regarding frequency is lost, making the assay less quantitative, and that in vitro cell expansion is required. A reflection of frequency can be regained if cytokine production is assessed using intracellular staining with anticytokine antibodies (Abs) and flow cytometric analysis. The ELISPOT assay was developed, in part, to combine metric analysis with high-speed cell sorting, functional assays can also be performed on sorted cells without in vitro expansion. In addition, by coupling flow cytometric analysis with high-speed cell sorting, functional assays can also be performed on sorted cells without in vitro expansion. The disadvantages are that it requires specialized imaging equipment for efficient use, may yield ambiguous results due to variability in the size of each spot in addition to the number of spots, and might not detect T cells that are present but nonfunctional.

A final in vitro monitoring technique utilizes specific peptide-MHC multimers that are used as a labeling reagent to identify antigen-specific T cells by flow cytometry. Two types of constructs have been employed, the first consisting of recombinant class I MHC molecules rendered tetrameric via conjugation to biotin and the second consisting of class I MHC molecules synthesized as a fusion protein with an immunoglobulin heavy-chain molecule, thus creating a flexible dimer. Each of these can be loaded with specific peptide, forming a ligand for T-cell receptors that recognize that peptide-MHC complex and allowing detection of those T cells by flow cytometry (1, 9, 10). The advantages of this approach are that it can assess the frequency of specific cells, can allow analysis of additional surface markers, and in many cases can detect relevant T cells without in vitro expansion. In addition, by coupling flow cytometric analysis with high-speed cell sorting, functional assays can also be performed on sorted cells without in vitro expansion. The disadvantages are that it requires a specialized reagent for every MHC molecule of interest and that the reagents preferentially bind to high-affinity T-cell receptors. Nevertheless, the most informative results to date have been obtained using this technology.

### PRELIMINARY EXPERIENCE WITH PEPTIDE-MHC MULTIMERS IN MELANOMA PATIENTS

Specific CTLs against the dominant HLA-A2-binding MELAN-A/MART-1 peptide have been detected at baseline in both normal individuals and patients with melanoma by using LDA (7). This apparently paradoxical observation has been explored more recently using MELAN-A-HLA-A2 tetramer. Interestingly, the MELAN-A-specific T cells in normal
individuals displayed a naive surface phenotype, whereas those from melanoma patients were CD45RAlo/ROhi (15), suggesting that a degree of activation had occurred in the melanoma-bearing individuals. Functional assays were also done with specimens from some subjects. LDA revealed CTL activity in both normal individuals and melanoma patients, although the frequency of positive cells was significantly underestimated compared to results obtained by tetramer binding. This result might be expected, because the LDA technique involves in vitro expansion, which may itself promote acquisition of lytic activity. In contrast, an ELISPOT assay for IFN-γ-producing cells revealed an absence of specific cells in the vast majority of subjects. Collectively, these results suggest that peptide-MHC tetramer staining can allow detection of both naive and antigen-experienced specific T cells but that functional assays have the potential to reveal whether effector activity is intact in the context of an established tumor.

A second report analyzed the frequency of MELAN-A-HLA-A2 tetramer-positive T cells from eight melanoma patients following in vitro expansion with peptide-loaded T2 cells (2). Frequencies of between 1/100,000 and 1/1,400 were observed after in vitro expansion. In three patients, T cells also were isolated from metastatic tumor deposits and were found to be present based on MELAN-A-HLA-A2 tetramer staining. These data suggest that CTL precursors can be present both in the blood and within the tumor site in patients with melanoma without leading to tumor regression, indicating that intrinsic tumor cell resistance to recognition or lysis may contribute to a failed antitumor immune response. However, as this study analyzed T-cell function after in vitro expansion, the possibility of a T-cell-unresponsive state in vivo overcome by restimulation in vitro cannot be excluded.

A third recent study analyzed the presence of MELAN-A- and tyrosinase-specific T cells by tetramer staining in 11 patients with stage III or stage IV melanoma (11). Positive cells were seen in six of those individuals, at frequencies of 0.014 to 2.2%. The characteristics of the tyrosinase–HLA-A2+ cells from the patient with the highest frequency were analyzed further. Interestingly, these cells had a mixed surface phenotype, being CD45RA+ and CD44lo (like naive cells) but CD11ahi, CD57hi, and intracellular perforin+ (like differentiated effector cells). In addition, positive sorting of those cells followed by direct functional analysis revealed a failure to produce the cytokines IFN-γ and tumor necrosis factor alpha, as well as a failure to lyse tyrosinase-expressing target cells. These results support the utility of peptide-MHC tetramer staining for identifying and characterizing melanoma antigen-specific T-cell populations and suggest that specific T-cell unresponsiveness might be induced in melanoma patients in vivo.

EXAMPLES OF MONITORING IN RECENT MELANOMA VACCINE STUDIES

The results of several pilot clinical trials exploring melanoma vaccines have recently been published, with a fraction of patients experiencing clinical benefit. Peptide-MHC multimers have not yet been widely used to characterize augmented immunity in response to vaccination. Although the assays that have been used to date have revealed useful information, there is no clear consensus on which technique for monitoring CD8+ T-cell responses might predict clinical response. The lack of correlation could be due to the inferiority of the immunologic assays employed or because the vaccine strategies explored to date have been suboptimal.

Vaccination with the MAGE-3 peptide presented by HLA-A1 mixed only in saline was performed with 39 patients with advanced melanoma, led by the group of Thierry Boon. Tumor responses were observed in seven individuals, most of whom had in transit metastases. Analysis of the immune response was performed with several patients by using a modified LDA technique. However, no increase in CTL precursor frequency was detected following immunization in any patient (12).

Several studies by Rosenberg and colleagues have investigated immunization with melanoma antigen peptides emulsified in incomplete Freund’s adjuvant. One trial utilized a peptide derived from MELAN-A/MART-1; no clinical responses were seen. T-cell activity was analyzed in 18 patients by using IFN-γ production or cytolytic activity measured from bulk cultures after in vitro expansion. Evidence of T-cell priming was seen in most patients despite a lack of clinical activity (5). In a second study, immunization with wild-type or mutated gp100 peptide was performed. No patients immunized with peptide alone experienced a clinical response despite the fact that the majority of individuals were successfully immunized based on detection of IFN-γ-producing T cells. However, in a second study cohort, patients were immunized with gp100 followed by treatment with IL-2. In this case, 13 of 31 patients displayed a clinical response, but only a minority showed the presence of peptide-specific T cells in the peripheral blood (17).

Monocyte-derived dendritic cells pulsed with melanoma peptides or tumor lysate, along with keyhole limpet hemocyanin as a helper antigen, were administered intralymphatically to 16 patients with melanoma. Five patients showed evidence of a clinical response. Evidence for T-cell priming was demonstrated in 11 patients by using DTH responses and examination of whether CD8+ T cells were recruited into the DTH challenge site (13). It is likely, however, that CD4+ T cells were largely responsible for initiating the DTH response.

The group at the University of Pittsburgh recently completed two melanoma vaccine clinical trials, one using MELAN-A, gp100, or tyrosinase peptide mixed in MF59 adjuvant and the other using dendritic cells loaded with five different peptides and administered intravenously (2a). One clinical response was seen among the 25 patients treated in the dendritic cell trial. T-cell monitoring was performed using a modified ELISPOT assay (2a). The assay conditions were developed carefully to confirm the reliability of the technique. With an antigen-specific CTL line as a standard, 1 CTL among 100,000 irrelevant peripheral blood lymphocytes was detected, and ELISPOT was found to give frequencies similar to those obtained with the more labor-intensive LDA technique. When clinical material was then analyzed, the ELISPOT was not sensitive enough to detect melanoma peptide-specific T cells in the majority of patients. Enriching for CD8+ cells as responders or resorting to one or two rounds of in vitro expansion appeared to increase the fraction of patients with detectable peptide-specific T cells. However, in only a few cases did the frequency of specific T cells increase in the postvaccination sample. It is likely either that the assay was insufficiently sensitive, that a T-cell-unresponsive state existed in these cancer patients in vivo, or that the immunization strategy employed was not powerful enough to augment T-cell responses to a detectable level.

We reasoned that a highly sensitive T-cell assay may not be as important as a more potent vaccine strategy to augment T-cell responses to the point of being detectable using current techniques. We recently completed a phase I study of immunization with MELAN-A or MAGE-3 peptide-loaded autologous peripheral blood mononuclear cells plus recombinant human IL-12, both delivered subcutaneously. By using a short in vitro expansion of CD8+ T cells and measurement of IFN-γ
CONCLUSIONS AND FUTURE PROSPECTS

Results from melanoma vaccine clinical trials obtained to date have not allowed determination of the ideal assay for verifying successful induction of tumor antigen-specific CD8+ T-cell responses. Some studies have shown clinical activity but no detectable CTL generation, while others have shown detectable T-cell priming without a clinical response. It is likely that these discrepancies are the result of several factors, including suboptimal vaccine strategies, varying assay sensitivities, and immunosuppression in the setting of cancer. The fact that primed tumor-reactive T cells can be detected in some cases without tumor regression argues that downstream factors may be important, such as T-cell survival in the tumor microenvironment and tumor cell escape through down-regulation of class I MHC and of antigen-processing machinery. The greatest amount of information regarding both the frequency of melanoma antigen-specific T cells and phenotypic and functional characteristics has been obtained using peptide-MHC tetramers. A comparison of biotin-based tetramers to immunoglobulin-based dimers has not yet been performed. The coupling of flow cytometry using peptide-MHC multimers with high-speed cell sorting and functional analysis of positive T cells without in vitro expansion should be explored in future studies of melanoma vaccines. In addition, increased attention should be given to defects that may lie downstream from T-cell priming, such as inhibition of effector function in the tumor microenvironment and tumor cell resistance to T-cell destruction.

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