
Immune Monitoring of T-Cell Responses in Cancer Vaccine Development

Ulrich Keilholz, Peter Martus, and Carmen Scheibenbogen

Abstract Monitoring cellular immune responses is one prerequisite for rational development of cancer vaccines. The primary objective of immune monitoring is to determine the efficacy of a vaccine to induce or augment a specific T-cell response. Further questions relate to the prevalence and functional relevance of spontaneous tumor-directed immune responses, the functional characteristics of T-cell responses, and, finally and most importantly, the relationship between immune monitoring assay results and clinical end points. The issue of T-cell monitoring has become more complex as different types and generations of assays have been adopted during the past decade and both standardization and validation of assays have often been insufficient. Because the development of assays parallel the clinical development of cancer vaccines, technical advances have been achieved simultaneously with broadening understanding of cancer immunity. Suitable animal models for immune monitoring are, however, lacking, because preclinical vaccine development in rodents does not allow serial immune monitoring of the peripheral blood, as is commonly used in patients. The current situation is characterized by a lack of universal standards for T-cell assessment, uncertainty about the association between immune monitoring assay results and clinical antitumor end points, and lack of knowledge regarding the contribution of different aspects of T-cell function to clinical efficacy. It is acknowledged that T-cell monitoring will have to be validated in large trials with clinically effective vaccines, but this necessity should not discourage the current application of novel assays within clinical trials of all stages.

In principle, there is general agreement that immune monitoring should guide the development of cancer vaccines. In early clinical trials, immune monitoring should guide the selection of vaccine preparations and schedules for advanced clinical testing (vaccine potency assessment), and in phase 3 clinical trials immune monitoring should define surrogate markers for clinical efficacy that will allow bridging of studies over time, in case vaccine preparations or production methods are modified. However, there are a number of limitations that need to be acknowledged.

One limitation is that in the clinical setting, only the peripheral blood compartment is generally accessible for serial analyses. Suitable animal models for immune monitoring are lacking because most preclinical vaccine development is done in small rodents, which are unsuitable for serial monitoring of peripheral blood or tumor compartments. Therefore, preclinical models cannot guide us regarding the suitability and the

type and magnitude of peripheral blood T-cell responses that may predict the clinical efficacy of a vaccine.

The lack of a successful cancer vaccine as a clinical paradigm further impedes development. Currently, the objective response rate in few clinical studies is >10%, preventing meaningful correlations of T-cell response rates with clinical responses (1). The lack of knowledge regarding the magnitude and type of T-cell responses required to achieve clinical effect has held back the field of cancer vaccines. More recently, induction of cancer antigen-specific T cells by peptide-based vaccination has been shown in most patients with minimal residual disease or limited tumor burden who were vaccinated in a series of trials, and there is evidence that vaccination may prevent relapses in high-risk patients (2–5). New potent adjuvants and immunostimulatory molecules have raised optimism about improvement in efficacy of therapeutic vaccination (6, 7).

Further, the contribution of individual aspects of immune function and of the more recently defined T-cell subsets are unknown (8). Based on the current knowledge, it can be assumed that immune protection against cancer is not a single-variable or single-compartment end point, but likely a balanced mosaic of immune responses of both cellular and humoral effectors. The capability of current flow cytometry to deliver multiparametric data enables a new biomarker-based approach for monitoring multiple markers of immune reactions, which will hopefully enhance our understanding of immune reactions associated with successful immunotherapy. Thus, we have to accept the challenges of the field when moving forward and

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selecting cancer vaccines for advanced clinical testing. This article summarizes advances in T-cell monitoring and discusses experimental strategies and statistical requirements for addressing the basic questions in vaccine development within clinical trials.

Three Generations of Assays Measuring T-Cell Responses

The first generation of assays capable of detecting immune responses was developed several decades ago, and included the proliferation assay in response to antigen exposure and the chromium release assay for cytotoxicity (Fig. 1). Both assays assess the T-cell response at the level of the entire cell population in the culture, thus requiring prior *in vitro* expansion. Therefore, the ability to detect T-cell responses is based on the proliferative potential of the cells, and the results are influenced by the *in vitro* stimulation procedures limiting the sensitivity and usefulness to detect immune responses operational *in vivo*.

The emergence of second-generation *ex vivo* T-cell assays during the last 10 years has significantly improved our ability to measure T-cell responses to vaccines, because they are based on detecting single-cell events and provide quantitative results (Fig. 2). The second-generation flow cytometry assays detect T cells either by staining the T-cell receptor with MHC peptide multimers (often tetramers), measuring the affinity of the T-cell receptor to a given epitope, or measuring production of cytokines (most commonly IFN- γ) in response to antigens. Principal limitations of the tetramer (or multimer) analysis include the required knowledge of a defined epitope, the availability of suitable tetramers for the respective epitope/HLA allele, unspecific binding of the tetramers, and the lack of information about the functionality of cells. ELISPOT and cytokine flow cytometry analyses provide functional information (i.e., the capability to produce the cytokine analyzed); however, only the subset of specific T cells producing the respective cytokine is detected. They have the advantage that a suitable negative control (e.g., an irrelevant peptide) can be included. This

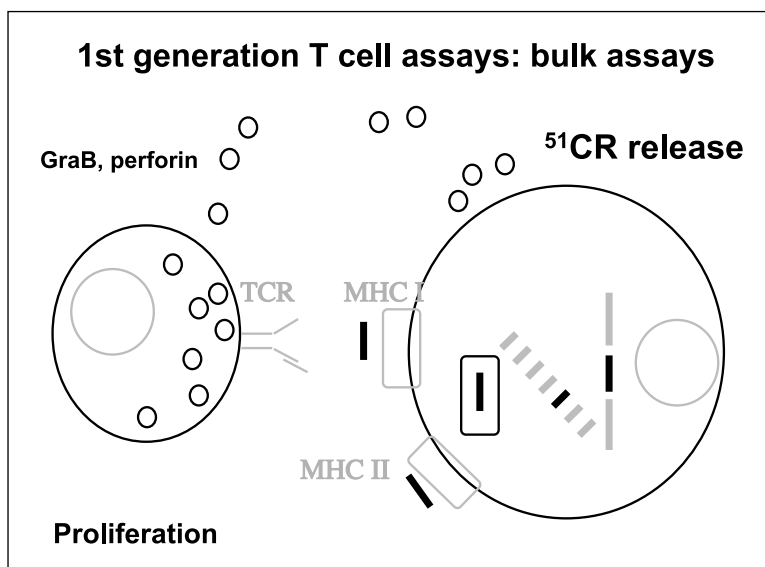
technology is limited by the fact that tumor-specific T cells frequently produce much smaller amounts of cytokines as virus-specific T cells, which may be below the detection limit of the ELISPOT assay (9, 10). Further, functional assays are much more sensitive to variations in assay conditions and often nonspecific background production of cytokines may limit sensitivity of these assays. The second-generation assay technology and applicability have been the major focus of an International Society for Biological Therapy workshop, entitled Immune Monitoring, and held in Bethesda, Maryland, in 2001 (11). The recommendation of that workshop to perform monitoring preferentially with two of these second-generation assays has been largely followed for cancer vaccine trials. If material is scarce, one functional assay may suffice, especially in case of a comparative clinical study.

The third-generation T-cell assays have been developed to provide a detailed analysis of the phenotype and functional repertoire of antigen-reactive T cells (Fig. 3). In principle, these assays are based on detection of specific T cells based on tetramers or cytokines and their further characterization by additional phenotypic or functional markers as listed in Table 1. This type of analysis can yield information on many facets of the nature of T-cell responses, including T-cell differentiation subsets, cytokine production repertoire, proliferative and cytotoxic capacity, and migratory potential (12–16). The specific role of any of these T-cell characteristics for clinical vaccine efficacy, however, has not yet been established in the setting of clinical trials.

Questions Addressed by Immune Monitoring of Cancer Vaccine Trials and Specific Methodologic Considerations

Is there evidence for spontaneous tumor-directed T-cell responses? The analysis of spontaneous T-cell responses before initiation of clinical development with a given cancer vaccine provides valid information. First, the presence of spontaneous functional immune responses suggests absence of immune tolerance due to selected T-cell receptor repertoire or T-cell energy. Second, spontaneous immune responses in the absence

Fig. 1. First-generation of T-cell assays. Bulk assays measuring overall T-cell proliferation after exposure to target (standard readout [^3H]thymidine uptake) or target lysis after exposure to effector population (standard readout ^{51}Cr release from target cells into the supernatant).



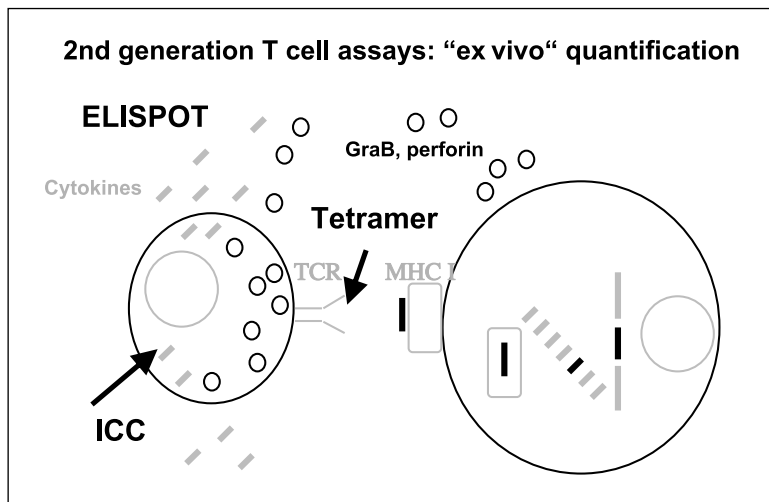


Fig. 2. Second-generation T-cell assays using single cell – based analysis of T-cell properties. T-cell receptor affinity to major histocompatibility β_2 M peptide multimers or cytokine production capacity of T cells after exposure to antigen (readouts cytokine accumulation in T cells with agents blocking secretion, cytokine flow cytometry assay, or secretion of cytokines captured by antibodies bound to cell surface, cyclosporin A assay, or plate, ELISPOT assay). GraB, granzyme B; ICC, intracellular cytokine; TCR, T-cell receptor.

of autoimmune phenomena may indicate that vaccine-induced T-cell responses do not target normal tissue. Third, investigating the nature of spontaneous T-cell responses in the absence or presence of tumor may elucidate mechanisms of immune response and immune escape.

Such single-point T-cell response analysis should be done preferentially with a second-generation functional T-cell assay, because it is often of low frequency and there will be some background staining with most tetramers. When performing tetramer analyses, the inclusion of a sufficient number of healthy controls is necessary. Because spontaneous T-cell responses are often a close-to-threshold phenomenon, adjustment of statistics to the variability of the given assays at close-to-threshold signals is necessary. There are a number of studies with second- and third-generation assays reporting spontaneous T-cell responses in a proportion of patients to various tumor-associated antigens and in tumors of various histotypes, including melanoma, colorectal cancer, myeloma, and leukemia (17). Results from various studies suggest that metastatic spread to the lymph nodes is a prerequisite for the occurrence of circulating tumor-specific T cells (18, 19).

The clinical relevance of spontaneous T-cell responses in patients has thus far only been addressed in few studies. No association of the presence of T-cell responses to tumor-associated antigens was found in two studies in melanoma or colorectal cancer patients (20, 21). In a recent study that analyzed the chemokine receptor profile of melanoma-specific T cells in patients with spontaneous or vaccine-induced melanoma antigen-specific T-cell responses, the presence of CXCR3-expressing tumor-specific T cells was, however, associated with increased survival (22).

Regulatory T cells have recently gained much interest as potential suppressors of tumor-specific effector T-cell responses. A first study showed the presence of specific regulatory T cells directed against the cancer-testis antigen LAGE in melanoma tissue (23). Recent achievements in the characterization of regulatory T cells will allow the direct detection of antigen-specific regulatory T cells and it will be of great interest to study the prevalence and functional role of tumor antigen-specific regulatory T cells in patients (24).

Does a vaccine induce or augment specific T-cell responses?
The primary objective of early clinical trials of novel vaccines,

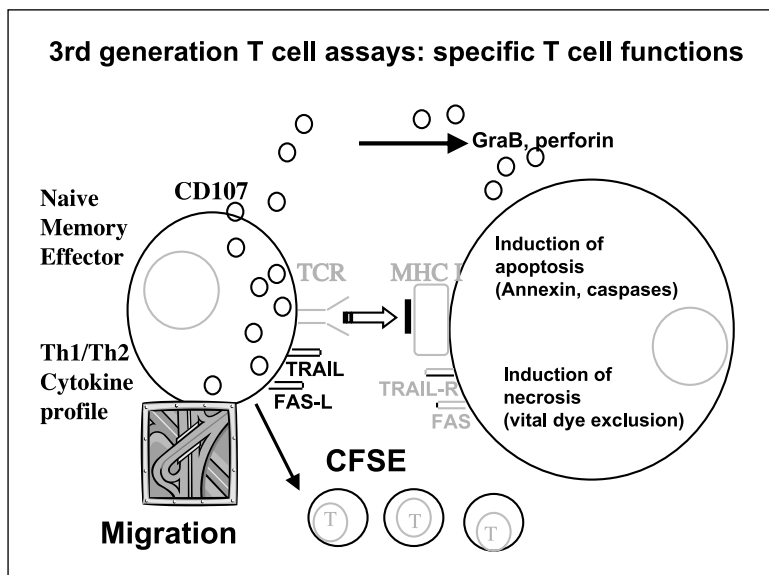


Fig. 3. Third-generation T-cell assays determining characteristics of antigen-specific T cells in a two-step principle: detection of specific T cells by CFC or tetramers and quantitative characterization of specific T cells according to their properties. CFSE, 5- (and-6)-carboxyfluorescein diacetate succinimidyl ester; TRAIL, tumor necrosis factor – related apoptosis-inducing ligand.

Table 1. T-cell properties assessed by third-generation assays

Property	Readout	Detection of specific T cells	Selected key references
Cytokine profile	Accumulation of type 1/type 2 cytokines, including IFN- γ , IL-2, TNF α /IL-5, and IL-13	CFC	(12)
Memory/effector phenotype	Surface expression of CD27, CD28, CD45RA, CCR7	Tetramers or CFC	(13)
Cytotoxic capacity	Perforin, induction of CD107 surface expression	Tetramers or CFC	(14)
Proliferative capacity	CFSE dilution	Tetramers	(15)
Migratory phenotype	Chemokine receptors, adhesion antigens	Tetramers or CFC (prior staining before antigen incubation may be required)	(16)
Functional TCR affinity or avidity	Response to serially diluted antigen concentrations	Tetramer dilutions CFC with antigen dilutions	(29)
Regulatory T cells	CD25, FOXP3, IL-10	Tetramers (yet to be studied)	(24)

Abbreviations: CFC, cytokine flow cytometry; TCR, T-cell receptor; CFSE, 5- (and-6) -carboxyfluorescein diacetate succinimidyl ester.

vaccine preparations, or vaccination schedules is the assessment of the immunologic efficacy, also termed vaccine potency. Vaccine potency assessment has become standard in vaccines eliciting antibody responses to microbial pathogens because antibodies can reliably be detected in peripheral blood and protective titers are well established for many pathogens. In contrast to antibody responses, specific T-cell responses may not always be present in peripheral blood and their presence in the blood is of undefined relevance for clinical efficacy. Few studies have also monitored T-cell responses in vaccine site draining lymph nodes (2) and in tumor compartments of selected patients (25), suggesting higher-frequency T-cell responses in lymph nodes, but these studies are inherently difficult to perform. Therefore, most currently available information comes from serial monitoring of peripheral blood samples.

The appropriate study methods are straightforward at first sight and consist of prevaccine to postvaccine comparisons applying regular clinical phase 2 trial statistics to estimate the immunologic response rate, just as clinical antitumor response rates are generally estimated. Several challenges, however, need to be considered. Because of the weak immunogenicity of many cancer antigens, the responses observed after a limited number of vaccinations are often close to the limit of detection, where assay reproducibility is of concern. Several studies have reported augmentation of the immune responses to levels more easily detected by subsequent vaccinations, but tumor progression often precludes continued vaccination in a proportion of patients.

Uncertainty remains about how to acknowledge augmentation of preexisting T-cell responses, and in some patients even the disappearance of preexisting T-cell responses from peripheral blood early during vaccination (26). Several studies have used rather arbitrary thresholds for defining an augmentation of a T-cell response (e.g., >1.5- or 2-fold) and combined the proportion of patients with induction and augmentation of immune responses for estimation of immunologic response rates, but it remains unclear whether this combined analysis is appropriate.

Are there differences in the nature of immune responses between different vaccines? With the advent of multivariable cytometry, characterization of antigen-reactive T cells is possible and these analyses may greatly enhance our understanding of vaccine-induced, tumor-directed immunity. T-cell properties analyzed with currently available assays are listed in Table 1. Different vaccines or vaccine adjuvants may induce different T-cell subsets with differences in cytotoxic or proliferative capacity or long-term memory, all characteristics that may be crucial for clinical effects. Further, different T-cell subsets may have different migratory or homing characteristics and compartment-specific distribution of T-cell subsets may occur, resulting in their selective accumulation in lymph nodes, bone marrow, or tumor tissue (2, 25, 27). The most dramatic example in this context is the rapid and within several hours almost complete disappearance of activated lymphocytes from the peripheral blood in patients receiving systemic interleukin 2 treatment (28).

The statistical analysis of results from analysis of multiple T-cell variables will be descriptive, unless a solid primary hypothesis can be formulated. A primary hypothesis, however, should have a sound scientific rationale and has to be prospectively defined, rather than be driven by data of the study, because the latter would foster unreliable conclusions because of the large number of statistical comparisons within multiparametric studies. Alternatively, multivariable statistical analysis methods, as have been developed for the more complex analysis of microarray data, may be used provided large enough patient numbers are available.

Is there a link between immune monitoring assay results and clinical end points? A positive answer to this question would show the relevance of peripheral blood immune end points as surrogate markers for clinical end points. The problem in attempting to correlate T-cell responses with objective clinical responses is that most phase 2 vaccination studies reported thus far had an objective response rate of <10%, making any correlations difficult given the small numbers that result. There is some evidence, however, from a few vaccination trials with

higher clinical response rates that there is a correlation between vaccine-induced T-cell responses and clinical effects (2).

A meaningful investigation of potential correlations between immune and clinical end points, however, carries certain requirements. First, sufficient numbers of patients and sufficient vaccine efficacy are needed. Second, we have to assume that only a fraction of patients will experience tumor regression in case of a relevant immune response, and the magnitude of this fraction has to be considered for sample size calculations. Further, robust analysis requires either one primary vaccine potency assay or a predefined hierarchy in case results of two or more assays with potentially conflicting results are considered. Table 2 provides examples of sample size calculations under defined assumptions, also taking into account that in case nonclassic clinical efficacy variables are considered, such as stable disease, the likelihood of this end point being reached (i.e., stable disease) in patients without immune response has to be considered. Because the assumptions listed in Table 2 currently are purely hypothetical, the accumulating aggregate of rigidly analyzed published clinical studies, and increasing vaccine efficacy, may allow more realistic future predictions.

Future Prospects

Modern technology (especially ELISPOT assays and multi-variable cytometry) has provided a refined and detailed analysis of the characteristics of tumor antigen-specific T-cell responses and permits the rational development of immunologic approaches to treatment efficacy correlations. It is acknowledged that for the third-generation assays, the conditions still have to be optimized and assay results validated through

correlation with clinical observations. This necessity should not discourage the application of novel assays within clinical trials, especially within current trials comparing different vaccines, vaccine schedules, and adjuvants. Further, it is expected that clinical success is likely to depend on a complex set of immune response and tumor characteristics, and predicting immune protection requires discovering its correlates, not only for identifying the most promising vaccines, but also for patient-by-patient monitoring of immunologic responses.

Open Discussion

Dr. Sondak: One of the premises of peptide vaccines that has become ingrained in our thinking is that administering a modified peptide will lead to an immune response to the native peptide in the host. As we get more sophisticated monitoring, is there evidence that this is true?

Dr. Keilholz: I've seen examples in both directions, although those examples have been on single patients only. This is a question about frequency. If it's 50%, it may be okay. If it's 20% recognition of the native peptides, it's a problem. If it had been 90%, it would have been great. So, I think it is a quantitative, not a qualitative, question.

Dr. Hwu: If you clone the population of T cells generated from modified peptides, to look at all the reactivities, most will recognize the wild-type peptide if you've modified the anchor residues, but there are some T-cell clones that will recognize just the modified peptide antigen. There's work being done in other groups to modify peptide residues that face the T-cell surface. It's much more dangerous to modify those because you may get reactivity against just the modified peptide, but anchor residue

Table 2. Sample size calculation for assessment of correlation between immune monitoring results and clinical end points (two-sided χ^2 test, α error 0.05, β error 0.8)

Example	Immunologic response rate (%)	Clinical end point	Clinical response rate in patients with T-cell response (%)	Clinical response rate in patients without T-cell response (%)	Expected overall clinical response rate (%)	Required sample size
1	25	CR/PR	50	0	12.5	32
2	25	CR/PR	25	0	6.25	74
3	25	CR/PR	10	0	2.5	203
4	50	CR/PR	50	0	25	29
5	50	CR/PR	25	0	12.5	69
6	50	CR/PR	10	0	5	190
7	80	CR/PR	50	0	40	51
8	80	CR/PR	25	0	20	132
9	80	CR/PR	10	0	8	378
10	25	CR/PR/SD	70*	20	32.5	46
11	25	CR/PR/SD	45*	20	26.25	157
12	25	CR/PR/SD	30*	20	22.5	811
13	50	CR/PR/SD	70*	20	47.5	37
14	50	CR/PR/SD	45*	20	32.5	124
15	50	CR/PR/SD	30*	20	25	626
16	80	CR/PR/SD	70*	20	60	63
17	80	CR/PR/SD	45*	20	40	228
18	80	CR/PR/SD	30*	20	28	1,158

Abbreviations: CR, complete response; PR, partial response; SD, stable disease.
*Including the 20% spontaneous stabilization rate.

modification generally results in the majority of T cells recognizing the wild-type antigen.

Dr. Sondak: When you look at those other parameters, does a T cell immunized to a modified peptide really recognize the native peptide and produce a high efficiency immune response?

Dr. Keilholz: Early on, the T-cell clones may be very different from the clones you have after 15 vaccinations. After 15 vaccinations, you may tease out the clones that may have less avidity to the native peptides because you constantly vaccinate with the optimized peptides.

Dr. Mier: Independent of the behavior of specific clones, has anyone simply done spectratyping of a tetramer population, which you get with wild-type immunization versus modified peptide vaccination? If you got identical distributions of all the tetramer reactive cells, it would argue that the peptides are interchangeable. If they're skewed, then it would argue that there may be some differences.

Dr. Haluska: We've done that in our dendritic cell trials with 289 V modified peptide. When you do spectratyping, you don't see clonal populations although our tetramer data would suggest that we are essentially uniformly successful in inducing immunity to both the 280 and the 289 V.

Dr. Atkins: What is your view of the cellular microarray approach that the people at Stanford have been doing? Can we use those type of assays to look at what happens to the regulatory cells in the setting of a vaccine, as opposed to what is happening to the effector cells?

Dr. Keilholz: The cellular microarray assays may be another form of third-generation assay because you still look for specificity of the T cell and then characterize. That gives much more data, and it adds to the complexity and makes the statistics of the analysis almost impossible. It is a question of how to use this in a clinical study and then how to interpret the data. You learn a lot, but in a clinical trial that is being done to induce immunity and correlate with clinical efficacy, it's not that easy to use all this information in a way that we can make sense of it.

Dr. Kirkwood: Is it possible that the assays we might develop would ultimately be able to measure the trafficking cells to the appropriate locations?

Dr. Keilholz: It may be even more complex to get the cells where you want them to be. You may have to vaccinate them differently or produce a different set of immune cells to eliminate a tumor that is in the skin compared with tumor in the gastrointestinal tract compared with tumor in the lymph nodes. You may have to do assays of each of those compartments to see

how well you're immunizing, and you may only be effective in treating certain compartments with certain approaches.

Dr. Hwu: The T-cell migration pathways that have been described have been to skin, bronchus, and the gastrointestinal tract, where you'd imagine a lot of pathogens are. But the question is "did we even evolve a pathway for T cells to migrate to other areas?" Melanoma, being from the skin, probably gets exposed to antigen-presenting cells in a peripheral lymph node. Do we have a higher repertoire of cells that go to the skin to begin with? Is that why 50% of patients with in-transit disease respond to interleukin 2 compared with patients with liver and lung metastases?

Dr. Kirkwood: My philosophy is that because this tumor begins in the skin, it is the best place for immunization. What do you take from the experience of late-disease patients, who are refractory to multiple therapies, who got the ALVAC gp100 vaccine, and then got their usual transient paltry immune response without any clinical efficacy? Also, what about the patients in Canada who were treated with IFNs that seemed to repolarize the whole system, perhaps in the wrong direction. Is it possible that we can still pull back from the fire when things have headed in the wrong direction?

Dr. Keilholz: It's fine to induce immunity, but if that is not working there may be different ways to make it efficient. The first step in that study was to induce some immunity. Induction of immunity may not be sufficient to elicit an effect in heavy tumor burden patients. But that is why I still have hope that in low-volume disease vaccine may work differently than in patients with heavy tumor burden, where you may need other things to turn off immunosuppressive effects of the tumor.

Dr. Kirkwood: We should not give up on the original vaccination, because there are many things that could be done to retrieve, recall, and repotentiate those responses, and IFN is just one of them. Have you looked at your patients with the vaccinations that you've done, down the road, even with transient responses to see if they can be retrieved?

Dr. Slingluff: We haven't done anything specifically to try to retrieve them, although a fair number of patients have received 12 class 1 peptides and then have subsequently been enrolled into this helper peptide trial to see whether adding that subsequently makes a difference. I think that doing more systematic evaluations like that is worthwhile. What we ought to do next is a systematic study, where you have the power to answer the question, "Is this going to make a difference?"

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