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# Immune responses in the draining lymph nodes against cancer: Implications for immunotherapy*

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**Abstract** Regional lymph nodes are the first site for melanoma metastases. The sentinel node (SN), on the direct lymphatic drainage pathway, which usually harbors first metastases, demonstrates significant suppression in its ability to respond to antigenic stimulation. This downregulation of SN immunity is likely the basis of its susceptibility to tumor metastases, suggesting a potential role of the immune system in the control of malignant tumors. Despite immune dysfunction in the SN, phase II trials of systemic post-operative immunotherapy with a polyvalent melanoma vaccine developed at the John Wayne Cancer Institute showed improved 5-year overall survival in patients with melanoma metastatic to regional nodes. However, most immunotherapy clinical trials have failed to demonstrate a significant clinical response, and analyses of immune responses to tumor-associated antigens that correlate clinical responses have not been established. Therefore, refinements in assay methodologies and improvements in vaccine designs are critical to the success of cancer immunotherapy. Antigen presentation by dendritic cells (DCs) is the most potent means to initiate a T cell immunity. Dendritic cell-based immunotherapies have been vigorously attempted in the past decade. To improve the immunogenicity of cancer vaccines, we recently generated heterokaryons of DCs and tumor cells by electrofusion. The fusion hybrids retained their full antigen-presenting capacity and all natural tumor antigens. In pre-clinical animal experiments, a single injection of the DC-tumor fusion hybrids was sufficient to mediate the regression of tumors established in the lung, skin and brain. Most interestingly, successful therapy required the delivery of fusion hybrids directly into lymphoid organs such as lymph nodes. A clinical trial is now being carried out to test the immunogenicity and therapeutic effects of fusion hybrids for the treatment of metastatic melanoma.

**Keywords** Sentinel lymph nodes · Immunosuppression · Active immunotherapy · Dendritic cells · Electrofusion · Immune monitoring

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## Introduction

Although the draining lymph nodes (LNs), situated directly in the lymphatic drainage pathway from primary melanoma are often the site of metastases, they are also known to be the first lymphoid organ to respond to the antigenic stimulation that usually precedes the establishment of systemic immunity [1]. The pivotal role of draining LNs in the development of immunity that could mediate the rejection of malignant tumors has been unequivocally documented. Early studies [2, 3] demonstrated that excision of the immunization site

two days after injections had little impact on the eventual development of systemic immunity, whereas an intact draining LN was necessary for 7 to 9 days in order to immunize an animal to reject subsequent tumor challenges. While these experiments demonstrated that initiation of an immune response occurred in the draining LN, melanoma metastases in the SN suggest the inability of this LN to elicit effective anti-melanoma immunity [4]. This review will summarize experimental evidence indicating that selective down-regulation of immune function does occur in the SN.

Immune dysfunction in the SN is not indicative of generalized immune suppression. In melanoma patients whose tumors have invaded regional LNs, lymphadenectomy is often performed. The efficacy of such surgery depends in part on the patient's immune response, which can be modulated by postoperative active immunotherapy, using a polyvalent formulation developed at the John Wayne Cancer Institute [5]. Active immunotherapy resulted in a significantly higher overall survival in Stage III, tumor-free melanoma patients. However, its effects on patients with existing tumor metastases (Stage IV) appears to be marginal. To achieve therapeutic effects against cancer metastases, vaccine design must be based on a greater appreciation of the mechanisms by which tumor-specific cellular immune responses are initiated and elicited. In the past couple of decades, the mechanisms of activating, resting, naïve T cells *in vivo* have been increasingly understood. It has been well documented that dendritic cells (DCs) are the principal initiators of antigen-specific immune responses. Many factors appear to contribute to the unique potency of DCs in activating T cells [6]. These cells express abundant MHC molecules on their surface, providing ample peptide/MHC ligands for T-cell receptor engagement. They also express high levels of important adhesion and costimulatory molecules to facilitate vigorous T-cell activation. Based on these findings, delivery of antigens with DCs should stimulate powerful immune responses against tumors. DC vaccines have entered clinical testing for a variety of metastatic lesions. In most cases, autologous DCs have been loaded with tumor antigen preparations such as MHC class I-restricted peptides, proteins, and tumor lysates, or transfected with tumor RNA. Despite the theoretical attraction of DCs, a recent review of DC vaccines in $>1000$ patients indicated that the overall response rate was 8.9% for melanoma [7].

There are many possible mechanisms that may account for the therapeutic failure of DC-based immunotherapy. Effective vaccine design should be able to overcome many obstacles such as tolerance, anergy and immunosuppression. Given the superiority of DCs' antigen-presenting function, we have been interested in DC-based immunotherapy utilizing a live fusion product composed of tumor cells and autologous DCs. Compared with other antigen-loading methods, fusion is appealing because it should induce a polyclonal immune response, including both CD4 T helper cells and CD8 cytotoxic lymphocytes against an array of both known and undefined natural tumor-associated antigens. In the literature, fusion cells were functionally active in stimulating T cells and eradicating established tumors [8–10]. However, the traditional method of polyethylene glycol (PEG) fusion is plagued by toxicity, poor efficiency and poor reproducibility. In fact, close scrutiny of previously published studies raised considerable concerns as to whether physical fusion of cells had occurred [11–13]. To improve the technique of somatic fusion, we recently developed a large-scale process where cells are fused by exposing them to electric fields. The electrofusion allowed the generation of verifiable and reproducible fusion hybrids from both animal and human cells. In this review, we will summarize recent highlights of immunological analyses and therapeutic efficacies of DC-tumor fusion heterokaryons.

Finally, *in vitro* analyses of T cell reactivities from patients undergoing immunotherapy have demonstrated the generation of antitumor T cells based on surrogate or subjective endpoints. However, the scarcity of clinical responses in these patients has made it difficult to validate the *in vitro* assay results as a useful prediction of clinical response. Refinements in assay methodology and improvements in cancer vaccine design will allow for more precise quantitation of the frequencies of responding cells and more reliable elucidation of their phenotype. In addition, direct sampling of tumor site and draining LNs may allow comparisons of local vs. systemic immune responses as well as suppressive effects of the progressive tumor.

## Tumor-induced immune modulation and establishment of metastases in regional draining lymph nodes

Most patients with cutaneous melanoma never develop metastases. Patients who do develop metastases most often first present in the ipsilateral regional nodes [14] and preferentially in the sentinel node (SN), the first LN on the direct lymphatic drainage pathway from a primary melanoma [15]. This may simply reflect the flow dynamics of lymph that travels from the area of the primary site to the SN and may transport, within the lymph, viable tumor cells with the capacity to establish metastatic colonies. Alternatively, the SN may be selectively permissive to colonization by and expansion of metastatic tumor cells.

In early studies, before the current and popular approaches to the identification and assessment of the SN were developed [16], we demonstrated that individual nodes of the regional node group were heterogeneous in the extent of their reactivity. Indices examined included microscopic reaction

patterns, frequency, density and dendriticity of paracortical dendritic cells (PDC) [17], T cell activation, transformability [18], lymphokine generation [19] and cytotoxicity against cultured melanoma cells [20] and frequency of suppressor cells [21]. Nodes that were located close to tumor showed most down-regulation of all these indices.

We have extended these studies to compare the architecture, cytology and cellular phenotype of metastasis-susceptible SN with non-sentinel nodes (NSN) from the same patient in melanoma [4, 22] and breast cancer [23]. Compared to NSN, SN are entirely or segmentally [24] down-regulated, as evidenced by a reduction in the aggregate area of the paracortex, the area of the node occupied by PDC, as well as the frequency, density, meshworking and dendritic complexity of PDC [25]. Thus, alterations in the SN affect the critical antigen transporting and presenting dendritic cells that migrate to the node from the peripheral tissues of tumor and tumor-associated skin. Also affected is the paracortical area within the nodes, which shows reduced T cell density and reduced expression of activation markers [26] by the cells that under normal circumstances are the clients of the antigen presenting cells. One effect of this immune suppression is a reduction in the availability of continuing supplies of tumor-directed cytotoxic T cells, cells that are viewed as having a major role in limiting the local evolution of the primary melanoma and inhibiting the establishment and expansion of metastases.

Since naïve T lymphocytes arrive in the paracortex for their encounter with dendritic cells by migrating through the endothelium of paracortical high endothelial venules (HEV), we have examined the vascular system of SN for differences in pattern, frequency and activation relative to NSN. We have demonstrated a reduced frequency of HEV in SN and evidence of reduced endothelial cell activation (absence or reduction of high profile endothelium and of expression of activation markers by endothelial cells) in paracortical venules. There is also a striking reduction in transendothelial migration of naïve T cells and an apparently associated reduction in the frequency of PDC-associated activated T cells [27]. SN immune down-regulation has been confirmed by others [28–33].

That these alterations are not fixed is shown by our observation that such alterations are lacking in the SN of patients treated preoperatively with intradermal peritumoral GM-CSF [34]. It is arguable that these observations indicate selective down regulation of the SN, likely by tumor-derived products such as gangliosides [35, 36] and cytokines [37, 38]. We consider that our findings and those of others active in this area support our hypothesis that tumor-induced down-regulation of SN immunity is the basis of the susceptibility of the SN to the survival and development of the tumor cells that lead to clinically significant metastases. Current efforts to reverse tumor-induced down regulation of nodal function may thus have some capacity to reduce the incidence of nodal metastases in melanoma patients.

## Prolonged survival of patients receiving Canvaxin™ immunotherapy after complete resection of melanoma metastatic to regional lymph nodes

Over the past decades, immunologic and molecular studies indicate that melanoma cells contain antigens capable of stimulating both humoral and cellular immune responses in patients. Several lines of evidence also suggest that the immune system may play a significant role in the host-tumor interaction. For example, spontaneous regression of melanoma has been documented, especially in children. Approximately 5% of patients with metastatic melanoma have an unknown primary tumor, suggesting spontaneous regression. Vitiligo-like leukoderma may be associated with destruction of both normal melanocytes and melanoma cells. In superficial spreading melanoma, it is not infrequent to observe areas of regression with tumor-infiltrating lymphocytes. In fact, increase in melanoma incidence in immunosuppressed renal transplant recipients provides additional clinical evidence for the role of immune surveillance in the evolution of melanoma. Therefore, augmentation of the immunological reactivity of melanoma patients against their own tumors is a scientifically sound rationale for the development of immunotherapy.

Identification and characterization of melanoma antigens have shifted attention from nonspecific immunostimulants to antigen-specific immunotherapy. Current melanoma immunotherapy utilizes vaccines ranging from complex antigen mixtures such as whole cell preparations, to purified single antigenic peptides. Complex vaccines are polyvalent and therefore can stimulate immune responses to potentially many tumor antigens, which increases the strength and diversity of the overall immune response. In addition, the antitumor reactivity of a polyvalent vaccine is less susceptible to antigen modulation, although immune responses to irrelevant antigens are a potential disadvantage. On the other hand, vaccines made from purified antigens are easier to manufacture and the patient's response to a single antigen is easier to analyze. However, single-antigen vaccines can be rendered ineffective by poor immunogenicity due to the lack of CD4 T-cell responses and/or by the emergence of resistant antigen-negative tumors or clones.

Viable whole tumor cells inactivated by irradiation so that they are not capable of growth are the most effective immunogens in syngeneic animal tumor model studies. These whole-cell vaccines have been extensively studied in human trials of active immunotherapy. There are two forms of whole-cell vaccine preparations: autologous and allogeneic. Autologous vaccine is patient-specific and its production depends on the

availability of tumor cells from that patient. Thus it is difficult to standardize in terms of potency and consistency. Nevertheless, a nonrandomized clinical trial demonstrated a 12.5% regression rate after vaccination with autologous tumor cells [39].

Because melanoma is probably one of the most immunogenic solid tumors by virtue of a large number of defined antigens, allogeneic tumor cell vaccine preparations may offer a well-characterized and uniform product that can be used for different patients. The availability of recombinant antigens allows the characterization of antigen-specific humoral and cellular responses in vaccinated patients. Correlation of these responses with clinical outcomes should identify the most immunologically relevant antigens. Efforts then can focus on augmenting clinical responses by presenting the appropriate antigens as components of cellular, protein or even peptide vaccine.

Canvaxin$^{TM}$, an allogeneic tumor vaccine, is the result of more than 30 years of experience in melanoma immunotherapy. Canvaxin$^{TM}$ is composed of 3 cell lines (M10, M24 and M101) specifically selected from 150 melanoma cell lines started and stored in the John Wayne Cancer Institute's tumor-cell freezer bank. At that time, these cell lines were unique and important because of their high content of several antigens ($GM_2$, $GD_2$, $GD_3$ and O-acetylated gangliosides, 69.5 Kd and 90 Kd glycoproteins, and 180 Kd lipoprotein) defined by antibody reactions [40]. Since then, many additional protein antigens of melanoma have been molecularly defined and cloned, including gp100, MART-1, MAGE-1 and MAGE-3, tyrosinase and TRP glycoprotein 75. Each of these Ag has subsequently been found to be present in Canvaxin.

In clinical trials, patients received biweekly intradermal vaccination for 8 weeks. The first two vaccinations were given with bacille Calmette-Guerin (BCG) as adjuvant. Patients then received monthly vaccination for an additional 4 months. The toxicity associated with this immunization is negligible. A minority of patients reported some mild fatigue, musculoskeletal discomfort and hyperpyrexia associated with the first two vaccinations and BCG. Occasionally, patients developed an intense local inflammatory skin reaction to BCG that was self-limiting, but in some patients, required local wound care.

Phase II trials for AJCC Stage III melanoma revealed a significantly higher median survival for 283 patients receiving immunotherapy after complete resection of regional metastases than for historical controls receiving other postoperative adjuvant therapies (Fig. 1) [5]. The two groups had similar prognostic factors, including a similar distribution of tumor-involved LN. Five-year and ten-year overall survival rates were 52% and 47%, respectively, in patients receiving Canvaxin, vs. 36% and 31%, respectively, for control patients. A recently reported phase III multicenter trial of Canvaxin in patients with stage III melanoma has been unable to confirm these phase II trial results. At the dose and schedule used in the phase III trial, Canvaxin was no more active than a placebo.

## Dendritic cell-based cancer vaccine: Development of electric field-induced somatic hybridization of dendritic and tumor cells

Central to the induction of cellular immune responses to antigens are professional antigen-presenting cells. Among various antigen-presenting cells, DCs are the principal initiators of antigen-specific immune responses. In a host of preclinical experiments and clinical trials over the past decades, DCs have been used to process and present tumor antigens. However, despite a heightened immunity in response to vaccination, clinical responses are disappointing [7]. Improvement of cancer vaccines thus holds the key to the success of cancer immunotherapy.

One of the promising strategies for inducing therapeutic immune responses against malignancies is the use of DC and tumor fusion cells. In addition to the presentation of a whole array of unaltered tumor antigens, fusion hybrids should have the functional ability to provide the strongest T cell stimulation signals. The idea of fusion in vaccine design is not new, but the commonly used PEG fusion technique failed to generate consistently verifiable heterokaryons. An alternative technique for generating hybrid cells is to expose them to electric fields [41, 42]. Cells in suspension are first exposed to an alternate electric current (ac) of low intensity that induces an oscillating dipole on the cells, leading to the formation of tight membrane contacts in a pearl-chain-like fashion. Following this "alignment", a direct current (dc) pulse with relatively high intensity disrupts the bilipid layer of the cell membrane temporarily. After cessation of electric pulses, membrane resealing occurs between adjacent cells, forming multi-nucleated hybrid cells. To verify that true cell fusion had occurred, dually fluorescent cells were clearly observed using FACS, fluorescence microscopy, cytospin preparations and DNA analyses [43]. In addition, fusion hybrids expressed a mature DC-like phenotype. They stimulated both CD4 and CD8 tumor-specific immune T cells to secrete interferon-gamma (IFN$\gamma$) *in vitro*.

In pre-clinical therapy experiments, a standard protocol of 3-day established experimental pulmonary metastases was used to evaluate the therapeutic efficacy of DC-tumor fusion cells. We have demonstrated that a single vaccination resulted in a significant antitumor response in four antigenically and histologically distinct murine tumors, including the D5LacZ melanoma, MCA205 fibrosarcoma, GL261 glioma and 4T1 breast cancer [44–46]. However, repeated experiments indicated that the therapeutic effects required the direct delivery of fusion cells into lymphoid organs (spleen or LNs) and the

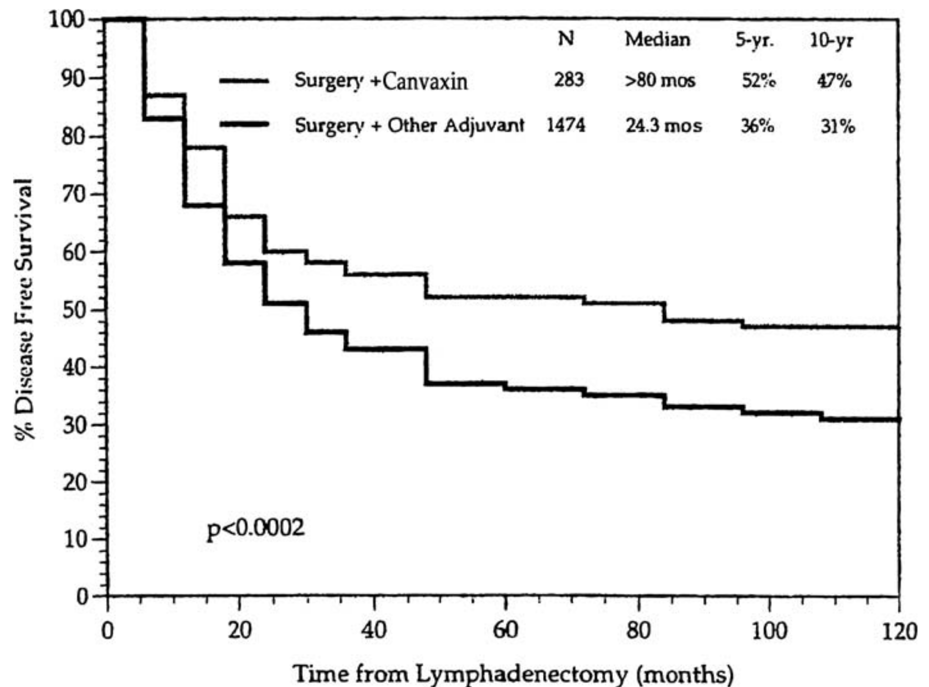


**Fig. 1** Disease-free survival of patients receiving Canvaxin vs. other adjuvant therapy or no adjuvant therapy after complete resection of Afcc stage III melanoma (*CA Cancer J Clin* 46: 225–244, 1996)

co-administration of an adjuvant or a third signal cytokine such as agonist OX-40, 4-1BB monoclonal antibodies, or IL-12.

The antitumor effects of fusion cells were not confined to the treatment of pulmonary metastases. Early tumors established in the skin and brain were equally susceptible to the fusion immunotherapy [43, 45]. In a recent report [47], fusion cell immunotherapy was demonstrably effective for advanced intracranial tumors when the GL261 and MCA205 sarcoma were established for 7 days before immunotherapy. In this treatment protocol, mice with brain tumors also received local cranial radiotherapy. Seven days after vaccine with fusion cells, treated mice demonstrated robust infiltration of both CD4 and CD8 T cells that was exclusively confined to the brain tumor. Consistent with previous demonstrations, cured mice developed a systemic immunity against further lethal tumor challenges.

Electrofusion is a physical reaction, and most mammalian cells should be susceptible to the effects of electric fields. We therefore attempted to fuse human DCs with tumor cells. Using 22 well-characterized melanoma cell lines, we successfully generated DC-tumor heterokaryons ($\geq$10%) in 18 cases. Figure 2 depicts FACS analyses of 18 fusions where tumor cells were pre-labeled with the green fluorescence dye, carboxyfluorescein diacetate succinimidyl (CFSE), and after fusion, hybrid cells were detected by staining with PE-conjugated anti-CD11c monoclonal antibodies. We also evaluated the ability of human DC-tumor fusion cells to present tumor-associated epitopes in the context of both MHC class I and class II molecules [48]. Allogeneic DCs expressing HLA-A2, HLA-DR4, and DR7 were fused with the 888 mel cells that do not express any of these MHC molecules, but do express multiple melanoma-associated antigens (MART-1, gp100, tyrosinase and tyrosinase-related protein 2). As evaluated by specific cytokine secretion, the DC-tumor hybrid cells interacted specifically with the 6 well-characterized CTL cell lines. Furthermore, these hybrids were specifically recognized by CD4 T cell lines reactive with gp100 epitopes in the context of HLA-DR4 and DR7. These results demonstrated that electrofused human DC-tumor hybrids elicited both CD4 and CD8 T cell responses. Currently, the immunotherapeutic potential of these hybrid cells is being evaluated in a human clinical trial where metastatic melanoma patients will be treated with autologous DC-tumor fusion hybrid cells to test the therapeutic efficacy of this immunotherapy.

## Monitoring immune responses in patients treated with tumor-specific vaccines

*Ex vivo* assays of global and antigen-specific immunity have been refined and streamlined over the last several years, so that even responses of very low magnitude can be precisely and reliably detected [49, 50]. Global immunity can be measured by stimulating responsive cells with mitogens such as phorbol myristate acetate (PMA) + ionomycin, anti-CD3 + CD28 antibodies, or superantigens such as Staphylococcal enterotoxins (e.g., SEA or SEB). Antigen-specific immunity can be measured by stimulating responsive cells with tumor cells, tumor cell lysates, defined proteins, peptides, or

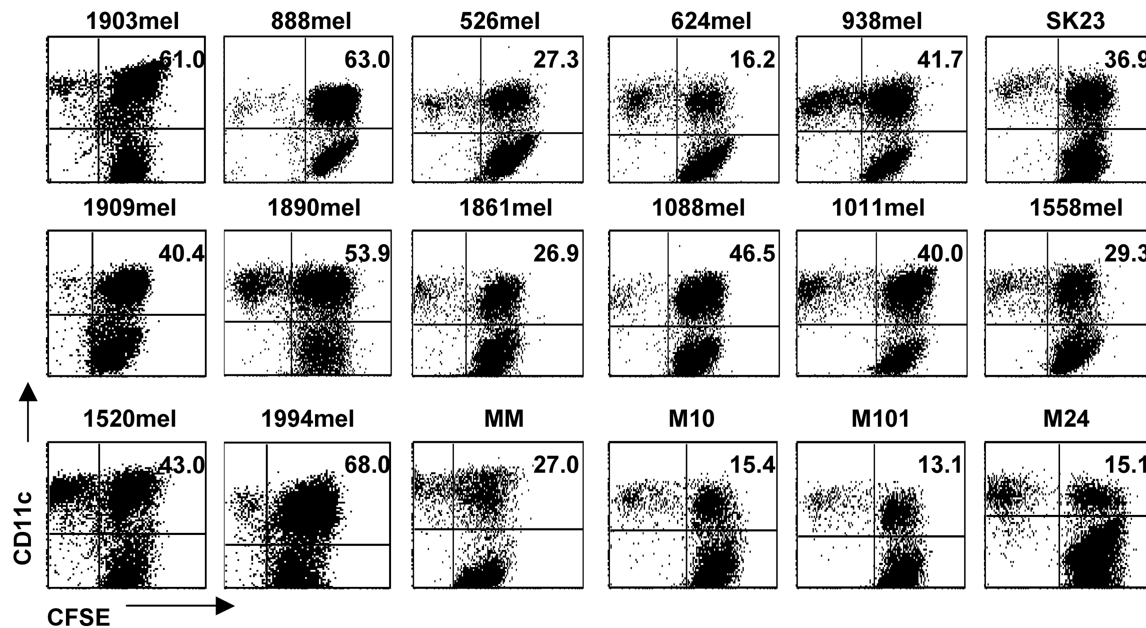


**Fig. 2** Electrofusion of human DCs with melanoma cell lines. Tumor cells were labeled with CFSE before fusion. After fusion and overnight culture, adherent cell populations were stained with PE-anti-CD11c mAb. Numbers in the quadrants represent percentages of double-positive fusion hybrids. 18 melanoma cells of 22 tested yield ≥ 10% fusion rates

peptide mixtures [51]. Assays for both global and antigen-specific immunity have traditionally been bulk assays, such as $^3$H-thymidine incorporation (to measure proliferation), $^{51}$Cr release (to measure cytotoxicity), or ELISA (to measure cytokine secretion). However, newer assays have recently emerged that measure responses on a single-cell basis. These include enzyme-linked immunospot (ELISPOT), intracellular cytokine staining (ICS), and MHC-peptide multimer staining. Of these, MHC-peptide multimer staining is perhaps the simplest technique, but it is limited in terms of detecting only T cells specific for single defined peptide epitopes in the context of specific MHC molecules [52]. ELISPOT and ICS, while more cumbersome in requiring cell stimulation, have broader applicability to a variety of antigens, without regard to MHC restriction [53]. Refinements in these assays to make them more amenable to clinical trial use are discussed below, along with examples of how these assays can and have been used to monitor immune responses.

## ELISPOT assays

The ELISPOT technique relies upon the capture of secreted cytokines by antibodies coated on filter-bottom plates. After incubation of PBMC (or isolated T cell subsets) with stimuli, usually for 24–48 hrs, the plates are washed, and bound cytokine is detected with an enzyme-labeled detector antibody and colorimetric substrate [54]. Kits with pre-coated plates and standardized reagents are now available to make this assay easier and more reliable. Perhaps most importantly, automated spot readers can be used to greatly increase the throughput and standardization of the analysis. Further automation to handle the plate set-up and washing steps is being pursued by some groups (G. Ferrari, personal communication). A major limitation of the ELISPOT assay, however, is the inability to determine which cell subsets are producing cytokines, unless specific cell subsets are first isolated, which is time-consuming and prone to error and ambiguity. Also, the ELISPOT assay has proven difficult to move from single-parameter detection to detection of multiple cytokines, without loss of accuracy in quantitation (G. Ferrari, J. Cox, personal communication).

## Intracellular cytokine staining assays

The ICS technique also uses stimulation of PBMC or other responsive cells with antigens, although usually for a shorter time period (e.g., 6 hr) [55]. It relies upon the ability of secretion inhibitors such as brefeldin A to allow accumulation of cytokines intracellularly, where they can be detected with fluorescent-labeled antibodies in multiparameter flow cytometry. This addresses the two limitations of the ELISPOT assay mentioned above, because the cell subsets producing cytokine can now be easily defined with other antibodies, and multiple cytokines can be detected in a single stimulation. In fact, the multiparametric nature of flow cytometry is unparalleled for determining which cell subsets are producing particular cytokines. However, it is also a platform that

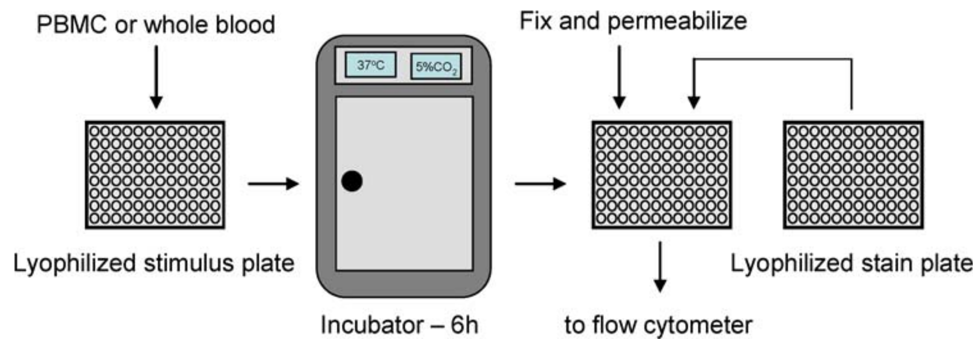


**Fig. 3** ICS assays can be streamlined by using 96-well plates, and further standardization and ease of use can be achieved with pre-configured lyophilized plates containing stimuli and staining antibodies. PBMC or whole blood is added directly to the stimulus plate, followed by incubation, fixation, and permeabilization. The antibodies in the staining plate are then hydrated and added to the plate containing cells. After being stained and washed, the samples can be directly acquired on a plate-loading flow cytometer. Adapted after [5]

has traditionally not lent itself to high-throughput analysis. The development of plate-based protocols and plate-reading flow cytometers, along with the ability to lyophilize antigens and staining antibodies in multiwell plates, begins to address this concern (Fig. 3) [56].

## Patient subsetting

One use of these cellular assays is in subsetting cancer patients on the basis of their immune response, either global or cancer-specific. For example, patients who are globally immunosuppressed would likely not be good candidates for an immunotherapeutic vaccine. Alternatively, patients who are not immunosuppressed, or who have a pre-existing anti-tumor immune response, might have a better prognosis and could be spared highly aggressive chemotherapy. Before contemplating such uses of immunological assays for patient subsetting, one needs to ask two fundamental questions: (1) To what degree are cancer patients globally immunosuppressed?, and (2) Is there any evidence that the degree of immune suppression is correlated with prognosis?

Regarding the first question, a recent report examined global immune competence in a large cohort of newly diagnosed breast cancer patients, prior to any therapy [57]. The degree of immune suppression, as seen by using ICS assays for both Th1 and Th2 cytokines, was quite profound. PBMC from the majority of the cancer patients had significantly fewer CD4+ and CD8+ T cells making IFN$\gamma$, TNF$\alpha$, IL-2, and IL-4 in response to PMA + ionomycin stimulation. Findings such as this suggest that global immune suppression can be seen even in untreated, early-stage cancer patients, and might therefore be worth measuring as an indicator of their prognosis or suitability for particular types of therapy.

Regarding the second question, there is much less evidence to demonstrate that immune responses in cancer patients do in fact have prognostic value. Leong et al. [58] showed a correlation of immune responses with lack of micrometastases in lymph nodes of melanoma patients, as well as an increase in IL-10 production in those lymph nodes that were positive for micrometastases. However, direct correlations of immune responses with clinical outcome have not yet been established.

## Patient monitoring

Another potential use for assays of tumor-specific cellular immunity is in the monitoring of patients undergoing vaccines or other immune-based therapies. In such settings, there is a need to determine: (1) Whether a particular vaccine is immunogenic [49]; (2) if the vaccine is potentially efficacious [50], and (3) whether a particular patient is responsive or not. There is a need for standardized, validated methodology in the assays used for both purposes, in order to compare different vaccine trials or to compare various patients and patient time points. Such standardization is beginning to be addressed for ELISPOT [59] and ICS assays [56].

If there is to be any use in monitoring patient immune responses with the above assays, one might first ask whether there have been any immune responses to cancer vaccines that have been clearly demonstrated by ELISPOT or ICS. The answer to this question is definitely "yes", although the magnitudes and frequencies of such responses vary dramatically. For example, consider two vaccine studies in which immune monitoring was done by ICS. One study, using gp100 peptide vaccination of melanoma patients, showed a detectable CD8 T cell response in 28 of 29 patients, with several responses above 1% in magnitude [60]. Another study, using idiotype + KLH-pulsed DC vaccination of multiple myeloma patients undergoing bone marrow transplant, showed a CD4 T cell response to either idiotype or KLH in only 3 of 9 patients [61]. These results are undoubtedly influenced by the immune competence of the patient population. In fact, in the myeloma study, the three responsive patients were those who also had the highest response to the positive control, SEB, suggesting that global immune competence may have been a determinant of the ability to mount a tumor-specific response.

In any case, these two studies suggest that current methods of immune monitoring, such as ICS, can easily detect responses under favorable immunization conditions.

A final question with regard to patient monitoring is whether responses, as measured by ICS for example, will be predictive of clinical outcome. While some studies have suggested such a correlation for infectious diseases [62], the preponderance of data suggests that the magnitude of a cytokine response (e.g., IFN$\gamma$), while it may be a necessary prerequisite for clinical protection, is unlikely to be sufficient [63]. This is likely true for cancer as well as infectious diseases [64]. As such, searches for better immune correlates of protection will continue, and are likely to include affinity of response [65], functional markers [66, 67] and phenotypic attributes of the responsive cells [68]. The measurement of tumor-specific immunity by a method such as ICS, which is amenable to multiparametric measurements, will be pivotal to defining such correlates of protection.

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