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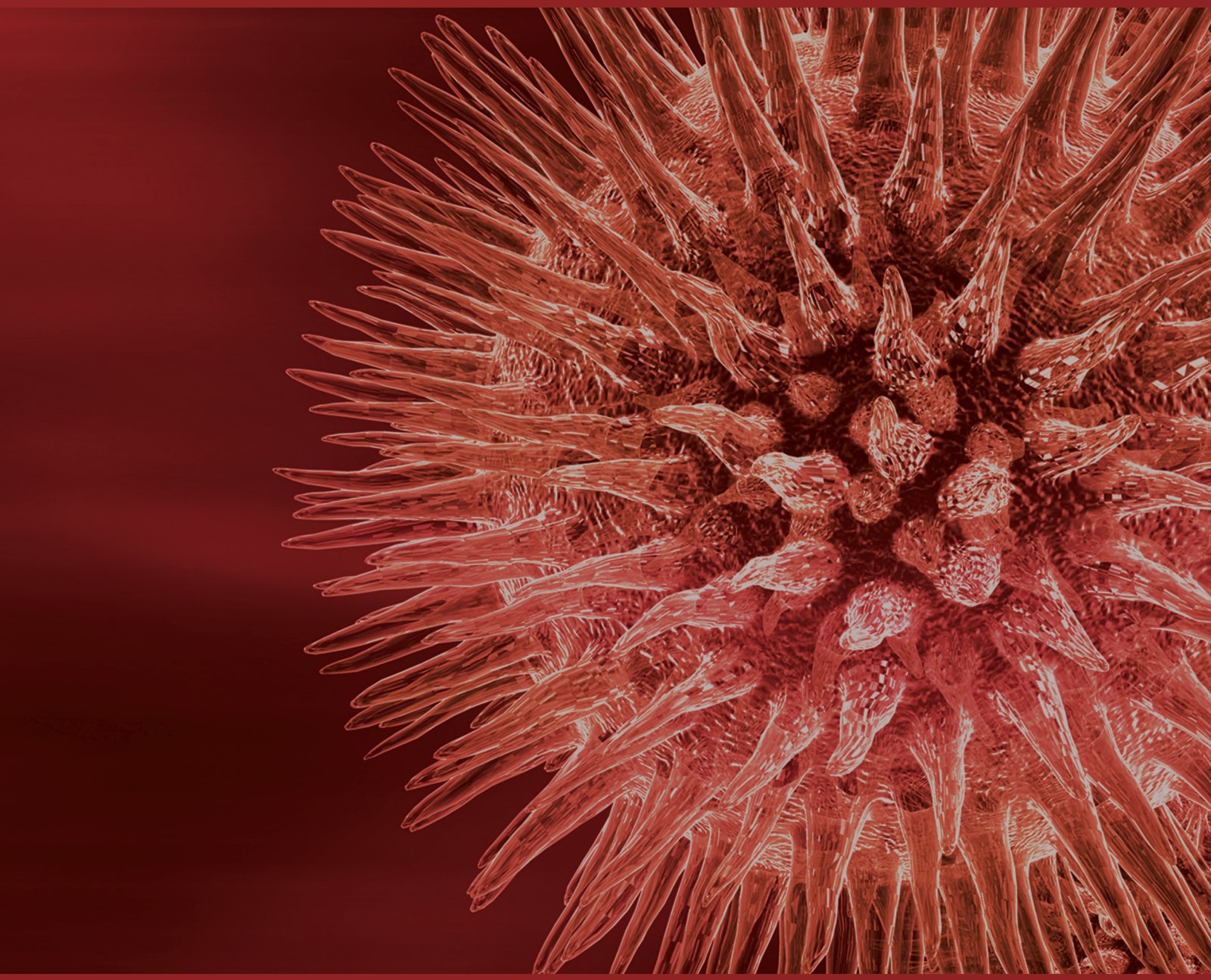
Immunologic Monitoring of Cellular Immune Responses in Cancer Vaccine Therapy

Guest Editors: Theresa L. Whiteside, James L. Gulley, Timothy M. Clay, and Kwong Yok Tsang



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Editorial

Immunologic Monitoring of Cellular Immune Responses in Cancer Vaccine Therapy

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Immunotherapy for cancer is based on the concept of inducing the generation and expansion of immune cells that can attack and eliminate cancer. Immunotherapy with therapeutic cancer vaccines aims specifically at inducing tumor antigen-specific T cells. The immune system is a complex, multifaceted cellular network that is not fully understood. Monitoring cellular immune responses is essential for rational cancer vaccine development. The primary objectives of immune monitoring after vaccination are to document the induction of vaccine-specific and tumor-specific immune responses and to correlate the presence and magnitude of vaccine-induced immune responses to clinical outcomes. Immune monitoring could also be used to (a) define the ability of a given vaccine to generate antigen cascade responses (i.e., epitope spreading); (b) compare the effects of vaccines of different potencies; (c) evaluate the ability of a given cytokine, drug, adjuvant, and so forth. to enhance or hinder vaccine-induced immune responses; (d) define appropriate patient populations for vaccine studies; (e) study the presence and activity of inhibitory/suppressor cell populations.

Despite advances in the development of immune monitoring assays during the past decade, it has been difficult to establish significant correlations between vaccine-induced immune responses and clinical outcomes. This lack of correlation could reflect the methodological limitations of immunologic assays or the postvaccination absence of antitumor responses sufficiently robust to induce disease-free or overall survival. A wide portfolio of monitoring assays is currently available. However, these assays fail to define surrogate markers that could be used as predictors of clinical response and thus serve to advance vaccine development. The immune monitoring assays currently used in cancer immunotherapy trials (such as enzyme-linked immunospot assays, tetramer-based assays, intracellular cytokine flow cytometry, antibody tests, proliferation assays, reverse transcription polymerase chain reaction, and serum cytokine and chemokine profiles) have limited usefulness as surrogate markers of clinical efficacy. There is general consensus that further studies are needed to account for the difficulties in establishing the correlation between different aspects of T-cell function and clinical efficacy.

Vaccine-induced immune responses against cancer depend on a balance between immune responses of various subsets of effector and suppressor T cells. Because tumor antigens are mostly self-antigens, this balance is shifted toward tolerance in cancer patients, so that generating effective antitumor responses requires breaking of tolerance. Although preclinical data have shown that it is possible to break tolerance to tumor-associated self-antigens, human clinical trials employing cancer vaccines have mostly failed to do so. In an immunocompetent cancer patient, the immune system suppresses attacks against self-antigens, including tumor-associated antigens, particularly in the tumor microenvironment. Recent studies have focused on defining the role of the suppressive component of the antitumor immune response in breaking tolerance and steering the immune system toward autoimmunity. In this

respect, monitoring assays that measure the extent of cancer-induced suppression may be especially important.

The suppressive compartment of the immune system includes a group of heterogeneous immune cells, including regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs). Increased numbers and/or enhanced functionality of these cells have been detected in the peripheral blood mononuclear cells, tumor microenvironment, and tumor-draining lymph nodes of patients with hematologic malignancies and various types of solid tumors. One of the major problems in characterizing these cells is their extreme plasticity. Cells normally committed to activating an immune response can transiently acquire suppressive characteristics, as often happens in cancer. While suppressor cells represent an important mechanism by which the immune system fine-tunes specific immune responses, expansion of these cells in cancer patients interferes with antitumor immunity. In humans, it has been difficult to establish a definite phenotype for these cells, and assessment of their functional status has been a special challenge as they are minor lymphocyte subsets lacking well-defined surface markers. Greater understanding of the mechanisms that regulate the homeostasis of these suppressive cells could lead to the development of more effective cancer immunotherapies and better immune monitoring of patients receiving cancer vaccines.

Several studies have demonstrated that Treg depletion can efficiently enhance vaccine-mediated antitumor immunity in cancer patients. For example, in a randomized placebo-controlled multicenter phase II trial, 125 patients with metastatic castration-resistant prostate cancer were treated with a poxviral-based vaccine containing the transgenes for prostate-specific antigen and 3 costimulatory molecules (PSA-TRICOM). This trial demonstrated a direct correlation between the post-vaccination frequency and function of Tregs and overall survival. Treg function and/or phenotype, as well as the ratio of effector to CTLA-4^+ Tregs, could potentially be used to monitor immune function (the balance between immunostimulatory and immunosuppressive factors) in patients enrolled in clinical trials of therapeutic cancer vaccines [1, 2]. Analysis of Tregs in real time as part of the immune monitoring of patients could also help to identify the subpopulation of patients who would most likely benefit from vaccine therapy.

Similar to Tregs, MDSCs are a heterogeneous cell population that has been difficult to monitor in humans. MDSCs are composed mainly of myeloid progenitor cells that do not completely differentiate into mature macrophages, dendritic cells, or granulocytes. A recent study demonstrated that a subpopulation of monocytic MDSCs, phenotypically defined as $CD14^+HLA\text{-}DR^{-/lo}$, is significantly expanded in patients with metastatic melanoma, hepatocellular carcinoma, glioblastoma, and prostate cancer. Increased circulating MDSCs have also been correlated with tumor stage and metastatic spread in different types of tumors [3, 4]. Moreover, it has been demonstrated that MDSCs can be differently affected by standard-of-care therapies such as sunitinib, doxorubicin-cyclophosphamide, and docetaxel, as well as some immunotherapies. These findings suggest a potential use for these cells in immune monitoring of cancer patients receiving immunotherapies.

Recent clinical studies have demonstrated a correlation between increased numbers of TAMs and poor prognosis for esophageal, bladder, prostate, endometrial, breast, and lung cancers [5–8]. These data suggest a possible use of TAMs in the immune monitoring of cancer patients enrolled in clinical trials employing therapeutic vaccines.

While the major focus of post-vaccination monitoring is assessment of tumor antigen-specific immune responses, it is important to note that innate immunity mediated by natural killer cells, macrophages, dendritic cells, or granulocytes could significantly contribute to beneficial clinical outcome. Vaccines that engage and promote adaptive and innate antitumor responses appear to be most effective. Thus, at least some aspects of innate immunity should be monitored in clinical trials of antitumor vaccines.

The increasing number of immunotherapy clinical trials that use immunologic parameters as primary or secondary endpoints, as well as the availability of an increasing number of monitoring assays, highlights two aspects of immune monitoring. First, selection of assays that are most likely to correlate with clinical outcomes is a critical factor. This should be an informed and hypothesis-driven choice, considering factors such as cost and the need for serial monitoring with multiple assays. As understanding of the mechanisms of immune regulation increases, additional biomarkers will be identified that, hopefully, can be used as surrogate markers for immune responses to cancer vaccines. Second, quality control and assurance are essential for successful immune monitoring of cancer vaccines. All immune monitoring assays need to be standardized for reliability and consistency in order to establish their limitations and overall performance standards. Immune monitoring assays should be performed according to Good Laboratory Practice guidelines, such as those recently formulated based on recommendations from the iSBTc-SITC/FDA/NCI Workshop on Immunotherapy Biomarkers [9]. Only standardized monitoring assays are likely to be useful for defining surrogate endpoints of clinical response to antitumor vaccines.

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James L. Gulley
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Review Article

Immunologic Monitoring of Cellular Responses by Dendritic/Tumor Cell Fusion Vaccines

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Although dendritic cell (DC)- based cancer vaccines induce effective antitumor activities in murine models, only limited therapeutic results have been obtained in clinical trials. As cancer vaccines induce antitumor activities by eliciting or modifying immune responses in patients with cancer, the Response Evaluation Criteria in Solid Tumors (RECIST) and WHO criteria, designed to detect early effects of cytotoxic chemotherapy in solid tumors, may not provide a complete assessment of cancer vaccines. The problem may, in part, be resolved by carrying out immunologic cellular monitoring, which is one prerequisite for rational development of cancer vaccines. In this review, we will discuss immunologic monitoring of cellular responses for the evaluation of cancer vaccines including fusions of DC and whole tumor cell.

1. Introduction

The mechanism of action for most cancer vaccines is mainly mediated through cytotoxic T lymphocytes (CTLs). We are now gaining a clear understanding of the cellular events leading to an effective CTL-mediated antitumor immunity. The antigen-presenting cells (APCs) most suitable for cancer vaccines are dendritic cells (DCs), which can be distinguished from B cells and macrophages by their abundant expression of costimulatory molecules and abilities to initiate a strong primary immune response [1, 2]. DCs are specialized to capture and process tumor-associated antigens (TAAs), converting the proteins to peptides that are presented on major histocompatibility complex (MHC) class I and class II molecules [3]. After TAAs uptake and inflammatory stimulation, immature DCs in peripheral tissues undergo a maturation process characterized by the upregulation of costimulatory molecules [2, 3]. During this process, mature DCs migrate to T-cell areas of secondary lymphoid organs, where they present antigenic peptides to CD8+ and CD4+ T cells through MHC class I and class II pathways, respectively, and become competent to present antigens to T cells, thus initiating antigen-specific CTL responses [4]. Antigen-specific CTLs in turn can attack tumor cells that express cognate antigenic determinants or can provide help for B-cell responses that produce antibodies, which can also lead to tumor cell death in some cases [5]. Thus, the mechanism of action for cancer vaccines, based on harnessing host immune cells to infiltrate tumors and to exert CTL responses, is quite different from that of a traditional cytotoxic chemotherapy [6].

2. DC-Based Cancer Vaccines

A major area of investigation in induction of antitumor immunity involves the design of DC-based cancer vaccines [7]. DCs derive their potency from constitutive and inducible expression of essential costimulatory molecules including

B7, ICAM-1, LFA-1, LFA-3, and CD40 on the cell surface [1, 8, 9]. These proteins function in concert to generate a network of secondary signals essential for reinforcing the primary antigen-specific signals in T-cell activation. Therefore, many strategies have been developed to load TAAs onto DCs and used as cancer vaccines. For example, DCs are pulsed with synthetic peptides derived from the known antigens [10], tumor lysates [11], tumor RNA [12, 13], and dying tumor cells [14] to induce antigen-specific antitumor immunity. Although the production of DC-based cancer vaccines for individual patients with cancer has currently been addressed in clinical trials, a major drawback of these strategies comes from the limited number of known antigenic peptides available in many HLA contexts. Moreover, the results of clinical trials using DCs pulsed with antigen-specific peptides show that clinical responses have been found in a small number of patients [15, 16]. To overcome this limitation, we have proposed the fusions of DCs and whole tumor cell (DC/tumor) to generate cell hybrids with the characteristics of APCs able to process endogenously provided whole TAAs [17]. The whole tumor cells may be postulated to serve as the best source of antigens [17–21].

3. DC/Tumor Fusion Vaccines

The fusion of DC and tumor cell through chemical [17], physical [22], or biological means [23] creates a heterokaryon which combines DC-derived costimulatory molecules, efficient antigen-processing and -presentation machinery, and an abundance of tumor-derived antigens including those yet to be unidentified (Figure 1). Thus, the DC/tumor fusion cells combine the essential elements for presenting tumor antigens to host immune cells and for inducing effective antitumor responses. Now, it is becoming clear that the tumor antigens are processed along the endogenous pathway, through the antigen processing machinery of human DC. Thus, it is conceivable that tumor antigens synthesized de novo in the heterokaryon are processed and presented through the endogenous pathway. The advantage of DC/tumor fusion vaccines over pulsing DC with whole tumor lysates is that endogenously synthesized antigens have better access to MHC class I pathway [18–21]. Indeed, it has been demonstrated that DC/tumor fusion vaccines are superior to those involving other methods of DC loaded with antigenic proteins, peptides, tumor cell lysates, or irradiated tumor cells in murine models [18–21]. The efficacy of antitumor immunity induced by DC/tumor fusion vaccines has been demonstrated in murine models using melanoma [24–32], colorectal [17, 30, 31, 33–41], breast [42–47], esophageal [48], pancreatic [49, 50], hepatocellular [51–55], lung [22, 41, 56–59], renal cell [60] carcinoma, sarcoma [61–66], myeloma [67–74], mastocytoma [75], lymphoma [76], and neuroblastoma [77]. The fusion cells generated with human DC and tumor cell also have the ability to present multiple tumor antigens, thus increasing the frequency of responding T cells and maximizing antitumor immunity capable of killing tumor targets such as colon [78–84], gastric [85, 86], pancreatic [87], breast [47, 88–93], laryngeal [94], ovarian [95–97], lung [85, 98], prostate [99, 100], renal cell [101, 102], hepatocellular [103–105] carcinoma, leukemia [106–111], myeloma [112, 113], sarcoma [114, 115], melanoma [116–119], glioma [120], and plasmacytoma [121].

4. Monitoring of DC/Tumor Fusion Cell Preparations

Despite the strong preclinical evidences supporting the use of DC/tumor fusions for cancer vaccination, the results of clinical trials so far reported are conflicting [18–21]. One of the reasons is the evidence for fusion cell formation used as clinical trials is not definitive [23]. The levels of fusion efficiency, which can be quantified by determining the percentage of cells that coexpress tumor and DC antigens, are closely correlated with CTL induction in vitro [82, 83]. Another reason is immunosuppressive substances such as TGF-β derived from tumor cells used for fusion cell preparations [35, 47]. Although tumor-derived TGF-β reduces the efficacy of DC/tumor fusion vaccines via an in vivo mechanism [35], the reduction of TGF-β derived from the fusions inhibits the generation of Tregs and enhances antitumor immunity [47]. Moreover, the therapeutic effects in patients vaccinated by DC/tumor fusions are correlated with the characteristics of the DCs used as the fusion vaccines [82, 83]. Indeed, patient-derived fusions show inferior levels of MHC class II and costimulatory molecules and produce decreased levels of IL-12 and increased levels of IL-10, as compared with those obtained from fusions of tumor cell and DC from healthy donors [87, 103]. However, the fusion vaccines induce recovery of DC function in metastatic cancer patients [103]. Therefore, it is important to assess the phenotype and function of DC/tumor fusion cell preparations used in each vaccination.

5. In Vivo Monitoring

The delayed-type hypersensitivity (DTH) is an inflammatory reaction mainly mediated by CD4+ effector memory T cells that infiltrate the site of injection of an antigen against which the immune system has been primed by cancer vaccines [122]. Actually, soluble proteins, peptides, or antigens pulsed DCs have been injected intradermally, and the diameter of erythema or induration after 48–72 h is measured. CD4+ effector memory T cells that recognize the antigens presented on local APCs mediate the immune responses by releasing cytokines, resulting in an increased vascular permeability and the recruitment of monocytes and other inflammatory cells in the site. CD8+ T cells less frequently also mediate similar responses [123]. It has been reported that antigen-specific T cells can be readily detected in skin biopsies from DTH sites, much less in abdominal lymph nodes, and not in peripheral blood and tumor site [124]. Moreover, there is a significant correlation between favorable clinical outcome and the presence of vaccine-related antigen-specific T cells in biopsies from DTH sites [122]. Indeed, the increased DTH reactivity against tumor antigens has been observed in clinical responders by DC/tumor fusion vaccines [125]. In almost patients with cancer, T cells from lymph nodes and the tumor site itself are not readily available for

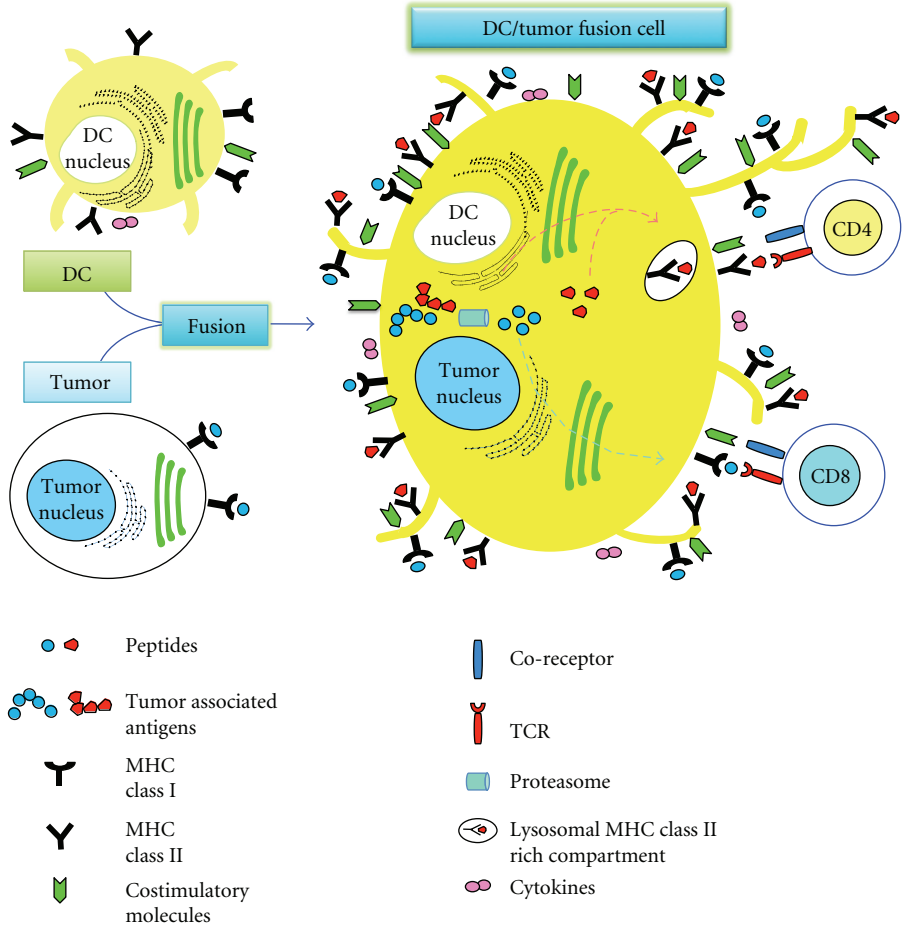

Figure 1: A model of antigen processing and presentation by DC/tumor fusion cell. DC/tumor fusion cell expresses MHC class I, class II, costimulatory molecules, and tumor-associated antigens. Tumor-associated antigens can be processed and presented through the antigen processing and presentation pathway of DC.

monitoring purposes. Therefore, functional assessment of antigen-specific T cells from such DTH sites may serve as an additional strategy to evaluate antigen-specific antitumor immune responses [122, 126, 127].

6. T-Cell Monitoring In Vitro

The mechanism of cancer vaccines, based on inducing CTLs, infiltrating tumors, and exerting T-cell-mediated cytotoxic effects, is quite different from that of cytotoxic chemotherapy. As cancer vaccines do not work as quickly as chemotherapy which has a direct cytotoxic effect, the Response Evaluation Criteria in Solid Tumors (RECIST) and WHO criteria [128, 129], designed to detect early effects of cytotoxic chemotherapy, cannot appropriately evaluate the response patterns observed with cancer vaccines. The RECIST criteria are highly dependent upon measurement of tumor size. They presume that linear measures are an adequate substitute for 2-dimentional methods and register four response categories: CR (complete response), PR (partial response), SD (stable disease), and PD (progressive disease). However, in the solid tumors, there exist not only antigen-specific CTLs but also immune suppressive cells such as myeloid-derived suppressor cells (MDSCs) [130], immunosuppressive tumor-associated macrophages (TAMs) [131], and cancer associated fibroblasts (CAFs) [132] (Figure 2). After vaccination, the solid tumors may become heavily infiltrated by immune-related cells resulting in an apparent increase in size of lesions, which is, at least in part, due to the infiltration of CTLs induced by cancer vaccines. Therefore, the development of new response criteria, including immunologic cellular monitoring, is of great importance in the development of cancer vaccines.

In clinical trials, the peripheral blood T-cell responses are generally accessible for serial analyses. The currently used methods of assessing T-cells from patients treated with cancer vaccines are T-cell proliferation, cytokine profile, cytotoxic T lymphocyte assays (CTL assays), CTL-associated molecules (CD107, perforin, granzyme B, and CD154), multimer analysis, T-cell receptor (TCR) gene usage, and immune suppression assays (Table 1). While these assays can be also used for monitoring cellular immune responses induced by DC/tumor fusion vaccines, none has been standardized. As DC/tumor fusion vaccines can induce defined

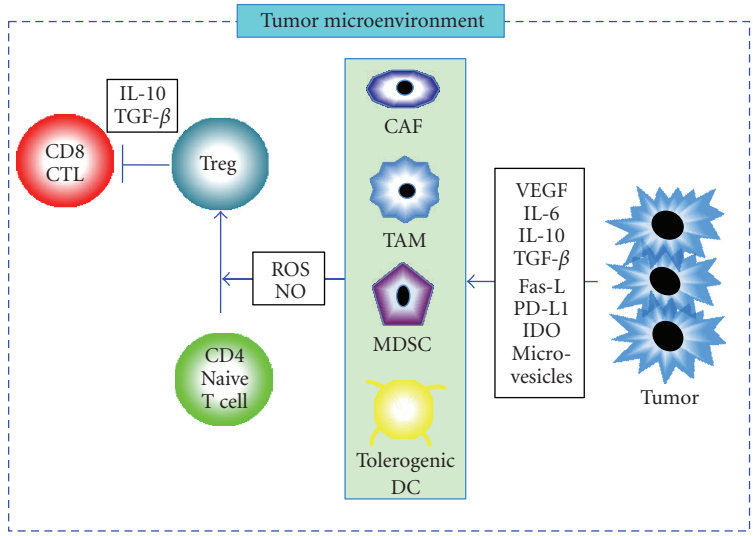

Figure 2: Immune suppressive responses at the tumor microenvironment. Tumor cells secrete various factors such as VEGF, IL-6, IL-10, TGF-β, Fas-L, IDO, PD-L1, and microvesicles, all of which promote the accumulation of heterogeneous populations of tumor-associated macrophage (TAM), myeloid-derived suppressor cell (MDSC), or tolerogenic DC. These immunosuppressive cells inhibit antitumor immunity by various mechanisms, including elaboration of reactive oxygen species (ROS) and nitrogen oxide (NO). The tumor microenvironment also promote the accumulation of regulatory T cell (Treg) that suppresses CD8+ CTL function through secretion of IL-10 or TGF-β from Tregs and tumor cells.

and undefined antigen-specific antitumor activities, immunologic cellular monitoring for the fusion vaccines is much more complex. Furthermore, as immune responses induced by DC/tumor fusion vaccines are a balanced mosaic of both immune stimulatory and suppressive responses [92], multiple monitoring assays for the clinical efficacy parameters may be needed to evaluate the antitumor immune responses.

6.1. T-Cell Proliferation. T-cell proliferation assay assesses the number and function at the level of the entire T-cell population in the culture. Therefore, the ability to detect T-cell responses is based on the proliferative potential of the cells in response to antigens. The most commonly used in vitro method for measuring antigen-specific T-cell proliferation is the assessment of T-cell clonal expansion following incubation of T-cells with antigens in the presence of a radiolabeled nucleotide (e.g., [^{3}H] thymidine) in vitro. CFSE (5-(and-6)-carboxyfluorescein diacetate succinimidyl ester) staining can be also used to directly detect proliferative responses of T-cells [82]. Because CFSE is partitioned equally during cell division [133], this technique can monitor T-cell division and determine the relationship between T-cell division and differentiation in vitro and in vivo. The extensive T-cell proliferation can be demonstrated by the few undivided T-cells left and from proper accumulation of activated T cells, as shown by the increase in T-cell counts correlating with the decrease in CFSE label for each division. The CFSE-based assays are equivalent to traditional measures of antigen-specific T-cell responsiveness and have significant advantages for the ability to gate on a specific population of T-cells and the concomitant measurement of T-cell phenotype [134]. After vaccination, DC/tumor fusion cells can migrate to the T-cell area in the regional lymph nodes and form clusters with CD8+ and CD4+ T cells [34]. Simultaneous recognition of cognate peptides presented by MHC class I and class II molecules on DC/tumor fusion cell is essential in the induction of

Table 1: Immunologic monitoring.

Inflammatory skin reaction	DTH
T-cell proliferation	[^{3}H] thymidine uptake
	CFSE dilution
Cytokine profile	ELISPOT assay
	Secretion of cytokines
	Intracellular cytokines
CTL assays	^{51}Cr-release assays
	Flow cytometry-based cytotoxicity assays (Caspase-3, Anexin-V)
CTL-associated molecules	Perforin
	Granzyme B
	CD107a and b expression in CD8+ T cells
	CD154 expression in CD4+ T cells
T cell phenotype	Multimer analysis
	TCR analysis
Immune suppression assays	CD25, FOXP3, IL-10, TGF-beta
	DTH; delayed type hypersensitivity
	CFSE; 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester
	TCR; T-cell receptor

efficient CTLs. Therefore, measuring antigen-specific CD8+ and CD4+ T-cell proliferation is essential to evaluate the induction of vaccine-specific immune responses. Although T-cell proliferation assay is usefulness to detect immune responses in vitro, the results are strongly influenced by the in vitro stimulation procedures. Stimulation of naive T cells from healthy donors with DC/tumor fusions in vitro results in potent proliferation of CD4+ and CD8+ T cells [34, 80]. Therefore, to assess DC/tumor fusion vaccines, antigen-specific CD4+ and CD8+ T cells need to be expanded by stimulation with autologous tumor lysates [103]. In addition, the frozen peripheral blood mononuclear cells (PBMCs) obtained before and after vaccination must be processed in the same set of experiments [103, 135, 136]. As T-cell proliferation assay is biologically irrelevant and imprecise for the reasons stated above, this assay may not be emphasized in future studies.

6.2. Cytokine Production. There is a currently great interest in the assay of polyfunctional T cells, secreting multiple cytokines (e.g., secreting IFN-γ and TNF-α rather than either alone), or expressing multiple surface markers. As the release of Th1 cytokines such as IFN-γ and TNF-α is important to determine long-lasting antitumor immunity, a shift to Th1 response by cancer vaccines is essential for therapeutic potential in murine models [36, 37, 67, 77, 137, 138]. Therefore, it is important to test whether cancer vaccines can induce a Th1 response in the tumor-specific T cells, and what impact might this have on the clinical responses. Cytokine production by T cells in response to antigens can be detected in individual T cells by enzyme-linked immunospot (ELISPOT) assay [18–21, 139]. Moreover, production of IFN-γ captured by antibodies bound to T-cell surface can be detected by flow cytometry analysis [96, 140]. The actual state of antigen specific T-cell reactivity directly from peripheral blood T cells can be quantified by IFN-γ ELISPOT assay and flow cytometry analysis [18–21, 141]. As the IFN-γ ELISPOT assay shows highly reproducible results among different laboratories, the ELISPOT may be an ideal candidate for robust monitoring of T-cell activity [18–21, 142]. Coculture of CD4+ and CD8+ T cells from healthy donors with DC/tumor fusions results in high levels of IFN-γ production and low levels of IL-10 production [50, 54, 80, 143]. Therefore, to assess DC/tumor fusion vaccines precisely, T cells obtained before and after vaccination might be directly quantified with stimulation of autologous tumor lysates in vitro [103]. In effective clinical responders, comparable levels of IFN-γ production in response to tumor lysates may be detected in PBMCs obtained before vaccination. A correlation between IFN-γ ELISPOT outcome and effective clinical responses (period free of relapse or survival) has been found in patients treated with cancer vaccines including DC/tumor fusions [103, 135, 136, 144].

6.3. CTL Assays. For immune monitoring of cancer vaccines, T-cell-mediated CTL assays are appealing because measurement of the ability of CTL to kill tumor targets is thought to be a relevant marker for antitumor activity. It has been assumed that the cytotoxicity has been measured in ^{51}Cr-release assays in vitro. One drawback to the CTL assays is their relative insensitivity. Instead of ^{51}Cr release assays, flow cytometry-based methods have been developed to assess CTL activity [145, 146]. Flow cytometry CTL assays can be predicated on measurement of CTL-induced caspase-3 or annexin-V activation in target cells through fluorescence detection, which are more sensitive to conventional ^{51}Cr release assays [145–147]. These assays show increased sensitivity at early time points after target/effector cell mixing and allow for analysis of target cells in real time at the single-cell level. However, it is unusual to detect antigen-specific killing by T cells directly isolated from the patients vaccinated with DC/tumor fusions even with the use of flow cytometry-based CTL assays [103, 148]. Therefore, there is a need to stimulate and expand the antigen-specific T cells in vitro for several days. These stimulations may distort the phenotype and function of the T-cell populations from tumor state. Moreover, it is difficult to obtain sufficient numbers of viable tumor cells from primary lesion due to the length of culture time and potential contamination of bacteria and fungus [79]. Thus, semiallogeneic targets with shared TAAs and MHC class I molecules are necessary instead of autologous targets. Importantly, a majority of the antigen-specific CD8+ CTLs in peripheral blood may not be tumor reactive due to various mechanisms such as downmodulation of MHC class I molecules on tumors and presence of Tregs at the tumor site. Indeed, cytotoxic activity against autologous targets has been observed in peripheral blood T cells from patients vaccinated with DC/tumor fusions by CTL assays [103, 148], but the clinical responses from early clinical trails in patients with melanoma, glioma, gastric, breast, and renal cancer are muted [103, 130, 134, 135, 142, 143, 148–154]. The defects of the clinical responses may be caused by the immunosuppressive influences derived from the local tumor microenvironment [103]. In addition, therapeutic antitumor immunity depends on highly migratory CTLs capable of trafficking between lymphoid and tumor sites [155]. Therefore, localization of antigen-specific CTLs demonstrated by analysis of biopsy samples from tumor sites may be directly associated with clinical responses [155].

6.4. Tumor-Specific CD8+ and CD4+ T Cells. The population of CD8+ CTLs can destroy tumor cells through effector molecules (e.g., perforin and granzyme B) [156]. Degranulation of CD107a and b is a requisite process of perforin/granzyme B-dependent lytic fashions mediated by responding antigen-specific CTLs. These perforin/granzyme B-dependent lytic fashions require degranulation of CD107a and b in CD8+ CTLs [5]. Therefore, measurement of CD107a and b, perforin, or granzyme B expression by flow cytometric analysis can be combined with intracellular IFN-γ staining to more completely assess the functionality of CD8+ CTLs [83, 87]. Moreover, autologous tumor-induced de novo CD154 expression in CD4+ T cells is highly sensitive for tumor-specific Th cells [157]. The coupling of CD154 expression with multiplexed measurements of

IFN-γ production provides a greater level of detail for the study of tumor-specific CD4+ T-cell responses. Although DC/tumor fusion vaccines have abilities to induce CD107+ IFN-γ+ CD8+ T cells and CD154+ IFN-γ+ CD4+ T cells upon autologous tumor encounter in vitro [83, 87], it has now been unclear the correlation of the assay with clinical outcome.

6.5. Multimer Assays. Now, it has become possible to analyze antigen-specific CD8+ and CD4+ T cells by flow cytometric analysis using multimeric MHC-peptide complexes, measuring the affinity of the TCR to a given epitope [158]. The MHC-peptide multimer analysis is more sensitive to conventional CTL assays [158]. Although DC/tumor fusion vaccines can induce defined and undefined antigens-specific CD8+ and CD4+ T cells, the multimer analysis can only be used to detect immune responses against defined antigenic epitopes expressing in tumor cells [21]. MHC-peptide multimers stably bind to the TCR exhibiting a certain minimal avidity. Hence, there are principal limitations of the multimeric analysis including the suitability and specificity of multimers and the lack of information about the functionality of multimer-positive T cells [158]. The specific role of the multimer-positive T cells for cancer vaccine efficacy has not yet been well established in the setting of clinical trials. Recent studies suggest that effective cancer vaccines not only stimulate CTL activity, but also sustain long-term memory T cells capable of mounting strong proliferative and functional responses to secondary tumor antigen challenge [159]. Therefore, it is more important to assess whether multimer-positive T cells are effector or effector-memory cells. Moreover, the combined use of multimers and functional assays such as IFN-γ analysis may have provided some insight into the functional activity of these cells. It has been demonstrated that cryopreserved PBMCs from melanoma patients vaccinated with gp100 peptide show that the majority of multimer-positive CD8+ T cells had either a long-term "effector" (CD45RA+ CCR7−) or an "effector-memory" (CD45RA− CCR7−) phenotype [160]. Interestingly, after vaccination, the resected melanoma patients can mount a significant antigen-specific CD8+ T cell immune response with a production of IFN-γ and high proliferation potential [160]. To date, no studies have evaluated the functional activity of multimer-positive T cells in the blood after vaccination with DC/tumor fusions.

6.6. TCR. Only T cells having a TCR specific for a given antigen are triggered by interaction with cancer vaccines. This activation results in the clonal expansion of antigen-specific T cells that can be followed by TCR Vβ gene usage. Recently, the availability of a large panel of monoclonal antibodies against TCRs, mainly Vβ epitopes, allows one to study the TCR repertoire by flow cytometry [161]. PCR techniques can also be used to detect a restricted TCR repertoire from small amounts of T cells without biases caused by ex vivo expansions [162]. Although DC/tumor fusion vaccines have resulted in selection and expansion of T-cell clones [87], the generation of antitumor immunity by CTLs has not correlated with clinical responses. Tumors may evade surveillance of CTLs by distinct mechanisms. Immunogenic tolerance to a particular set of antigens is the absence of an immune response to those antigens, which can be achieved by processes that result in T-cell anergy (antigen-specific unresponsiveness), T-cell unresponsiveness (generalized dysfunction), and T-cell deletion (apoptosis) [163]. Future fusion vaccine studies should be designed to determine whether T-cell dysfunction correlated with clinical outcome.

6.7. Immune Suppression Assays. Although antigen-specific CTLs can be generated and detected in the circulation of vaccinated patients, these do not usually act against the tumor. It has been documented that immune suppressive cells can counteract antitumor immune responses. In tumor microenvironment, there are not only CTLs but also many immune suppressive cells such as CD4+ CD25high+ Foxp3+ Tregs [103, 164], MDSCs [130], TAMs [131], and CAFs [132] (Figure 2). Moreover, tumor cells produce immunosuppressive substances such as transforming growth factor β (TGF-β) [165] vascular endothelial growth factor (VEGF) [166], IL-6 [167], IL-10 [167], soluble Fas ligand (Fas-L) [168], programmed death-1 ligand (PD-L1) [169], indolamine-2,3-dioxygenase (IDO) [170], and microvesicles [171]. Type 1 regulatory T cells (Tr1) expressing CD39 may mediate suppression by IL-10, TGF-β, and adenosine secretion, and whereby accumulation strongly correlates with the cancer progression [172]. The mechanisms that suppress the immune system provide a fundamental reason why cancer vaccines fail to induce consistently robust antitumor immune responses. In DC/tumor fusion vaccines, CD4+ CD25high+ Foxp3+ Tregs were promoted in the presence of the local tumor-related factors in vitro [103]. Moreover, increased CD4+ CD25high+ Foxp3+ Tregs impaired the effector function of CTLs induced by DC/tumor fusion vaccines [103]. Therefore, monitoring of immune suppressive cells in cancer patients vaccinated with DC/tumor fusions is also essential.

7. Conclusion

The development of assays for detecting immune responses associated with clinical outcome has been limited. A variety of assays had been introduced to provide monitoring tools necessary for following changes in the frequency of antigen-specific CTLs and to assess the impact of cancer vaccines on the immune system. As the mechanisms of immune response that cause tumor regression are not simple, the currently available assays may not actually measure a function with direct relevance to how tumors are actually attacked immunologically in cancer patients. A high reproducibility of results among different laboratories leads to the conclusion that cytokine flow cytometry or ELISPOT may be an ideal candidate for robust and reproducible monitoring of T-cell activity in vivo. However, the widely used ELISPOT assay often does not correlate the best with clinical outcome [173]. Therefore, it may be important to use a functional assay like

cytokine flow cytometry or ELISPOT in combination with a quantitative assay like multimers for immune monitoring. Furthermore, it is necessary to understand the immune responses seen in peripheral blood versus the responses at the tumor site. Monitoring of antigen-specific CTLs at the tumor site may be directly associated with clinical responses [155]. However, T cells from lymph nodes and the tumor site itself are not readily available for monitoring purposes in almost all patients. Therefore, the ability to assess the function of antigen-specific T cells from DTH site may serve as an additional strategy to evaluate cancer vaccines [122, 126, 127]. In our opinion, monitoring of multimer-positive CD8+ (effector or effector memory) T cells from the DTH sites or PBMCs with IFN-γ production by flow cytometry may be sensitive markers particularly associated with overall survival. In addition, the DC/tumor fusion vaccine studies should be designed to determine whether T cell dysfunction in the tumor microenvironment correlated with clinical outcome. This informations may help us more fully understand the mechanisms of cancer vaccines and its potency to hasten the progress of efficient cancer vaccine strategies into the clinic.

Disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the paper.

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Review Article

Profile of a Serial Killer: Cellular and Molecular Approaches to Study Individual Cytotoxic T-Cells following Therapeutic Vaccination

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T-cell vaccination may prevent or treat cancer and infectious diseases, but further progress is required to increase clinical efficacy. Step-by-step improvements of T-cell vaccination in phase I/II clinical studies combined with very detailed analysis of T-cell responses at the single cell level are the strategy of choice for the identification of the most promising vaccine candidates for testing in subsequent large-scale phase III clinical trials. Major aims are to fully identify the most efficient T-cells in anticancer therapy, to characterize their TCRs, and to pinpoint the mechanisms of T-cell recruitment and function in well-defined clinical situations. Here we discuss novel strategies for the assessment of human T-cell responses, revealing in part unprecedented insight into T-cell biology and novel structural principles that govern TCR-pMHC recognition. Together, the described approaches advance our knowledge of T-cell mediated-protection from human diseases.

1. Introduction

Despite major advancements in the fields of molecular and cellular biology, and the improved understanding of cancer formation and progression, the surgical removal of tumors remains the most effective therapeutic strategy against cancer. While radiation therapy and chemotoxic drugs are often employed to successfully prolong the disease-free survival or to slow down tumor progression, their limited specificity in targeting neoplastic cells is often responsible for a wide spectrum of common clinical side effects. In this respect, immunotherapy is a promising therapeutic alternative to avoid such side effects by activating the patient's own immune system against tumor cells. In this Paper we focused on selected aspects of current vaccination strategies against melanoma, as well as new and sophisticated tools employed by immunologists to analyze cellular immune responses by "zooming in" on single tumor-specific lymphocytes.

1.1. Melanoma. Melanoma arises from the pigment-producing melanocytes. It is the major cause of mortality among skin malignancies. The incidence is steadily increasing at rates over 3% per year, with many hundreds of new cases per 100 000 inhabitants, and mortality rates ranging from 0.1 to >10% [1]. These figures, however, differ largely depending on risk factors such as sun exposure depending on the local climate. Melanoma is one of the most antigenic and immunogenic cancers with a high percentage of tumors expressing well-characterized tumor-associated antigens. Immunotherapy targeting one or several of these tumor-expressed antigens has shown promising results over the past years in enhancing antitumor immune responses.

It is now well established that spontaneous tumor antigen-specific T-cell responses are generated in melanoma patients that can be detected both in the circulation as well as at the tumor sites. While spontaneous T-cell responses have been reported against cancer-germline antigens encoded by the MAGE family, and against NY-ESO-1, T-cell frequencies are generally very low (10^{-7} to 10^{-4}) [2]. An exception to this is the natural immune response observed in most melanoma patients against the Melan-A/MART1 differentiation antigen presented in the context of the MHC class I molecule HLA-A2. In untreated patients, frequencies of T-cells specific for this antigen usually range from 0.01% to 0.1% of total circulating $CD8^{pos}$ cells [3, 4]. These frequencies are often much higher in metastatic lymph nodes and other metastases of melanoma patients [5, 6]. In fact unusually high frequencies (10^{-3} to 10^{-4}) of A2/Melan-A-specific T lymphocytes are already found in the blood of newborns and healthy A2+ individuals. This population of self-peptide specific T-cells is preferentially selected in the thymus, presumably due to cross reactivity to unknown self-peptides. Consequently, large numbers of such cells are released from the thymus into the periphery as mature, naive precursor T-cells [7, 8]. Thus, the activation and expansion of this population of Melan-A-specific CD8 T lymphocytes to induce clinically relevant tumor cell lysis represents an important target of immunotherapy.

1.2. Therapeutic Immunization Strategies. The aim of an efficient cancer vaccine is to activate *de novo* the immune system against tumor cells and/or to enhance the preexisting tumor-specific response. An ideal vaccine would induce expansion of large populations of cytotoxic T-cells, with potent antitumor effector functions, both at the tumor-site but also as a systemic immune surveillance for long periods of time. The choice of adjuvants and antigenic peptides used, their combination, and timing are important factors. Currently there are three major approaches of immunotherapy: antigen-based vaccines, adoptive cell transfer of efficient antitumor T-cells, and stimulation of the immune system in an antigen-nonspecific manner.

Optimal vaccines consist of live or attenuated microbes. However, for many infectious diseases, and for cancers in general, such vaccines are not available. Therefore, synthetic vaccines are developed generally following the rational-based microbe-induced immune mechanisms. Synthetic vaccines are composed of at least two basic components: antigen and adjuvant. The rational of using antigens for cancer immunotherapy is based on the relatively large consensus that immune protection against malignant disease requires antigen-specific (adaptive) immune responses including T-cells. Some experts argue that stimulation of the innate immune system alone may be sufficient to generate tumor-specific immunity, since cancer tissue often produces tumor antigens allowing some activation of antigen-specific immune responses. Therefore, an increasing number of novel immune therapies are developed without taking advantage of (synthetic or recombinant) tumor antigens, essentially because this approach simplifies drug production and application. However, tumor cells produce only low amounts of antigen, which is often not present at the optimal location and/or time. Therefore, immune responses triggered by naturally expressed antigens are not sufficiently timed, neither strong nor anatomically focused to protect from tumor progression. In addition, immunotherapy without antigen often requires high and in part toxic drug doses, in contrast to vaccines containing synthetic antigens that can have powerful biological effects even at low doses. For these reasons we propose that synthetic cancer vaccines should include tumor antigens.

Besides antigen, the second essential vaccine component is the so-called immunological "adjuvant." Adjuvants are immune stimulating agents, which are important because immune responses remain poor when antigens are administered alone. For many years, adjuvants have been developed empirically, without significant progress in the understanding of their molecular nature and mechanisms of action. The discovery of dendritic cells (DCs) and of their central role in linking innate with adaptive immune responses was key for progress. Besides regulating central mechanisms of the innate immune system, DCs are the most effective antigen-presenting cells for enabling antigen-specific T- and B-cell responses. But how are they put in action? Only about 15 years ago it was discovered that DCs become activated due to triggering of pathogen recognition receptors (PRRs). These receptors enable the innate immune system to sense microbes. The best-characterized families of PRRs are the Toll-like receptors (TLRs) that bind microbial products [9–12]. Drugs that stimulate TLRs are promising adjuvants, particularly CpGs that bind to TLR9. CpGs are oligonucleotides containing CpG motifs similar to those observed in bacterial DNA. Our group has shown that vaccination with emulsions with IFA (Incomplete Freund's Adjuvant), containing antigenic peptides and CpG, rapidly induces strong human $CD8^{pos}$ T-cell responses (0.5%–8% of circulating $CD8^{pos}$ T-cells) in melanoma patients [13]. Moreover, it is the first synthetic vaccine formulation to consistently induce *ex vivo* detectable T-cell responses even when using a natural tumor peptide, that is, the natural Melan-A/MART-1 sequence [14].

A novel concept of lymphodepletion was recently introduced in the field of immunotherapy, with the aim of making "immunological space" and freeing up access to cytokines such as IL-7 and IL-15 for the tumor-specific T-cells to optimally expand. The rational for this is based on observations made from viral systems indicating that protective immune responses require large frequencies of antigen-specific T-cells. Recently, Rosenberg and colleagues [15, 16] have shown promising results with adoptive cell transfer of autologous melanoma-specific T-cells, combined with high-dose IL-2, in late stage melanoma patients, following transient lymphodepleting chemotherapy regimen. Although highly successful (objective antitumor response of 50% for stage IV melanoma), such a treatment is not easily available for a large number of patients, due to the requirement of highly specialized laboratories to support the delicate procedures of isolation and *in vitro* expansion of autologous T-cells, and the intensive care units necessary for the clinical management of the side effects caused by high-dose IL-2.

1.3. Insight into the Efficiency of a Vaccine Requires Detailed Analysis of the Immune Response. An increase in the number and frequency of antigen-specific T lymphocytes is often observed following peptide vaccination alone, thus achieving the first aim of candidate cancer vaccines. Unfortunately, the majority of these patients still experience tumor progression, despite increased numbers of tumor-specific T lymphocytes. It is believed that in the small number of patients showing disease stabilization or even tumor regression following immunotherapy, tumor-specific T-cells may succeed in infiltrating tumor tissue, destroying some of the tumor cells and temporarily reversing the local immunosuppressive environment. They would thus act like a spark for the activation, proliferation, and antitumor effector function of already infiltrated or newly activated tumor-specific T-cells.

It can thus be implied that a successful vaccine may not solely depend on the induction of large numbers of tumor-specific T lymphocytes but rather on the activation of a population of effector T-cells with potent antitumor activities and resistance to the local immunosuppressive environment (reviewed in [2]). In order to improve the clinical efficacy of such vaccines, a better characterization of the tumor-specific immune responses is required. Specifically each vaccine-induced immune response should be analyzed in terms of phenotype, function, selection of specific T-cells from the repertoire, and their affinity/avidity for the MHC-peptide complex. Importantly, one also needs to examine the changes and the degree of stability of these parameters over time and following repeated rounds of vaccination. Furthermore, a better understanding of the mechanisms that allow long-lasting memory T-cell survival and of strategies to increase T-cell migration and effector functions at the tumor site is needed.

1.4. Cancer versus Infection Models. From an immunological perspective, tumors can be compared with persistent viral infections, since in both situations T-cells may successfully protect from disease. The constant presence of antigen stimulates the immune system by continuously triggering antigen-specific T-cells. The major difference between cancer and infection is that, in the latter, the immune response is highly efficient in controlling the virus and in providing long-lasting protection. Contrarily, antitumor responses show less robustness compared with antiviral responses. Some of the explanations for this may be (i) immune tolerance against self-antigens, (ii) local immune suppression induced by tumors, and (iii) tumor escape mechanisms which render the existing immune responses less efficient. Nevertheless, chronic infections with genetically stable viruses such as Epstein-Barr virus (EBV) and cytomegalovirus (CMV) provide useful model situations for studying the generation and maintenance of highly efficient T-cell memory responses and for identification of T-cell correlates of protection in humans.

We divided this paper into six sections, each describing a particular aspect of current methodological approaches and technical tools used to dissect antigen-specific CD8 T-cell responses. Our strategy combining both *ex vivo* and *in vitro* analyses, and utilizing molecular and cellular approaches is schematically depicted in Figure 1. Each major component of this outline corresponds to one section of the text, thus providing a guideline for the reader throughout this paper. The observed similarities and differences between viral- and tumor-specific T-cell responses will be discussed, since they not only reveal the progress of novel vaccine formulations against cancers like melanoma, but also the obstacles which need to be overcome in order to further improve clinical efficacy.

2. Multiparameter Flow Cytometry Analysis of Antigen-Primed T Lymphocytes

The development of designed fluorescent peptide/MHC multimers (formerly called tetramers), which bind stably to specific TCR molecules on the surface of T-cells [5, 17], has revolutionized the detection of viral- and tumor-specific T-cells. This approach makes it possible to directly carry out subset analysis at the antigen-specific T-cell level *ex vivo* without preliminary *in vitro* rounds of antigen stimulation (reviewed in [18]). Multimer technology combined with multiparametric flow cytometry analysis has allowed gaining a better understanding of the T-cell specific immune responses against viruses and tumors. Furthermore, the *ex vivo* analysis of antigen-specific populations can be accompanied by the isolation of T-cell populations of interest, as well as the use of peptide-MHC multimers as a stimulus in *in vitro* functional assays. Briefly, PBMCs from vaccinated melanoma patients are first enriched for $CD8^{pos}$ T lymphocytes using anti-CD8-coated magnetic microbeads. Alternatively, T lymphocytes can be extracted from resected metastatic lymph nodes (TILNs) or distant soft/visceral metastases (TILs) [19]. Purified CD8 T-cells are then incubated using PE-labeled HLA-A*0201/peptide multimers [5, 17], in combination with fluorescent antibodies (i) for extracellular differentiation markers (e.g., CCR7, CD45RA, CD28, CD27), (ii) for cytolytic molecules (e.g., perforin, granzyme B), and/or (iii) for cytokines (IFNγ, TNFα) (Figure 1).

2.1. Defining Human CD8 T Lymphocyte Subpopulations. Major efforts have been made in recent years to understand the relationship between different T-cell subpopulations. An important task has been to define molecular markers that readily identify and isolate T-cells sharing discrete stages of cell differentiation. Circulating naive T lymphocytes form a relatively homogenous population expressing a well-defined set of surface glycoproteins and are characterized by the null expression of effector mediators (e.g., IFN-γ, TNF-α, granzyme B, perforin, FAS/CD95) and by proliferative potential (e.g., long telomeres, high detectable levels of TREC copies). During the past decades, primed antigen-experienced T lymphocytes have mostly been classified into two distinct subsets, for example, effector and memory cells [20]. Effectors are presumably rather short-lived, produce cytolytic effector molecules, and are capable of migrating to the site of infection and of killing target cells directly *ex vivo*. In contrast, memory cells are long lived, persist after

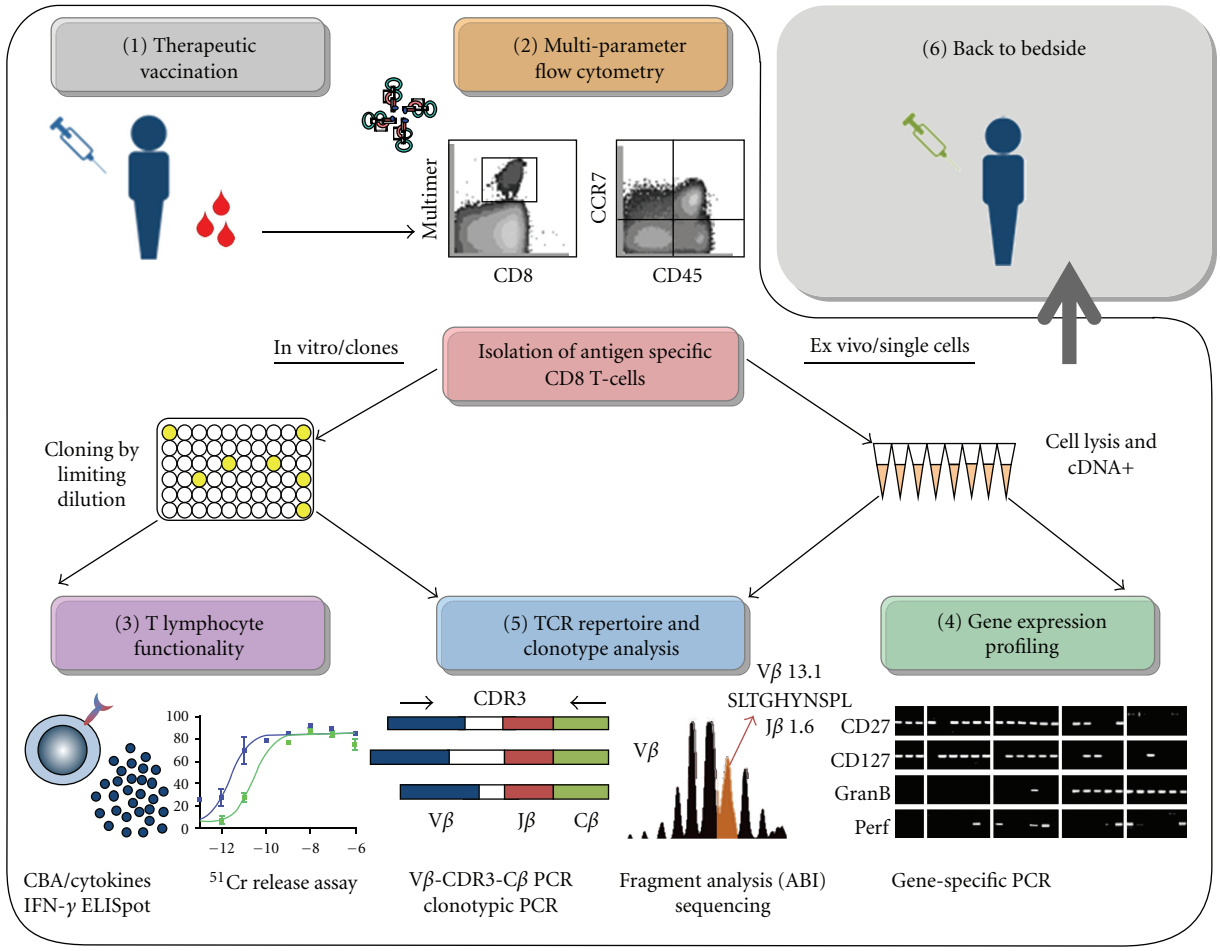

Figure 1: Cellular and molecular approaches for the characterization of cytotoxic T-cells following therapeutic vaccination. Each step (1 to 6) in the strategy bears the same name and section number as described in the body of text. (1) Patients undergoing therapeutic vaccination are monitored closely throughout the clinical study, and blood and tissue samples are collected at numerous time points. (2) Antigen-specific CD8 T-cell populations are first visualized by the use of fluorescent peptide-MHC multimers then analyzed for their phenotype and subset composition using multiparameter flow cytometry. T-cell populations of interest can subsequently be isolated for further *in vitro* [3, 5] or *ex vivo* [4, 5] studies. (3) *In vitro* generated T-cell clones can be subjected to a series of assays to determine their functionality, including target cell lysis (^{51}C release assay) and cytokine production (ELISpot). (4) *Ex vivo* sorted single cells are lysed and cDNA purified for gene-expression and TCR repertoire analysis. (5) Single cell samples or individual T-cell clones can be subjected to spectratyping, for the study of TCR repertoire diversity, selection, and clonotype composition. The unique signature of each T-cell can be identified, and its frequency determined. Furthermore, individual T-cell clonotypes can be followed across different T-cell compartments, and over time following therapeutic vaccination. (6) Conclusions drawn from this complete analysis of phenotype and functionality of vaccine-induced immune responses in melanoma patients can be taken back to the bedside. These results can be translated into improved therapeutic vaccination regimens aiming for more powerful immune activation and more efficient and specific antitumor responses.

pathogen clearance, and have increased survival properties and cell division capacities (reviewed in [21]). In line with this concept, human CD8 T-cells have been delineated with the help of two cell surface markers, the high isoform of the common lymphocyte antigen CD45RA and the chemokine receptor CCR7, and based on their anatomical location [22–24]. Central memory (T_{CM}) T-cells are characterized by the ability to repeatedly circulate into lymph nodes and eventually encounter antigen presented by incoming CCR7^{pos} mature dendritic cells, in contrast to effector memory (T_{EM}) cells which downregulate CCR7 and appear specialized in migrating to peripheral nonlymphoid tissues.

Although this two-marker procedure to identify functionally distinct CD8 T-cell subsets has proven popular, the recent technical improvements in the ability to dissect the immune response using multiparameter flow cytometry have indicated the existence of highly heterogeneous antigen-primed CD8 T subpopulations [25–38]. Importantly, the term of "effector" and "memory" CD8^{pos}cell may only apply for situations of acute and resolved infections after which the pathogen is cleared from the host, and not in situations of persistent latent or chronic active infections where it becomes more difficult to use this simplified view of defining primed T-cells (reviewed in [39, 40]). Indeed, in recent years,

a variety of other phenotypical and functional markers have been added allowing at present the discrimination of a wide spectrum of antigen-experienced CD8 T-cells with different phenotypes, functions, and half-lives and that may account for the heterogeneity of "memory" and "effector" cells observed following persistent chronic infections (reviewed in [40, 41]).

2.2. Phenotype of Tumor Antigen-Specific T-Cells following Therapeutic Vaccination. Since the fast advancing flow cytometry technology allows us to analyze as many as 20 fluorochromes simultaneously using 7 lasers, the difficult task now becomes the choice of the appropriate extracellular and intracellular markers, which would be the most informative in terms of the phenotypic and functional characteristics. Therefore, our own strategy of monitoring antitumor T-cell responses following peptide vaccination of melanoma patients relies on studies that we perform using well-established surface markers that allow to best correlate a particular phenotype of a T-cell with its function *in vivo*.

First, we uncovered additional heterogeneity among effector memory and effector CD8 T-cell subsets by studying the functional attributes of such T-cells distinguished on the basis of expression of the costimulatory receptors CD27 and CD28 [32, 34, 42]. Our studies show that these subsets can be subdivided into early differentiated ($CCR7^{-}CD45RA^{-}CD27^{+}CD28^{+}$) or late differentiated ($CCR7^{-}CD45RA^{+/-}CD27^{+/-}CD28^{-}$) T-cells. Early differentiated cells express low levels of effector mediators such as granzyme B and perforin and high levels of CD127/IL-7Rα, have a relatively short replicative history (long telomeres, detectable copies of TRECs), and display strong *ex vivo* telomerase activity. Therefore, these cells are closely related to central memory T-cells ($CCR7^{+}CD45RA^{-}CD27^{+}CD28^{+}$). Conversely, late differentiated cells have undergone additional rounds of *in vivo* cell division and share effector-type properties with increased expression of effector mediators and strong *ex vivo* cytolytic activity (Figure 2). In line with these data, our group more recently reported on the detailed analysis of CD8 T-cell responses specific for EBV and CMV viruses [43]. During chronic infection with EBV and CMV, $CD8^{pos}$ T-cell responses to A2/GLC and A2/NLV antigens, respectively, are composed of heterogeneous populations of T-cells of various differentiation stages, with EBV-specific responses being less differentiated than CMV-specific ones [25–38]. Although the sizes/proportions of these subsets varied between EBV and CMV responses, our recent observations revealed that all subsets, from early differentiated to late differentiated stages, were found in both viral-specific responses [43]. Remarkably, this finding was also observed for CD8 T-cells specific for the influenza matrix protein peptide (Flu-MA), where up to 20% of these cells were composed of effector T-cells, alongside the predominant memory cells [44].

Over the past decade, our group performed extensive work on antitumor responses against melanoma expressed antigens before as well as upon peptide vaccination [3, 13, 45–52], and we showed that vaccination is often found to increase the number of tumor antigen-specific T-cells and to induce their cell differentiation. Phenotypic analysis of Melan-A/MART-1 specific T-cells in vaccinated patients showed similarities to that of virus-specific (EBV and CMV) T-cells, in that they were comprised primarily of effector memory (T_{EM} or EM) cells containing both early (EM $CD28^{+}$ defined as $EM28^{pos}$) and late differentiated (EM $CD28^{-}$ defined as $EM28^{neg}$) T-cells. Compared with the viral system where we observed almost identical phenotypes among individuals (i.e., proportions of $EM28^{+}$ and $EM28^{-}$ for EBV-specific T-cells), the overall phenotype of Melan-A/MART-1 specific T-cells, specifically the proportion of $EM28^{pos}$ versus $EM28^{neg}$ T-cells, seems to be more variable and to depend on each patient and his/her vaccination history. Nevertheless, peptide vaccination has been shown to drive the Melan-A/MART-1 response towards cell differentiation with progressive upregulation of effector mediators and cytolytic activity. From this point of view, current vaccination strategies emerge as progressively successful in inducing tumor-specific T-cells at high frequencies and with similar phenotypic and functional characteristics as those associated with long-lasting protective responses (Rufer, Speiser, et al., *unpublished observations*).

2.3. Model of CD8 T-Cell Differentiation. Altogether, these in-depth analyses [32, 34, 45, 48] are in agreement with a model according to which there is a differentiation pathway with the stepwise loss of homing (CCR7), costimulatory (CD27, CD28), and cytokine (IL-7Rα) receptors, as well as concomitant upregulation of molecules involved in cell-cell adhesion and target cell destruction (Figure 2). Importantly, influenza-, EBV-, and CMV-specific T-cells follow the same pathway of cell differentiation, although CMV-specific cells are more frequently late differentiated than influenza- and EBV-specific cells [43, 44].

The pathway of T-cell differentiation described here (Figure 2) appears also to apply to self-/tumor-specific T-cells such as Melan-A/MART-1 and NY-ESO-1 in melanoma patients [45, 48], aside from being found in T-cells specific for persistent viruses [29, 43], in T-cells responding to acute viral infections like influenza [44], and in other types of T-cells ($\gamma\delta^{pos}$, $CD4^{pos}$) [53]. Essentially, following antigen stimulation, less differentiated cells like naive and central memory T-cells expressing CCR7 would initially differentiate into effector memory cells, then ultimately into highly differentiated effector type of cells (Figure 2). Sallusto and colleagues suggested that this differentiation process will depend upon the signal-strength of interaction between the T-cell and the antigen-presenting cell during the initial priming and expansion phase, specifically on factors such as the affinity of the TCR-MHC-peptide interaction, the concentration of antigen, and costimulatory molecules and cytokines [23, 54, 55]. In this model, T-cells that receive a weak signal will be unfit to differentiate and will rapidly die [56]. An intermediate signal or a strong signal that is followed by the clearance of antigen will result in the development of fit memory cells which can eventually give rise to precursors for rapid effector cells generation upon

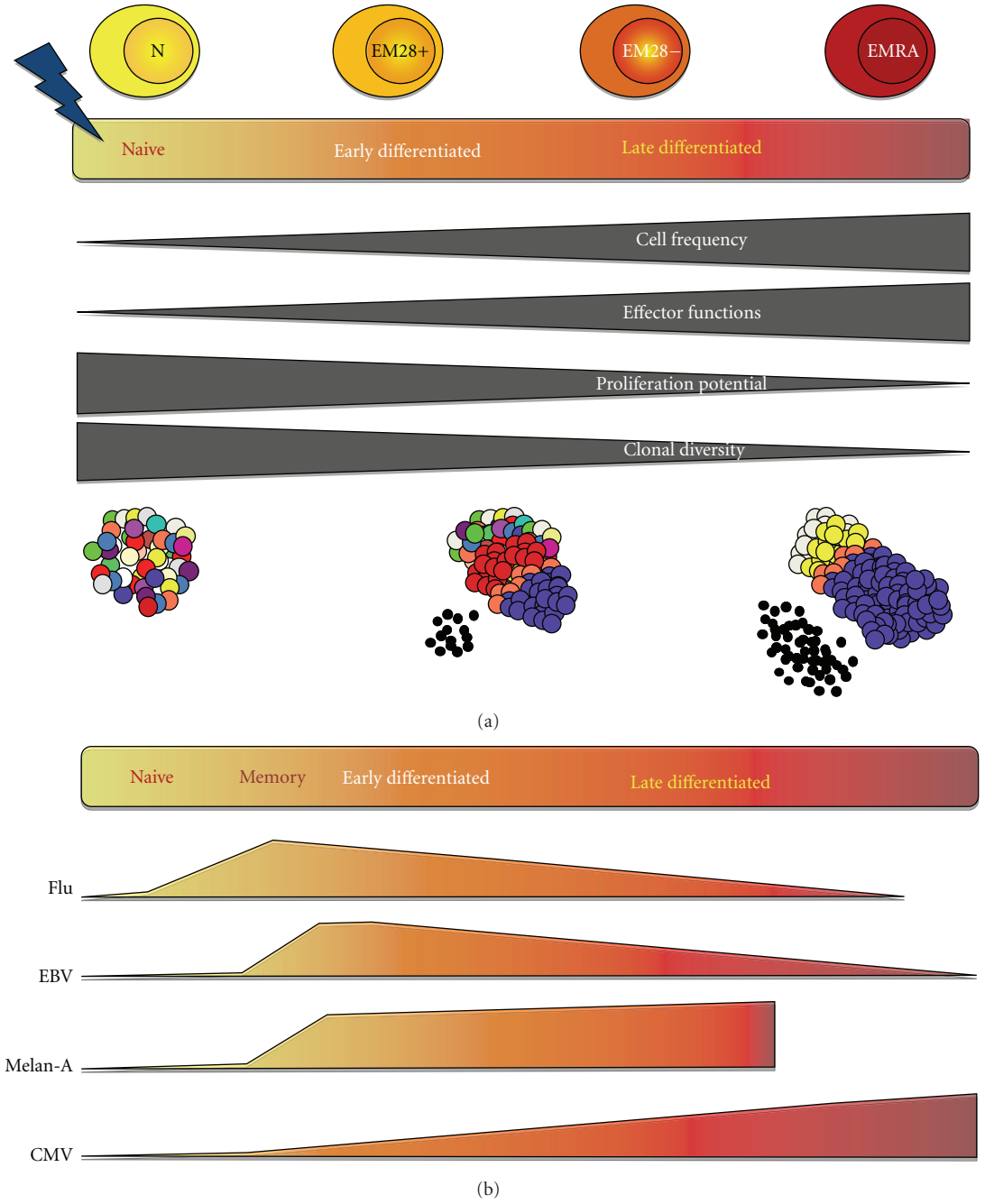

Figure 2: Model of CD8 T-cell differentiation. (a) The highly diverse naive T-cell compartment contains T-cell clonotypes of very low frequency (represented by one color), and null expression of effector mediators but high potential for proliferation in response to antigen. Following encounter with cognate antigen, CD8 T-cells are activated and begin a process of cellular differentiation characterized by a gradient of early- and late-differentiated cellular states, that can be described by changes within four major parameters as depicted by dark grey blocks. As a result of the massive cellular proliferation that ensues activation, there is an increase in the frequency of T-cells and a restriction in the TCR diversity, since the pool of primed differentiated T-cells is composed of a small number of clonally expanded T-cells (blue, white, yellow, and orange T-cells). Concomitantly, there is a decrease in proliferative potential from naive and early differentiated T-cell subsets towards highly differentiated subsets. Moreover the accumulation of effector functions (high production of effector molecules, small black dots) in these expanded populations of T-cells defines further maturation of differentiated T-cells. (b) The degree of differentiation of T-cells along this spectrum has been shown to vary with antigen specificity. As such, the antigen-specific responses against influenza virus and against persistent EBV and CMV viruses are compared with the tumor-specific response against the Melan-A antigen, while taking into account the four parameters described in (a). EBV-specific cells are more differentiated than influenza-specific cells but less so than CMV-specific cells which are composed primarily of highly differentiated CD8 T-cells. While both early and late differentiated subsets have also been identified in Melan-A specific responses, their proportions are highly variable between different patients following vaccination. In addition, tumor-specific responses contain a much lower fraction of highly differentiated EMRA (effector memory CD45RA+) cells compared with viral-specific responses.

antigen restimulation. When T-cells receive a strong signal, they differentiate towards effectors.

Thus, the presence and concentration of antigen would be one of the main factors determining at what point along this differentiation spectrum, each memory response would be found. In other words, the relative proportions of "memory-like" T-cell-subsets compared with the "effector-like" T-cell subsets would account for the observed differences between various immune responses. Specifically, in the presence of persistent antigen, EBV-specific memory cells will be driven towards an effector memory phenotype. Contrarily, several weeks or months after clearance of pathogens causing short-term acute infections, that is, in absence of antigen, the central memory compartment would be dominant, while a lower fraction of T-cells would exhibit an effector memory phenotype. We also propose that early (memory-like) and late differentiated (effector-like) cells serve important but distinct purposes to the overall antigen-specific immune response and in providing protection against different viruses. Such observations are in line with those made in the mouse model (reviewed in [40]), as it was shown that while immediate protection is better provided by differentiated effector-type T-cells present in peripheral tissues [57], early differentiated (T_{CM}-like) cells are most potent at protecting against systemic infection with lymphocytic choriomeningitis virus and respiratory challenge infections [57–59].

2.4. Recently Improved Strategies to Multiparameter Flow-Cytometry Analysis. The *ex vivo* characterization of T-cells is limited to T-cell populations with a frequency higher than 0.01% among total CD8 T-cells [3, 4]. This is more problematic for T-cell specific responses of rather low frequencies such as those generated against tumor antigens, in contrast to the relatively frequent viral specific T-cell responses. To address such problems, two groups have recently developed techniques for simultaneous analysis of T-cells with multiple different antigen specificity. The first consists in the combination of 4 fluorochromes for each T-cell specificity instead of a single one, allowing the detection of 15 different specificities in parallel [60]. The other strategy is to increase the number of peptide-MHC multimers labeled with a two-dimensional combination of fluorochromes using six different quantum dots (QD565, QD585, QD605, QD655,QD705, and QD800) and the two most intense fluorochromes PE and APC, which allows the visualization of T-cells with up to 28 different specificities [61]. While both strategies make it possible to simultaneously analyze numerous antigen-specific responses in a single step, they require careful setup of experimental controls. Furthermore, these approaches are currently limited on one hand by the development and application of sophisticated reagents and on the other by the thorough analysis of the data. Nevertheless, aside from decreasing the time of the experimental procedure, one of the most important advantages is that they allow the investigator to save precious material from patients and donors, since the sample does not have to be subdivided for multiple separate analyses [61].

The development of MHC class II multimers has been much less successful compared with MHC class I multimers due to several reasons. First, human MHC class II molecules are highly polymorphic. Second, peptide binding affinity is generally lower for class II as opposed to class I molecules, which is particularly evident for peptides derived from tumors. One of the most successful strategies has proven to be the initial production of HLA-DR molecules loaded with a "placeholder" peptide followed by a peptide exchange step using the peptide of interest. The efficient replacement by the peptide of interest remains one of the major limitations. Recently, Ayyoub and colleagues reported DR52b/NY-ESO-1 multimers using the strategy of His-tagged peptides that allows isolation of folded MHC/peptide monomers by affinity purification before tetramerization [62]. Using these molecules, they could detect *ex vivo* CD4 T-cell responses to the NY-ESO-1 peptide in patients undergoing a vaccination trial with recombinant NY-ESO-1 protein, Montanide ISA 51 (Seppic, France), and CpG ODN 7909 (Pfizer, USA). The application of this strategy to other tumor- and self-antigen-derived peptides may significantly accelerate the development of reliable MHC class II multimers to monitor antigen-specific CD4 T-cells.

2.5. Advantages and Limitations of the Multiparameter Flow Cytometry Analysis. The major advantage of the multiparameter flow-cytometric analysis is that it allows to *ex vivo* visualize the antigen-specific pool of lymphocytes and to divide it into numerous subpopulations. As discussed above, antigen-experienced T lymphocytes are highly heterogeneous and are composed of a wide range of discrete subsets. It is thus of high importance to elucidate the roles of these different subsets and to identify how they are regulated. In that regard, *in vivo/ex vivo* analyses remain the gold standard, and flow cytometry represents an instrumental technology to study antigen-specific T-cell biology *ex vivo*. Moreover, differences between various T-cell populations within the same response can only be revealed in such studies. On the contrary, the phenotypic analysis of a T-cell response as a whole can lead to false conclusions and biases induced by the predominant subset, while missing the presence of a less frequent yet biologically important population. Multiparameter flow cytometry technology allows the parallel detection of multiple activation markers, such as the expression of cytokines and chemokines by specific populations of cells allowing the fine characterization of particular profiles. Thereby it is possible to determine which parameters define distinct functional subsets in different antigen-specific T-cell contexts. For instance, Makedonas and colleagues recently showed that a polyfunctional response led by IL-2 upregulation was necessary for the control of EBV and the clearance of influenza, while conversely a strong perforin production was rather important in the clearance of CMV and adenovirus infection [63]. Lastly the technology allows to sort each of these populations of interest into various forms (tubes, plates), cell numbers (single cells), and for different purposes (*in vitro* cloning, *ex vivo* functional assays, and molecular assays) as will be discussed in the following sections.

3. Assessing Functionality of Antigen-Specific T Lymphocytes

It is evident that the analysis of phenotype of T lymphocytes using multiparameter flow cytometry can reveal a high degree of heterogeneity, especially among the antigen primed T-cell population. However, one should not only judge a book by its cover, not a T-cell solely on its phenotype since a high degree of heterogeneity exists even for T lymphocytes of similar phenotypes or alternatively, similar functions for T lymphocytes of distinct phenotypes. Thus, characterizing the functional capacities of each individual T lymphocyte is crucial to the understanding of their roles *in vivo* and to designing future strategies to enhance and prolong these functions through therapeutic vaccination. This section will discuss current methods to study T-cell function either directly *ex vivo* or following expansion *in vitro* and outline the lessons learned about the functionality of viral and tumor-specific T lymphocytes.

3.1. Current Strategies for Measuring T Lymphocyte Function. Helper CD4 T-cells have been shown to be important in the generation of efficient and long-lasting memory responses; however, cytotoxic CD8 T-cells remain the key effectors in the fight against most viral infections and tumors. The functionality of CD8 T-cells can be studied *in vitro* or *ex vivo*, whereby three parameters are often measured: cytotoxicity, cytokine secretion, and proliferation.

The first pathway of cytotoxicity involves the engagement of death receptors (TNF-α and Fas/FasL). The flow-cytometric analysis of the expression of these receptors by various T-cell subsets is possible; however, current *in vitro* assays are not sufficiently sensitive to correlate this with differences in cytotoxic capacities by the same cells (reviewed in [41]). The second pathway employed by CD8 T-cells is the granzyme-perforin-dependent cytotoxicity. This is a highly complex mechanism, which can be very difficult to assess using currently available approaches and extrapolated to the true capacities of a T-cell *in vivo*. Specifically, the cytotoxic potential of a T-cell *in vivo* will depend upon several factors, such as the level of constitutive expression of granzyme and perforin and storage into lytic organelles, the quantity released following activation, and the rate of regeneration of these molecules. These parameters may already differ significantly from cell to cell *in vivo* and may be further altered following *in vitro* stimulation [41].

Nevertheless, numerous assays exist to study CD8 T-cell cytolysis, the first being the degree of target cell death induced by CD8 T-cells in an antigen-specific manner. *In vitro* generated clones expanded in medium supplemented with human serum and recombinant human IL-2 are commonly used in such assays. One of the major advantages of this approach is that the periodic restimulation of these clones using phytohemagglutinin (PHA) and irradiated allogeneic PBMC as feeder cells allows the generation of a large number of cells and for extended periods of time. This opens the possibility for experiments to be repeated, as well as the simultaneous testing of several conditions (antigenic peptide concentrations, effector to target ratios). The classical method of determining the *in vitro* lytic activity of antigen-specific T-cell clones is through the 4 h ^{51}Cr release assays using antigenic peptide-pulsed T2 target cells (HLA-A2$^+$/TAP$^{-/-}$) [64]. In the case of tumor-reactive T-cells, melanoma tumor cell lines such as Me 275 and Me 290 (HLA-A2$^+$/Melan-A$^+$) and NA8-MEL (HLA-A2$^+$/Melan-A$^-$) can also be used as target cells in the presence or absence of exogenous Melan-A peptide [8]. The percentage of specific lysis is then calculated in terms of the observed level of ^{51}Cr released compared with the spontaneous and total possible release by the target cells. To quantify the efficiency of T-cell—target cell recognition, results can be expressed in terms of the amount of peptide required to reach 50% of the maximal lysis (EC50). Alternatively the lytic activity of a T-cell can be measured in a CD8 coreceptor independent setting, specifically using C1R target cells transfected with mutant HLA-A2 molecules that abrogate the docking on the CD8 molecule [43, 65]. When these results are compared with the level of lysis using targets expressing HLA-A2 wild type, such assays allow the determination of the degree of CD8 dependency, which can in turn be an indicator of the level of binding avidity of the TCR for the peptide-MHC molecule. While the ^{51}Cr-release assay has several practical advantages, it is not the most sensitive approach to detect small differences in lytic abilities of different clones, particularly since the prior *in vitro* expansion of T-cells may have dramatically altered their intrinsic cytotoxic machinery.

The alternative approach to study cell-mediated cytotoxicity is through the multiparameter flow cytometry analysis of markers associated with cellular death by the target cell or with the cytolytic potential of the effector T-cell. The most popular approaches for the former are based on the detection of caspase activation, annexin V binding to apoptotic cells, and uptake of propidium iodine (PI) or 7-amino-actinomycin D (7-AAD) by dead or dying target cells (reviewed in [66]). On the other hand, the most commonly used markers to detect potential killer T-cells versus non killers are granzymes A, B, and perforin. To overcome the constraint of having to fix the cells to allow intracellular staining of these proteins, Betts et al. described a novel strategy allowing the visualization of antigen-specific T-cells expressing the cell surface CD107 degranulation marker [67]. Accumulating evidence now shows that the expression of CD107 by CD8 T-cells correlates well with their cytotoxic potential and as a result CD107 is becoming routinely used in clinical trial monitoring alongside markers of activation and subset classification (discussed in [66]).

The Live Count Assay (LCA) was first described by Devêvre et al. It combines several much-needed parameters in T-cell functionality measurements. The LCA is a highly sensitive, *ex vivo* method of analyzing low numbers of antigen-specific T-cells for their cytolytic potential. Briefly a 1 : 1 mixture of specific and control target cells labeled with CMTMR is added to fluorescently sorted antigen-specific CD8$^+$ T-cells and incubated for 4 h in the presence of degranulation marker CD107a- and CD8-specific mAbs. Samples are harvested, resuspended in staining buffer containing DAPI and analyzed by flow-cytometry [68]. The optimization of the existing LCA protocol in order to

ensure minimal sample consumption during fluorescence sorting process is ongoing (Mahnke, Devevre, Speiser et al.; *manuscript in submission*).

Experimental approaches, which allow the determination of T-cell function along with T-cell quantification, continue to be of high interest. For instance the ELISpot assay allows the detection of T-cells having the capacity to produce IFN-γ, a major cytokine secreted by CD8 T-cells and having important actions in increasing CTL-mediated cytotoxicity. The high sensitivity of this method makes it suitable for *ex vivo* analyses of populations with low frequencies of antigen-specific T-cells and of precious samples (PBMC, TIL, TILN). Moreover it has been used for the characterization of fine antigen-specificity and cross-reactivity on Melan-A-specific T-cell clones [69] using a library of peptides predicted to be potentially cross-reactive with the Melan-A peptide [70]. The authors showed that increased differentiation of derived clones was associated with a decrease in TCR cross-reactivity and an increased specificity against the native Melan-A peptide [69].

The simultaneous detection of multiple cytokines using approaches such as the cytometric bead array (CBA from BD) or Luminex has significantly advanced the field beyond the commonly used ELISA technique. While these approaches are highly sensitive in detecting low amounts of expressed cytokines, they require prior stimulation using specific peptides and stimulating cells (DC or tumor cells). Alternatively the intracellular cytokine staining (ICS) allows the detection of cytokines expressed *ex vivo* by T-cells and their subsets. The major drawback of the ICS is that it requires fixation of the cells, meaning that viable cells expressing a cytokine of interest cannot be isolated and placed in culture to further determine its functional properties. Nevertheless this flow cytometry-based technique allows the simultaneous analysis of other intracellularly and extracellularly expressed markers, which has recently improved our understanding of the polyfunctionality of T-cells. Specifically, recent data suggest that cells capable of secreting multiple cytokines may play important roles in the control of HIV infection, since an immune response comprising such multifunctional cells was more frequently observed in individuals who maintained a low level of the virus and normal levels of the CD4+ T-cells for many years (reviewed in [41]).

Last but not least, a proliferative potential of fully functional cytotoxic T-cells is an equally important parameter to an efficient immune response. As with many other methods used to study functionality, the limitations of some of the approaches used to study proliferation (BrdU staining, CFSE dilution) involve an *in vitro* stimulation step prior to the analysis. These assays can be informative in making the distinction between cells with a high capacity for proliferation such as central memory cells versus cells with a lower capacity like highly differentiated cells, but may be less sensitive to small differences between more functionally similar subsets. Furthermore, a more accurate estimate of the *in vivo* proliferative potential and history of a T-cell may be provided by *ex vivo* strategies. Currently it is possible to determine proliferative activity using Ki67 staining of T-cells *ex vivo*, although this is also limited by an inability to keep such cells viable for additional tests. The degree of telomere shortening is another parameter, which can provide information about the extent of *in vivo* proliferation of a T-cell. Two of the most precise techniques currently used to measure telomere length are flow-FISH, based on the hybridization of telomere repeats using fluorescently labeled probes [71, 72], and STELA (single telomere length analysis) [73], a PCR-based technique of amplification of telomeric and subtelomeric regions in individual chromosomes. Future studies are thus also needed to get a more precise measurement of telomere length in small number of cells, and even single cells. However, since telomere dynamics does not seem to provide the only explanation for the persistence of T-cell clonotypes (discussed in [74]), other mechanisms of long-term survival *in vivo* should be investigated. Specifically, the roles of IL-7 and IL-15 survival cytokines should be further investigated in various T-cell populations across the differentiation spectrum, as are molecules involved in increased survival and resistance to apoptosis such as Bcl2. Finally, the replicative history of T lymphocytes can also be investigated by quantifying their content of TRECs, which are stable DNA episomes formed during TCR-α rearrangement and are diluted out with each cell division [75].

3.2. Future Targets for the Study of T Lymphocyte Functionality. The fast advancing field of multiparameter flow cytometry will continue to open many opportunities for the analysis of the functional profiles of phenotypically distinct subsets of antigen-specific T-cells in an *ex vivo* fashion. It is now even possible to observe the events of the downstream signaling cascades to the T-cell receptor or chemokine receptors at the single cell level. For example, the level of Phospho-Stat1 (pY701) and phospho-Stat5 (pY694) can be observed after 15-minute stimulation with either interferon alpha and gamma or IL-2/IL-15 on tumor antigen-specific CD8 T-cells [76]. In addition one of the downstream events of T-cell activation via the T-cell receptor is the phosphorylation of ERK1/2 (pYr202/pY204). Contact of antigen-specific peptide-pulsed antigen-presenting cells (APCs) leads to the phosphorylation of ERK1/2 which can be detected as a shift of the activated antigen-specific T-cells compared to unstimulated control cells [77]. Another parameter which can be useful in obtaining information about the capacity of tumor antigen-specific T-cells to react to an appropriate signal is the expression of the CD3zeta chain by intracellular staining which can be compared with the level expressed by total CD8 T-cells and viral antigen-specific T-cells [78, 79]. Importantly, additional markers allowing to select viable T-cells for further *ex vivo* cellular and molecular assays of T lymphocyte functionality are needed in the future, in order to enhance our understanding of the functional properties that correlate with immune protection.

3.3. The Functional Capacities of Tumor Reactive T Lymphocytes. It is now well established that an efficient antigen-specific CD8 T-cell response is one characterized by effector

cells with a strong ability to lyse antigen-specific target cells. This is highly important in viral disease, both for acute (e.g., influenza) as well as for chronic or persistent (e.g., herpes viruses) infections. It is now commonly accepted that T-cells specific for foreign (e.g., viral) antigens can give rise to strong protective immune responses, whereas self/tumor antigen-specific T-cells are thought to be less efficient. Therefore, it is of great importance to study the functional capacity, in particular the multifunctionality, of tumor-reactive T lymphocytes before and following therapeutic vaccination (Figure 1). We previously described a study on the functional and proliferative potential of a dominant CD8 T-cell response directed against NY-ESO-1 [48]. We could identify two functionally distinct populations of tumor-specific T-cells. The major population making up to 90% of the cells ($EM28^{neg}$) displayed the hallmarks of highly differentiated and active effector T-cells. These cells were able to mediate efficient *ex vivo* killing, produced IFN-γ, and still retained proliferative capacity upon antigenic stimulation, while up-regulating NK-like receptors such as CD57, CD94 as well as PD-1, an inhibitory receptor. The other relatively minor population ($EM28^{pos}$), representing between 5 to 10% of NY-ESO-1 reactive CD8 T-cells, was also differentiated with features consistent with a resting memory state ($CD28^{+}CD27^{+}CD127^{+}PD\text{-}1^{-}$), suggesting that they may serve to maintain effector cells [48]. These findings are in line with another study, in which we identified a single naturally primed T-cell clone that dominated the CD8 T-cell response to the Melan-A/MART-1 antigen [45]. The dominant clone expressed a high avidity TCR to cognate tumor antigen, efficiently killed tumor cells, and prevailed in the differentiated effector memory ($EM28^{neg}$) T-cell compartment. Other outstanding properties of this monoclonal T-cell population were the *in vivo* high frequencies, long-term persistence (>3.5 years), and efficient homing to metastases. Remarkably, during concomitant vaccination, we observed progressive enhancement of effector attributes, thus showing that repeated peptide vaccination together with IFA and CpG allows the induction of functionally competent tumor-reactive T-cells [45]. More recently, we took advantage of CpG oligodeoxynucleotides as a powerful vaccine adjuvant to induce and characterize T-cell responses upon vaccination with the natural Melan-A (EAAGIGILTV, "EAA") versus the analog Melan-A (ELAGIGILTV, "ELA") peptide antigen [14]. Compared with vaccination with analog peptide, natural peptide-induced T-cell frequencies were approximately twofold lower. However, T-cells showed superior tumor reactivity because of (i) increased functional avidity for natural antigen and (ii) enhancement of T-cell activation and effector function. Altogether, such studies are essential to characterize the functional requirements of tumor-specific T-cells upon vaccination. Moreover, they support further development of vaccine formulations with CpG and peptides in large-scale phase III trials.

It is essential that effector-type T-cells, once activated upon antigenic challenge, reach ("home to") affected tissues for the elimination of infected or malignant cells. In human cancers, current knowledge of the *in vivo* functions of tumor-specific T-cells has been until recently restricted to peripheral blood T-cells; however, studies on the functional activities of such cells at the site of tumor lesions are becoming possible and popular [80, 81]. At present, several reports have described that while circulating Melan-A-specific CD8 T-cells exhibited characteristics in common with the effector T-cells, tumor-specific T-cells that reside in metastatic lymph nodes and soft tissue and visceral metastases appeared functionally attenuated as compared with circulating T-cells, despite their accumulation at high frequencies in tumor lesions [50, 82, 83]. This coincided with a significant increase of $FoxP3^{+}$ regulatory T-cell activity within the tumor [50, 84]. Moreover, Melan-A-specific T-cells failed to produce IFNγ in both metastatic lymph nodes and nonlymphoid tissue metastases, but this defect was reversible in the presence of low dose IL-2/IL-7 cytokines *in vitro* [82], in line with gene expression profiling revealing downregulation of the interferon signaling pathway in T-cells from patients with metastatic melanoma [76]. Finally, Beyer and colleagues recently described that these nontumor reactive CD8 T-cells are characterized by a molecular program associated with the hallmarks of "division arrest anergy" [85]. There is still only limited data available regarding T-cell clonotypes, likely because this approach is labor intensive (see coming sections). Yet, clonotypic analyses provide promising results, mostly because T-cell clonotypes can be followed in a straightforward manner at any time and body location using the TCR as a clonotypic marker (reviewed in [74, 86]). For instance, further comparisons of the phenotype and T-cell clonotype composition between viral- and tumor-specific responses should be carried out between the blood and tumor site in patients where samples are available. Such analyses could extent previous studies while focusing on changes in the proportions of T-cell subsets and the potential selection of TCR clonotypes following migration to metastatic lesions [48]. This would also allow determining whether the expression of the tumor antigen at these sites negatively influences selection of tumor-specific TCR clonotypes compared with "bystander" viral-specific T-cells, which are not stimulated locally by their specific antigen.

4. Each T-Cell Is Unique: Gene Expression Profiling of Single Cells

An alternative strategy of avoiding the need for *in vitro* cultured T-cell clones is to analyze the gene-expression profile of *ex vivo* sorted cells. The standard microarray approach can be used for gene-expression profiling of T-cells of particular phenotypes; however, it is limited to the study of at least 100–1000 cells [87]. Nevertheless, the technique has evolved over the years to allow gene-expression profiling of coding and noncoding regions of the genome, which still makes it the goldstandard approach for identifying novel differentially expressed genes. Once these genes of interest have been identified, the ultimate goal is to perform detailed analyses on a limited number of antigen-specific T-cells and subpopulations. For this purpose, our group has developed a strategy consisting of cell lysis and cDNA synthesis in a single-step procedure, followed by a modified RT-PCR

protocol that relies on the detection of specific cDNAs after global amplification of expressed mRNAs [32, 88]. This method yields sufficient cDNA from as few as five cells. Thus, a major advantage is that it allows the analysis of gene expression and TCR repertoire analysis in small-purified subpopulations of antigen-specific cells even when a limited amount of material is available. Furthermore, since the global amplification is not selective and the amplified cDNA remains stable for long periods of time at −80∘C, this approach allows the amplification of genes of interest even at later time-points. We have previously employed this strategy to study the heterogeneity of CD8 T subpopulations. Five-cell aliquots isolated following multiparameter flow cytometric analysis were subjected to global amplification of cDNA, followed by the specific amplification by PCR of genes known to be differentially expressed at distinct stages of cellular differentiation (Figure 1). As such, the expression patterns of the CD27 costimulatory receptor, the IL-7Rα, and the expression of the cytotoxic molecules granzyme B and perforin have permitted the identification of a number of T-cell subpopulations, including pre-effector and effector memory subsets [32, 34, 88], as well as the fine characterization of tumor-specific CD8 T-cell responses in the context of therapeutic peptide vaccination (see [45, 48]; Rufer, Speiser et al., *unpublished observations*).

4.1. Towards Single Cell Profiling. During T-cell differentiation, the stochastic events become more prominent involving a set of modifications of multiple gene expressions inducing subtle or dramatic changes in the cell. Such changes incur variability among the antigen-primed T-cell populations and even among "naive" cells. CD8 T lymphocytes are a well-defined example of such a phenomenon. Such inevitable heterogeneity in biological systems emphasizes the need to determine this variation by analyzing single cells. Recently, we have optimized the above-described strategy of global cDNA amplification at the single cell level. This provides us with the added advantage of being able to determine the gene-expression profile of a single T-cell clone defined by its unique CDR3 sequence (i.e., a TCR clonotype). One drawback of this method is that low frequency TCR clonotypes require the isolation of numerous single cell samples for its detection. Nevertheless, it is highly powerful in that one can follow single TCR clonotypes over time before and after vaccination and compare their gene-expression profiles between (i) vaccination protocols, (ii) patients, (iii) T-cell subpopulations, (iv) anatomical localizations, and (v) over time. Furthermore, one can correlate the information gathered on the *ex vivo* gene-expression profile of a TCR clonotype of interest, with the functionality of a T-cell clone bearing the same unique receptor *in vitro*. Currently, our group is interested in understanding how the differentiation of melanoma-specific T-cells is affected by factors such as the type and dose of antigenic peptide used for vaccinating patients. The profiles of these cells will be subsequently compared against EBV- or CMV-specific T-cells, of which we know that they ensure efficient viral control *in vivo*, in spite of differences in phenotype, functionality, and TCR clonotype composition [43].

The cDNA global amplification technique provides information on the expression of genes at the mRNA level, and not at the protein level. However, the combination of the single cell gene-expression profiling and the multiparameter flow cytometry analysis using antibodies against the respective proteins can overcome this limitation. The disadvantage of the nonspecific amplification technique is that it is not quantitative, since the main principle of the technique relies on the nonspecific amplification of all expressed mRNAs present in the cell. Furthermore, particularly at the single cell level, this technique remains limited to the study of genes expressed at moderate and high levels within the cell, since genes expressed at very low levels may not be detected even following the global amplification procedure. Thus, overcoming this limitation may be a challenging task, although working with cDNA material of high quality and purity is indispensable. This requires a very clean and sterile working environment. All steps preceding the global amplification must be performed in an RNAse and DNAse free environment, free from potential sources of contaminant material.

4.2. Single Cell Profiling Strategies by Other Groups. Although for some time the direct quantification of gene expression a single cell was considered impossible since the amount of mRNA extracted from a cell is minute, there is now strong evidence of its feasibility, allowing efficient and reliable assessment of gene expression of individual cells [89–91]. Peixoto and colleagues reported of a method wherein they successfully quantified the expression of 20 different genes simultaneously from a single cell using reverse transcription of single cell mRNA with 3′-specific primer of each gene of interest [89, 90]. This cDNA was then amplified by a multiplex PCR of all 20 gene-specific 3′ and 5′ primers, followed by a seminested real-time PCR for each individual gene. Using this approach, Monteiro et al. investigated the heterogeneity of the $CD8^{pos}$ T-cell compartment, by first identifying several T-cell subpopulations using four extracellular markers (CCR7, CD45RA, CD27, CD28), followed by the analysis of mRNAs coding for chemokines, cytotoxic molecules, or effector cytokines expressed in single cells from these subsets [33]. This in-depth single cell profiling could demonstrate that certain T-cell subsets belonging to the memory compartment such as the EM T-cells expressing CD27 at high levels could exhibit similar functionality profiles as naive T-cells. Additionally $CCR7^{-}$ cells expressing both CD27 and CD28 showed similar profiles as central memory cells, in line with our own observations [34]. On the other hand, highly differentiated CD27 and CD28 double negative cells were highly similar in their gene-expression profiles regardless of CD45RA expression. The results obtained by this quantitative single-cell multiplex RT-PCR approach clearly depicted a high degree of heterogeneity with each cellular subset displaying characteristic patterns of gene expression, pointing to a hierarchical order of T-cell differentiation stages among the antigen-experienced cells [33]. Furthermore, even for individual cells belonging to the same subset, the gene-expression profiles showed variability, outlining the necessity to study multiple parameters in

order to characterize the uniqueness of each single T-cell. The main drawback of this strategy is that it can quantify multiplex PCR applied to only 20 preselected genes of interest expressed in a cell and thus fails to amplify high amounts of cDNA that can eventually be used to individually quantify any gene of interest, for example, TCR BV clonotype expression.

Since the study of single T lymphocytes remains one of the ultimate goals, other groups have developed approaches for this highly specific type of analysis. For instance, the Ampligrid platform by Beckman Coulter can be employed to sort single cells on unique hydrophilic/hydrophobic structured glass slides containing individual wells, which can then be directly used for PCR amplification in a very small volume allowing higher sensitivity. The chemically modified glass slide wells can be used as single reaction centers for cell growth and PCR all in one place [92] in which cells are stimulated, washed, and dried in situ, avoiding numerous handling steps and cellular stress [93]. This platform has already been used for the genetic analysis of circulating tumor cells in small-scale clinical trials [94] and is particularly advantageous for multiplex real-time PCR [95], robotic micromanipulations, and laser microdissection [96]. This method has a high sensitivity with a detection level threshold of 32 pg of purified DNA, allowing the minisequencing of mitochondrial DNA down to a single lymphocyte [97]. Yet, it also requires further optimization at the single cell level, since the efficiency may sometimes vary drastically from 20 to 80% [98] and a second seminested amplification may still be necessary for certain genes [99]. Furthermore, the optimal handling of minute volumes of numerous single cell samples should require robotized approaches.

5. T-Cell Receptor (TCR) Repertoire and TCR Clonotype Analysis

A better understanding of the structural principles that govern TCR-pMHC binding is essential to promote research and clinical applications. The quality of TCRs recruited during disease, or by vaccination, dominantly influences the potency of immune responses. To improve therapeutic immune interventions, TCRs need to be fully characterized, and the mechanisms for their recruitment and function must be elucidated. Results from such studies have a strong impact on the design of antigens for vaccination as well as on the choice of optimal TCRs for adoptive T-cell therapy, with or without TCR gene transfer.

Several strategies exist to assess the TCR repertoire diversity and clonal composition of various pools of antigen-specific T-cells. A straightforward approach is to combine the staining with peptide-MHC multimers with a panel of monoclonal antibodies (mAb) directed against the variable region of the TCR β chain (anti-BV). While this approach allows the direct and quantitative assessment of BV usage by tumor- and viral-specific CD8 T lymphocytes, it does not reveal whether the BV-restricted T-cell response analyzed is of monoclonal or of polyclonal nature (reviewed in [86]). The immunoscope or CDR3 spectratyping technique measures the size heterogeneity of the CDR3 region of the TCR and provides a powerful tool to characterize in-depth the level of the TCR repertoire complexity (reviewed in [86, 100]). Since most human TRBV segments can be amplified using 22 BV-specific primers as previously reported [101], fluorescently labeled primers specific for each TRBV gene segment and a primer specific for the constant region of the β chain of the TCR are used to amplify specifically the expressed CDR3β gene segment. The labeled PCR products are then run on an automated sequencer in the presence of fluorescent size markers to obtain the precise size of the CDR3 region (reviewed in [86]). Expansion of particular antigen-specific T-cell clonotypes is visualized as single dominant PCR peaks of a given size and PCR products are sequenced to obtain the nucleotide sequence of the CDR3 region. Analyses are then focused on determining the dominant clonotypes (defined as the presence of the same TCR BV-CDR3-BJ sequence in two separate subsets or time-points). They can be further divided into highly dominant or subdominant clonotypes, based on their frequencies in an immune response. Subsequently a comparison of the frequencies of a given clonotype can be made between different T-cell subsets, between different time-points, and even among different individuals (Figure 1).

5.1. Advantages and Limitations of the Spectratyping/Clonotyping Technique. Today, the spectratyping/clonotyping approach has proven to be a strong analytical technique, allowing both detecting oligoclonal T-cell expansion *ex vivo* and assessing the presence and frequencies of dominant T-cell clonotypes among virus- and tumor-specific CD8 T-cell subpopulations [43–45, 48, 49, 51, 52]. Yet, this approach has some limitations. The first one stems from the cross reactivity of the different TRBV oligonucleotide primers used. Due to the homology between several TRBV gene segments and the existence of several subtypes for numerous TRBV gene families (e.g., 14 variations of TRBV13 and 25 for TRBV6), the primer sequences utilized have a certain degree of cross-reactivity. In fact, the likelihood of unspecific recognition can be theoretically calculated for each primer set against all BV gene segments. Nevertheless, performing individual TRBV PCR (in contrast to multiplexing), sequencing of doubtful PCR amplicons, and careful analysis of the upstream sequences allows to accurately determine whether the PCR product corresponds to the correct amplified TCR BV family or is the result of cross-reactivity against another BV family. Another limitation is that there are instances where the TCR of one sample or clone cannot be identified despite repeated efforts. The more likely reason for this observation is that the 22 pairs of primers used detect an estimated >90% of all known TRBV families and subfamilies. Thus, although detection of the majority of all TRBV subfamilies is theoretically ensured, some TCR clonotypes among antigen-specific T-cell responses could express rare and particular BV segments and thus remain undetected by the spectratyping technique (own unpublished observations).

The spectratyping approach of analyzing T-cell clonotype composition requires a high number of samples to be sequenced. The strategy developed by our group involves the analysis of CDR3 sizes among T-cells population as

an early step, followed by the analysis of potential candidates for dominant clonotypes. This reduces the number of sequencing reactions of unique BV and unique CDR3 lengths. When the sequencing of several PCR products has yielded the same TCR sequence for a given BV, clonotypic primers can be designed to allow faster identification of these TCR clonotypes among the remaining samples. The forward and reverse clonotypic primers are designed to specifically recognize the unique CDR3 sequence of each clonotype and are used in combination with the BC primer, and BV primer respectively. In certain instances, clonotypic primers cannot be designed, because of lack of specificity or because CDR3β sequences are too similar. This is the case for most EBV-specific TCR clonotypes of the TRBV2 and TRBV4 families as they share highly similar CDR3 sequence motifs, sometimes differing by only one amino acid, and in these situations, all samples are being sequenced [43].

Finally, as the spectratyping technique is only semiquantitative, the amplification of cDNA from antigen-specific T-cell pools might introduce biases, leading to the nondetection of variations present within a particular CDR3β length (reviewed in [102]). Sequencing of antigen-specific T-cells at the single cell level should overcome most of these restrictions [103–105]. This aspect as well as the limitation in the determination of precise frequencies from individual clonotypes has prompted us to recently redefine our spectratyping/clonotyping approach towards the single cell level.

5.2. Clonotype Selection and Composition of Antigen-Specific T Lymphocyte Subsets. It has been proposed that the large heterogeneity that is observed among T-cell subsets may be influenced by the clonotype composition found within these subpopulations. Indeed, different TCRs, which vary in mechanism and strength of peptide-MHC binding, may lead to distinct activation and differentiation. However, it is evident that cellular heterogeneity exists even within the same T-cell clonotype (reviewed in [106]), as demonstrated in TCR-transgenic mice [106, 107], and in the elegant study by Stemberger and coworkers [108], in which it was shown that a single naive CD8 T-cell precursor can differentiate into different effector and memory subsets. Studies of the human TCR repertoire are somewhat conflicting, with some groups reporting that effector and memory are derived from identical, naive precursor cells [109, 110], while others suggest that they are recruited from distinct precursor cells [111].

Our group recently performed an in-depth analysis of the clonal TCR repertoire diversity and selection among healthy donors infected with persistent herpes viruses [43] or following influenza infection [44]. Sequencing of the TCR beta chains showed that the clonal composition was highly restricted for both influenza- and CMV-specific cells (between 1–3 dominant clonotypes), whereas it was relatively diverse for EBV-specific cells (on average 15 clonotypes). Importantly, these analyses revealed for the first time in a steady state of human T-cell memory that individual clonotypes can differentiate and be maintained within both the early- (memory) and late-differentiated (effector) T-cell subsets [44]. Data also indicate, in the EBV model, that the early differentiated memory subset is the most diverse and contains virtually all clonotypes found in the highly differentiated effector subset [43]. Yet, striking differences in the patterns of dominance can be observed among both subsets, since some clonotypes are selected with differentiation while others are not. Remarkably, clonotype selection and composition of EBV- and CMV-specific subsets upon differentiation are highly preserved over time, with the presence of the same dominant clonotypes at specific differentiation stages over a period of 4 years. Altogether, these observations demonstrate that T-cell clonotypes segregate with differentiation, but the clonal composition once established is kept constant for at least several years [43] (Figure 3).

Therapeutic vaccines against cancer aim to induce effective immune responses similar to protective antiviral responses. Previously, we showed that a potent vaccine formulation composed of low doses of Melan-A/MART-1 peptide, IFA, and low doses of CpG oligonucleotides induced *ex vivo* detectable T-cell expansions in virtually all melanoma patients reaching on average more than 1% of specific CD8 T-cells [13, 14]. Extensive analyses of the tumor-specific responses among peptide vaccinated patients revealed that the Melan-A/MART-1-specific T-cell responses were also relatively diverse comprising between 10 to 15 clonotypes, depending on individual patients (Rufer, Speiser et al., *unpublished observations*). These data are consistent with another report [49], in which we found that the number of dominant NY-ESO-1-specific T-cell clonotypes among patients was between 6 and 10 clonotypes (Figure 3). A striking observation is that tumor-specific T-cell responses can, in some rare patients, be dominated by single dominant clones [45, 49]. Collectively, a general observation is that, similarly to viral-specific T-cell responses, responses to Melan-A and NY-ESO-1 epitopes are also caused by selection and amplification of particular T-cell clonotypes, following T-cell differentiation (Figure 3). Specifically, the majority of the patients showed progressive TCR repertoire restrictions, from early differentiated to late differentiated T-cells, indicating the occurrence of oligoclonal T-cell expansions in the latter compartment. Thus, the $EM28^{neg}$ ("effector-like") subset consists primarily of codominant T-cell clonotypes, whereas the $EM28^{pos}$ ("memory-like") contains large polyclonal TCR repertoires with the presence of numerous nonclonotypic sequences (see [45, 48], Rufer, Speiser et al., *unpublished observations*). Finally, most tumor-specific clonotypes identified in the late differentiated $EM28^{neg}$ subset were also found within the $EM28^{pos}$ T-cells, in agreement with our recent observation made in CD8 T-cell responses against influenza [44] and herpes viruses [43]. Altogether, these results provide further evidence for a linear model of T-cell differentiation, in which a small number of clonotypes are selected to differentiate from a larger pool of less differentiated "memory" cells. Figure 3 summarizes the TCR diversity observed for tumor-specific CD8 T-cell responses compared with viral-specific responses. While larger numbers of codominant clonotypes are found for Melan-A/MART-1-specific T-cells, CMV responses involve low numbers of clonotypes. Nevertheless in all instances a

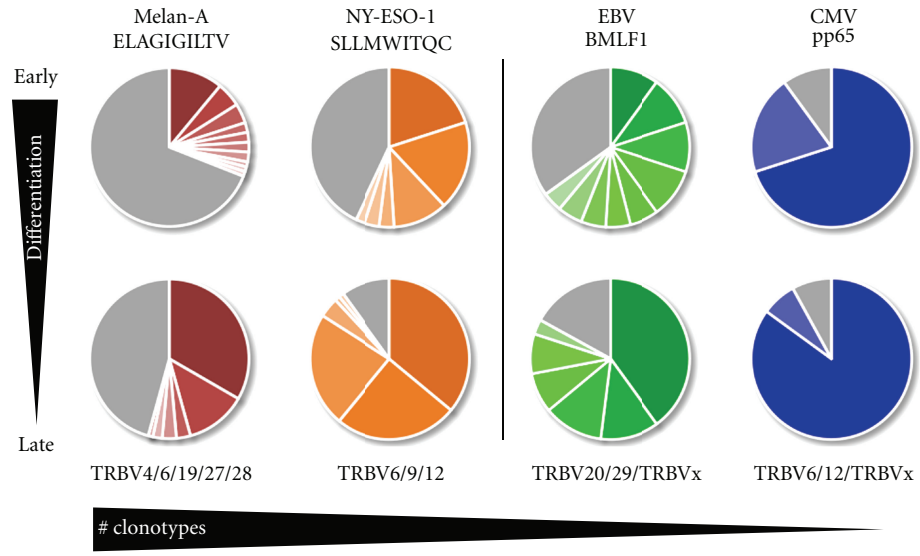

Figure 3: TCR repertoire restriction and TCR clonotype selection are driven by differentiation. Analysis of TCR repertoire diversity and clonotype composition between tumor-specific responses (left side of the figure) and viral-specific responses (right side of the figure) suggest that all antigen-specific T-cell responses undergo selection along with progressive cellular differentiation (top to bottom of figure). As such, early differentiated subsets show a higher degree of clonal diversity relative to their more differentiated subsets. Nevertheless, dominant clonotypes are also found in the former but make up significantly larger proportions of the more differentiated compartments (defined as TCR clonotype selection) supporting a linear model of differentiation. In addition, viral-specific responses which are composed of highly differentiated cells (EMRA T-cells) also show a higher degree of TCR repertoire restriction compared with tumor-specific responses, evident not only in the late but also already in the early differentiated subsets (indicated by a decrease in numbers of BV families and numbers of clonotypes from left to right). Each colored slice of pie represents a unique TCR clonotype, while the grey slices represent nondominant TCRs, that is, the sequences identified only once within the antigen-specific TCR repertoire. Melan-A (ELAGIGILTV) and NY-ESO-1 (SLLMWITQC) peptide sequences are indicated for the tumor-specific responses; virus specific T-cells were analyzed for the epitope EBV $BMLF1_{280–288}$ (GLCTLVAML) and for the CMV epitope $pp65_{495–503}$ (NLVPMVATV). All responses are HLA-A2 restricted. Preferential TRBV family usage is depicted based on IMGT's nomenclature [112].

clear selection is observed with T-cell differentiation, such that only a small number of TCR clonotypes are found to dominate the highly differentiated subsets.

5.3. Alternative Strategies to Study TCR Repertoire Diversity and Composition of Antigen-Specific CD8 T-Cell Responses. Koup, Douek, and coworkers have developed over the past decade an alternative approach to address the complexity of the TCR repertoires in several antigenic systems [113]. Its principle is based on the use of a template switch-anchored RT-PCR with primers specific for the 3′ TCR constant region to amplify all expressed TRBV or TRAV gene products. Since no primers specific for the TRBV- or TRAV-gene segment are applied, this strategy does not introduce bias associated to particular Vβ or Vα families (see the above discussion). The resulting PCR amplicons are then ligated into a plasmid vector, and cloned and selected colonies are sequenced. This powerful strategy allows the analysis of TCRs at the single cell level and provides an absolute frequency of particular CDR3β usage. One of the major disadvantages of this technique is the relative high cost associated with the need to sequence thousands of individual CDR3 gene segments. Moreover, biases related to the efficiency of the ligation and transformation of bacterial cells may still be introduced. Finally, the design of clonotypic primers specific to identified dominant T-cell clones when combined to quantitative real-time PCR allows the quantification of clonotypes at any given time-point during longitudinal studies [113]. This approach has led several research groups to achieve major advances in the fine characterization of CD8 T-cell responses during SIV [114–117], HIV [118–123], CMV and EBV [124, 125] infections as well as in patients suffering from melanoma [126, 127] or of hematological malignancies [128]. Describing in detail these publications is beyond the scope of this paper and, therefore we have chosen to discuss some aspects of this extensive work that is more closely related to ours.

An important question concerns the frequency and the functional properties of public versus private clonotypes and the *bona fide* clinical benefit obtained after their *in vivo* expansion following viral infection or therapeutic vaccination. Public clonotypes are defined by the presence of the same identical TRBV-CDR3-BJ and/or TRAV-CDR3-AJ sequences found in two unrelated individuals or animals. Recently, Price et al. have described the protective activity mediated by public Gag- specific TRBV clonotypes in vaccine-induced SIV-specific CD8 T-cell responses, and observed that the number of public clonotypes correlated inversely with the virological outcome after SIV challenge [114]. Compared to private vaccine-induced SIV-specific

T-cells, T-cell populations with public clonotypes exhibited greater levels of cross-reactivity to epitope variants, thus indicating the need to preserve some degree of cross-reactivity to contain viral escape [114]. Similarly, public TRBV sequences that are shared between HLA-matched individuals have also been described within the TCR repertoire specific for viral epitopes that do not mutate as in the case of EBV and CMV [43, 125, 129]. Venturi and colleagues have proposed that the sharing of TCR-beta chain between healthy individuals is strongly associated with TCR-beta production frequency and that a process of convergent recombination facilitates the more efficient production of those TRBV sequences [115, 130, 131]. We recently identified public TRBV sequences isolated from 15 melanoma patients, and unlike viral-specific TCR repertoires, such public TRBVs were primarily expressed by nondominant and infrequent T-cell clonotypes [51]. This highly contrasted with private CDR3β signatures frequently found in T-cell clonotypes that dominated repertoires of individual patients. Clearly, common TRBV motifs have been selected after vaccination in different patients, but at much lower frequency compared to distinct and private TCR structures. Yet, both public and private antigen-specific TCRs recognized tumor cells with comparable avidities suggesting that neither had a clear functional advantage [51]. Future directions involve understanding the structural and biophysical impact of public and private TCRs in the process of recognition of the pMHC complex, T-cell activation, and subsequent T-cell functionality.

The characterization of T-cell clonotypes bearing particular TCR CDR3 motifs, of their frequencies in well-defined phenotypic populations, as well as of their related cytolytic functions has revealed the need to understand the basis of the structure-function relationship of a given TCR to its pMHC molecule. This requires detailed studies performed at the molecular level of the TCR and the delineation of multiple parameters involved in the TCR signaling (e.g., CD8 coreceptor), combined with the characterization of intrinsic biophysical factors of the TCR molecule (e.g., CDR structural loops and binding kinetic rates). In that regard, the recent crystal structure of the Melan-A-MHC-TCR complex by Cole and colleagues brought important insights into the key elements within the TRAV and TRBV gene segments that are involved in the recognition of the Melan-A antigen [127]. They showed that only CDR1α, CDR3α, and CDR3β loops make contacts with the antigen and propose that this unusual pattern of antigen recognition may explain the unique characteristics and extraordinary frequencies of $CD8^{pos}$ T-cell responses to this epitope. Moreover, Asn92 is the only residue from the CDR3α that interacts with the antigen [127], and since it is commonly expressed by TRAV12-2 (or TCR Vα2.1) positive T-cell clonotypes [51], this finding may provide some explanation for the preferential segment usage of the TRAV12-2 in Melan-A-specific T-cell responses [132, 133].

Peptide-based cancer vaccines are often prepared with altered "analog" peptide antigens that have been optimized for HLA class I binding, in order to enhance their immunogenicity. An obvious but crucial point is that structural modifications of peptides should not alter TCR$\alpha\beta$ repertoires or TCR binding properties, to ensure that vaccination-primed T-cell clonotypes remain highly specific for the natural antigen and efficiently recognize tumor cells. Recently, we demonstrated fine specificity differences and enhanced recognition of naturally presented antigen by T-cells after vaccination with natural Melan-A/MART-1 peptide as compared with analog peptide [14]. These data highlight the importance of careful re-evaluation of studies using analog antigens with regard to the risk of activating T-cells with "imprecise" antigen specificity or low TCR affinity. We recently addressed the question whether the observed fine peptide antigen specificity could be explained by structurally different TCRs recruited and promoted by vaccination with natural versus analog Melan-A/MART-1 peptide. In this study [51], we compared the TCR primary structures of 1489 HLA-A*0201/Melan-$A_{26–35}$ specific CD8 T-cells derived from patients after vaccination with the natural or the analog peptide. Collectively, our data indicate that T-cell repertoires generated against natural or analog Melan-A peptide exhibited slightly different but otherwise structurally conserved TCR features, strongly suggesting that the differences in binding affinity/avidity of TCRs towards pMHC observed between both cohorts of vaccinated patients are caused by subtle structural TCR variations [51]. Very recently, Cole and colleagues [126] evaluated the intraindividual clonotypic responses to both analog and native Melan-A peptide following *in vitro* peptide stimulation, thus allowing comparison between samples derived from the same naive T-cell pool. They found that despite a clear bias towards particular TRBV gene usages and the presence of the GXG public motif in the CDR3β loop, similarly to previous reports [51, 134], the majority of the clonotypes were nonoverlapping between the two TCR repertoires [126]. These data further illustrate (i) the importance to monitor T-cell responses at the clonotype level and (ii) emphasize the need to the careful evaluation of such heteroclitic peptide-based immune interventions to ensure efficacy in the clinic.

6. Applying Improved Knowledge to Clinical Studies

Progress in basic immunology has led to a better understanding of immune protection against cancer. Although there is still much more to learn, we now know how to activate the immune system and which cellular and molecular components are key for immune protection. The knowledge is currently applied in clinical studies, with the aim of introducing immunotherapy against solid cancers in clinical routine in a few years from now (Figure 1).

The design of potent antitumor vaccines currently faces several obstacles, which will need to be addressed in order to improve their efficacy. One of them is the choice of optimal tumor antigens, as reviewed by Boon et al. [2]. For instance, mutated antigens, although unique to tumor cells, may not be shared among different individuals, thus raising the problem of individualized versus universal tumor-vaccines. In contrast, differentiation antigens (such as Melan-A/MART-1) are expressed by tumors from many individuals.

However, their expression in normal cells poses targeting problems thus toxicity, and of breaking immune tolerance. Since these concerns are found on opposite sides of the balance, addressing both of them will prove difficult. The use of potent adjuvants may be successful in stimulating immune responses at a cost of autoimmune effects, as for example observed in melanoma patients who developed vitiligo following vaccination [135].

Recent technological advancements now allow dissecting immune responses and compare the phenotypic and functional characteristics of various antigen-specific T-cell populations. An increased understanding of the generation and maintenance of memory T-cells in virus-specific responses will also help to better determine T-cell correlates of protection. This is important in the design of tumor-specific vaccines, as well as in determining which T-cell subset may be most optimal for adoptive cell transfer. Furthermore, recent studies have shown that vaccination with peptide analogues [14] with enhanced binding to MHC may result in the selection of unfavorable T-cells [51, 126]. Therefore, the stability of the peptide-MHC complex should always be taken into account when assessing affinity and avidity of TCR-mediated recognition.

Aside from the fact that tumor-/self-antigen-specific T-cells are less frequent than T-cells specific for pathogens, their capacity to recognize and interact with cognate antigen bearing cells is inferior. This is at least in part explained by the lower TCR affinity, resulting in reduced functional avidity. With a few exceptions, the so far best human cancer-specific TCRs have been isolated from patients with extraordinary strong spontaneous CD8 T-cell responses, often associated with unusually favorable clinical histories [45, 48]. Interestingly, T-cells isolated from metastases, particularly from tumor infiltrated lymph nodes, usually bear better TCRs than T-cells from peripheral blood, reflecting enrichment of specific T-cells in the tumor microenvironment. Unfortunately, TCRs isolated after vaccination with tumor antigens are often of lower avidity [45, 136–138], indicating that current generations of tumor vaccines are suboptimal for the selection of the best T-cells. It may even be possible that many of the TCRs isolated after immunotherapy have been primed earlier and were thus not primed but only boosted by the applied therapy. This issue needs to be clarified, for a full characterization of vaccination driven selection of T-cell clonotypes. It is necessary to improve vaccines in this regard, for example, by reducing vaccine antigenicity (with e.g., lower antigen doses or weaker antigens), or by limiting antigen presentation to professional antigen-presenting cells. However, it remains possible that patient's tumor specific TCR repertoires are fundamentally deficient. In this case, gene therapy with improved TCRs may be a valid approach (reviewed in [139]). Current and future developments in this regard [140, 141] will show whether this is a critical point. Finally, competent TCRs may exist in cancer patients but T-cell precursor frequencies may be too low, limiting the potential to generate strong and long-lasting T-cell responses. If so, gene therapy with naturally occurring TCRs, introduced in larger numbers of (naive) T-cells may be beneficial for improved clinical outcome.

In this paper we have focused on CD8 T-cell responses, which are important for cancer patients, since CD8 T cells can counteract tumor progression. However, tumors and their microenvironment include many more components. Tumors have been described as "wounds that never heal, that regularly reorganize their strategies for persistence and progression. Instead of stopping the disease, some components of the immune system can actually promote cancer, by provoking chronic inflammation and elaborating factors that may support tumor cell growth, survival, and angiogenesis. Furthermore, established tumors use immune-regulatory circuits to generate an immune-suppressive environment, which can act as substantial barriers to protective T-cell responses. Progress in our understanding of the molecular mechanisms governing the interaction between tumors and the immune system has been the basis for improvements of cancer therapy (reviewed in [142]). In view of the formidable challenge to fully understand the plethora of processes employed by tumors, it is evident that further work is needed to optimize cancer therapy, by targeting principal mechanisms of malignant diseases.

Abbreviations

CpG:	Deoxycytidylate-phosphate-deoxyguanylate oligodeoxynucleotide
EBV:	Epstein-Barr virus
CMV:	Cytomegalovirus
Influenza:	Flu
IFA:	Incomplete Freund's Adjuvant
FISH:	Fluorescence in situ hybridization.

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Research Article

Autologous Peripheral Blood Mononuclear Cell Recognition of Autologous Proliferating Tumor Cells in the Context of a Patient-Specific Vaccine Trial

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Metastatic melanoma patients who were treated with patient-specific vaccines consisting of dendritic cells loaded with autologous tumor cells had a 5-year survival of over 50%. Enzyme-linked immunospot (ELISPOT) has been used to detect antigen reactive T cells as a means of determining immune response. We wished to determine whether IFN-gamma secretion in an ELISPOT assay was prognostic or predictive for survival following treatment. Peripheral blood mononuclear cells (PBMCs) collected at weeks 0 and 4 were evaluated by ELISPOT assay for response to autologous tumor cells. Overall, there was slight increase in the number of tumor reactive lymphocytes from week 0 to week 4. Using >5 spots/100 K PBMC as the cutoff, a log-rank analysis revealed only a slight statistical significance in overall survival for patients who lacked tumor reactive PBMCs at week 4. The sensitivity of ELISPOT in the context of patient-specific cellular vaccines is unclear.

1. Introduction

Metastatic melanoma is generally considered to be incurable. Immunotherapy is a promising alternative treatment to chemotherapy, but challenges remain in determining response to therapy. Clinical trials that use adoptive immunotherapy regimens need surrogate markers to measure responsiveness. Currently, measuring changes in the level of serum cytokines by the enzyme-linked immunosorbant assay (ELISA) does not measure specific antigen responses. A novel-cell-based immune monitoring assay like the enzyme-linked immunosorbant spot (ELISPOT) assay may be a more precise and quantifiable measure of immune response.

Attempts to measure changes in serum cytokines involved in immune response (i.e., IL-10 and IL-12) by ELISA yielded no detectable levels in patients treated with the dendritic cell-based vaccine (unpublished observations). In light of this, we chose to use the ELISPOT assay because it is able to measure antigen-specific responses in very small subsets of effector cell populations. Such assays have been touted as a measure of whether a vaccine is enhancing immunity and as possible prognostic and predictive markers for melanoma patients receiving cancer vaccines made up of melanoma-specific peptides such as MART-1, gp100 and tyrosinase [1]. Cytokines that are secreted by immune cells in response to antigenic stimulation are rapidly captured by antibodies before they are diluted in the supernatant, captured by receptors of other cells, or degraded. The colored spot end product typically represents an individual cytokine-producing cell that can be counted. This allows ELISPOT assays to detect immune reactive cells that may be present at a frequency of less than 1/100,000 peripheral blood mononuclear cells. ELISPOT has proved to be a reliable assay for lymphocyte recognition of peptide antigens based on the secretion of cytokines such as interferon gamma.

We recently observed some remarkably long periods of progression-free survival and an encouraging 5-year survival rate of 50%, in patients with metastatic melanoma who were treated with patient-specific vaccines consisting of autologous dendritic cells loaded with interferon-gamma-treated and irradiated autologous tumor cells that had

been proliferating in culture [2]. The vaccinations were given once a week for three weeks and once a month for five months. The cell-based vaccine was administered in granulocyte macrophage-colony stimulating factor (GM-CSF) and injected subcutaneously.

In this series of experiments, we used an ELISPOT assay as a metric for postvaccination immune augmentation. The sensitivity of such assays in the context of our clinical trial is unclear since we were immunizing with antigens derived from tumor cells without awareness of which tumor-associated antigens might be most important in the immune response. In this case, we could not use an ELISPOT tetramer assay which employs the use of specific peptide to detect antigen specific T cells because we were not focusing on a single specific antigen [3]. We therefore had to rely on the release of gamma interferon from a population of autologous lymphocytes based on their exposure to cryopreserved autologous tumor cells that had been proliferating *in vitro*. We hypothesized that these cells were the best representatives for monitoring immune response for each patient since they represent the patient's own tumor cells and subsequently may contain all of the available tumor associated antigens.

2. Materials and Methods

2.1. Patients. Melanoma patients with recurrent or metastatic disease were enrolled in a phase I/II clinical trial [2]. All patients had to be off any immunosuppressive therapy for at least 4 weeks and meet ECOG (Eastern Cooperative Oncology Group) performance levels of 0–2 at the time of initial treatment. At the beginning of treatment, patients were tested for anergy to common recall antigens using available tests for Candida, trichophyton, and mumps but anergic patients were not denied treatment. Those patients with brain metastases not currently receiving pharmacologic doses of corticosteroids with therapy controlled tumors were eligible for enrollment. A total of 54 patients were clinically evaluable but only 42 were included in this study.

2.2. Autologous Dendritic Cell Generation. Dendritic cells (DCs) were generated by plastic adherence method as previously described [4, 5]. Briefly, peripheral blood mononuclear cells were isolated by ficoll-hypaque (GE Healthcare, Buckinghamshire, United Kingdom) density gradient separation from apheresis collections. The adherent populations were then incubated in AIM-V supplemented with 1000 IU/mL each of IL-4 (CellGenix, Freiburg, Germany) and GM-CSF (Invitrogen, Grand Island, NY) for 6 days prior to loading with autologous tumor cells.

2.3. Autologous Tumor Cell Line Generation. Pure melanoma tumor cell lines (defined as containing less than 5% fibroblasts), generated using techniques previously described [6–8], were incubated with 1000 IU/mL of IFN-γ (InterMune, Brisbane, CA) for 72 hours, irradiated with 100 Gy from a cesium source, and cryopreserved [9]. The tumor cells were recovered from cryopreservation, washed 3x with PBS, and then added to the *in vitro* cultivated DCs and incubated for ~24 hours. The antigen loaded DC were harvested by gentle scraping with a rubber policeman and cryopreserved at equal amounts in 9–11 aliquots.

2.4. Treatment Regimen. Aliquots of dendritic cells loaded with tumor cells were thawed in a 37°C water bath, washed 2x with AIM-V, combined with 500 ug/mL GM-CSF in saline and subcutaneously injected at weeks 1, 2, 3, 8, 12, 16, 20, and 24. Peripheral blood mononuclear cells (PBMCs) were obtained from peripheral blood samples at weeks 0 and 4 and were cryopreserved after separation of red blood cells by ficoll-hypaque density centrifugation.

2.5. IFN-γ Enzyme-Linked Immunospot (ELISPOT) Assay. Using BD ELISPOT Human IFN-γ ELISPOT Kit (BD Pharmingen, San Diego, Calif), thawed peripheral blood mononuclear cells (PBMCs) were rested for 24 hours and then cultured in four replicates at 2.0×10^5 viable cells with or without 4.0×10^5 viable autologous tumor cells per well in AIM-V in nitrocellulose backed 96 well plates. The PBMC : ATC ratio and incubation time were chosen based on work by Malyguine et al. [10]. Controls consisted of PBMCs stimulated with a combined cocktail of 3000 IU/mL IL-2, 2 μg/mL PHA, and 5 μg/mL OKT3 as a positive control, PBMCs or tumor cells only and AIM-V medium only as background and negative controls, respectively. After 48 hour incubation, plates were washed and stained as per manufacturer's instructions. Briefly, plates were washed twice with 200 μL/well deionized water and then three times with 200 μL/well Wash Buffer (provided by kit). 100 μL/well of 1 μg/mL mouse antihuman IFN-γ-biotinylated mAb (provided by kit) in assay diluent was added and the plates were incubated for 2 hours at room temperature. The plates were then rinsed, incubated with streptavidin-HRP solution for 1 hour and developed with aminoethylcarbazole (AEC) peroxidase substrate solution provided with the kits. The plates were then counted on an ImmunoSpot instrument (Cellular Technology Ltd. Cleveland, Ohio).

2.6. Statistical Analysis. Significant differences were defined by P-value $\leq .05$ using Student's t-test of two-tailed, two samples of equal variance. Log-rank calculations were used to determine whether there was a significant difference in survival curves. Progression-free and overall survival times were determined by time in months from first injection to last followup or patient expiration.

3. Results

The detection of antigen-specific peripheral blood T lymphocytes was determined by IFN-γ Enzyme-linked Immunospot (ELISPOT) assay using autologous tumor cells as the antigen source. Controls consisted of PBMC only (unstimulated), PBMCs exposed to a cytokine cocktail to induce the expression of IFN-γ (nonspecific stimulation), tumor cells only or media only (Figure 1). Neither the tumor cell only nor media controls resulted in any spotting

Table 1: Significant changes in tumor reactive T cells as measured by ELISPOT in patients receiving DC-based immunotherapy for metastatic melanoma.

Change in relative number of spots	Week 4 versus week 0 ($N = 42$)
Increase $P \leq .05$	7 (16.6%)
Decrease $P \leq .05$	4 (9.5%)
No change $P \geq .05$	31 (73.8%)

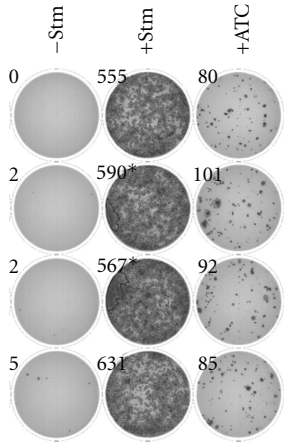

Figure 1: Peripheral blood lymphocytes were placed in the wells of ELISPOT plates in quadruplicate under three separate conditions: unstimulated (−Stm), stimulated with 3000 IU/mL IL-2, 2 μg/mL PHA, and 5 μg/mL OKT3 (+Stm), or combined with autologous tumor cells at a ratio of 1 : 2 (+ATC). After 48 hours, IFN-γ secretion was determined as per manufacturer's instructions. Numbers indicate spots counted by automated counter.

activity in all wells examined (data not shown). The levels of ELISPOT spotting were adjusted for nonspecific (unstimulated) IFN-γ secreting lymphocytes and the results are shown in Figure 2.

Out of 54 patients treated on the vaccine study, 42 were evaluable at weeks 0 and 4 for the ELISPOT testing. Although there was no significant change in the average number of tumor reactive PBMCs by week 4, there was trend toward slightly higher frequencies (week 0: 27.1 ± 6.9 spots/100 K and week 4: 30.9 ± 7.4 spots/100 K, $P = .708$). Only 7/42 (16.6%) demonstrated a significant increase ($P \leq .05$) in tumor reactive lymphocytes at week 4 versus week 0. Additionally, 4/42 (9.5%) demonstrated a significant decrease in tumor reactive lymphocytes at week 4 versus week 0 (Table 1). No correlations could be made with progression-free or overall survival in either group with significant changes in the number of spots between week 0 and week 4 either increasing or decreasing (data not shown).

Moodie et al. reported that an acceptable ELISPOT signal-to-noise ratio of 2 : 1 to 3 : 1 results in a detection limit of 4 to 6 spots/1 × 10^5 PBMC, respectively, for determining response to the applied antigen [11]. We applied this methodology to our assay and adjusted for any background

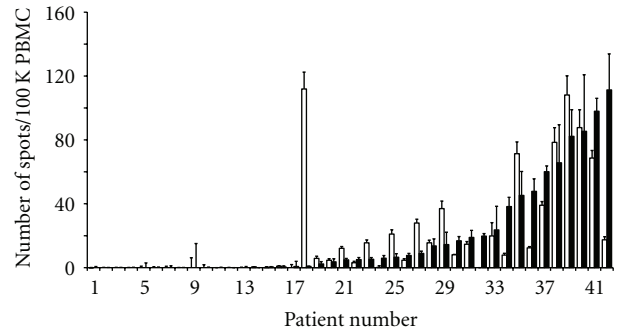

Figure 2: Results of induction of IFN-γ secreting T lymphocytes detected by ELISPOT in response to autologous tumors cells after DC-based immunotherapy for melanoma. Peripheral blood lymphocytes collected (PBMC) at week 0 (open bars) and week 4 (filled bars) were coincubated with purified autologous tumor cells at a ratio of 1 : 2, respectively, and incubated for 48 hours. Data shown is sorted by average number of spots/100 K PMBC ± SD. Dashed line approximates the 5 spots/100 K PBMC threshold.

by subtracting the activity of the nonstimulated PBMCs (Figure 2). At week 0 or baseline, 20/42 (47.6%) showed a significant number of spots (>5 spots/1 × 10^5 PBMC) in response to ATC. A similar result was seen at week 4, with 20/42 (47.6%) patients demonstrating response to their autologous tumor cells. Many of the same patients had measurable responses at both weeks (17/20).

Patients were then divided into two cohorts based upon whether they demonstrated greater than or less than 5 spots per 100,000 PBMC in response to autologous tumor cells as means of defining responders versus nonresponders at that time in their treatment schedule. Table 2 describes the patient clinical characteristics at the time of treatment for these cohorts. Log-rank tests were calculated and Kaplan-Meier plots generated for progression-free and overall survival at either week 0 or week 4. Baseline activity of tumor reactive lymphocytes at week 0 failed to correspond to either progression-free or overall survival. But at week 4, tumor reactivity correlated unfavorably to overall survival but was not associated with progression-free survival (Figure 3). A log-rank comparison of the 17 patients that had sustained tumor reactive lymphocytes (>5 spots/100 K PBMC at both week 0 and week 4) did not indicate an association with progression-free or overall survival ($P = .177$ and $P = .156$, resp.).

Although it appears that patients in the >5 spots/100 K PBMC had significantly higher lactose dehydrogenase (LDH) serum values at both week 0 and week 4 (Table 2), no significant correlation to clinical performance was found based solely on LDH values ($P = .197$ and $P = .263$, PFS and OS, resp.). Additionally, the number of ECOG 1 and 2 patients in the >5 spots/100 K PBMC is higher (8 versus 4 in the <5 spots/100 K PBMC group) which may influence the survival results since it was noted that patients who have higher ECOG scores tend to not perform as well clinically.

Table 2: Comparison of patient characteristics in the two cohorts with ELISPOT results less than or greater than 5 spots/100 K PBMC in response to autologous tumor cells at week 0 and week 4 after receiving dendritic-cell-based antimelanoma therapy.

		All	Week 0 (<5 spots)	Week 0 (>5 spots)	Week 4 (<5 spots)	Week 4 (>5 spots)
Sex	M	26	14	12	15	11
	F	16	8	8	7	9
Age (years)		49.3 ± 14.1	46.0 ± 14.8	52.9 ± 12.8	46.7 ± 14.3	52.0 ± 13.8
Disease status	M1a/b	20	7	13	7	13
	M1c	22	15	7	15	7
Anergy		11	6	5	6	5
Prior treatment	RT	6	3	3	3	3
	Chemo	12	6	6	5	7
	IL-2	10	7	3	6	4
	IFN-alpha	15	9	6	8	7
	GM-CSF	18	9	9	9	9
	Vaccine	6	3	3	3	3
	BCG	2	1	1	0	2
	mAb	1	1	0	1	0
	Biochemo	16	11	5	11	5
DC phenotype	% CD80	38.6 ± 15.1	33.5 ± 15.2	44.4 ± 13.1	34.9 ± 14.7	42.8 ± 14.8
	% CD83	10.9 ± 7.4	12.2 ± 7.0	9.5 ± 7.7	12.5 ± 7.1	9.1 ± 7.5
	% CD86	82.5 ± 9.3	78.1 ± 13.5	69.6 ± 19.6	77.3 ± 13.0	70.4 ± 20.3
	% CD11c	90.6 ± 7.3	89.3 ± 7.9	92.0 ± 6.4	88.8 ± 7.7	92.7 ± 6.3
Doses 1–3	Cell no. $\times 10^6$	16.3 ± 7.0	16.2 ± 6.8	16.4 ± 7.2	16.1 ± 7.2	16.4 ± 6.7
	% Viability	80.4 ± 9.6	79.0 ± 9.6	82.0 ± 9.1	81.0 ± 9.6	79.8 ± 9.8
DTH	DTH+ (week 0)	0	0	0	0	0
	DTH+ (week 4)	4 (2 eqv)	2 (2 eqv)	2	1 (1 eqv)	3
ECOG status	0	30	18	12	18	12
	1	10	3	7	3	7
	2	2	1	1	1	1
serum LDH (ng/mL)	LDH (week 0)	578.6 ± 391.3	453.2 ± 93.7	716.6* ± 531.4	487.6 ± 162.3	678.7 ± 530.2
	LDH (week 4)	594.6 ± 504.9	441.7 ± 92.9	762.9* ± 695.5	496.1 ± 321.6	703.1* ± 642.0

Each value is average ± SD where applicable. M1a: distant skin, lymph nodes, M1b: lung, and M1c: brain, liver or other visceral organs. RT: radiotherapy, Chemo: chemotherapy (temodar, cisplatin, vinblasin, tamoxifen, etc.), Vaccine: allogeneic tumor cell/lysate or peptide vaccines, BCG: bacillus calmette guerin, mAb: MDX-010, Biochemo: IL-2 ± IFN plus chemotherapy, DTH: delayed type hypersensitivity, eqv: equivocal result, LDH: lactose dehydrogenase. $^*P \leq .05$ compared to <5 spots/100 K PBMC.

4. Discussion

Attempts to find a reliable surrogate for monitoring of immune response during the course of immune-based therapies for cancer continues to be a challenge. The generation of cytotoxic T lymphocytes (CTL) can be used as a measure of the effectiveness of antigen presentation by dendritic cells, but only in a minority of cases does antigen-specific CTL activity correlate with clinical outcome [12].

The ELISPOT assay is a convenient method to monitor immune response since the cells used in the assay can be cryopreserved during the course of treatment and assayed together at a later date thus avoiding plate-to-plate variability. Samples handled in this way, proved to be reliably accurate and there appeared to be no adverse functional effects on the peripheral blood lymphocytes due to cryopreservation as previously reported [13]. The use of whole tumor cells as the antigen source, even at significantly high concentration (4×10^5/well), did not contribute to significant background in the ELISPOT assay, indicating that even a heterogeneous population of autologous tumor cells can be used in this assay.

Other investigators have reported immune responses in the context of ELISPOT assays which correlated with either disease-free survival or overall survival [1, 14] but in those cases, the antigens used to induce immunity were specific peptides plus GM-CSF without dendritic cells. In studies that have involved the use of dendritic cells, the correlation between ELISPOT results and survival have been mixed [15] and only a few reports have been published that use autologous tumor cells as the antigen source to measure immune recognition which were found not to correspond to outcome [16]. In addition, the extent of disease burden of patients enrolled in a study in which

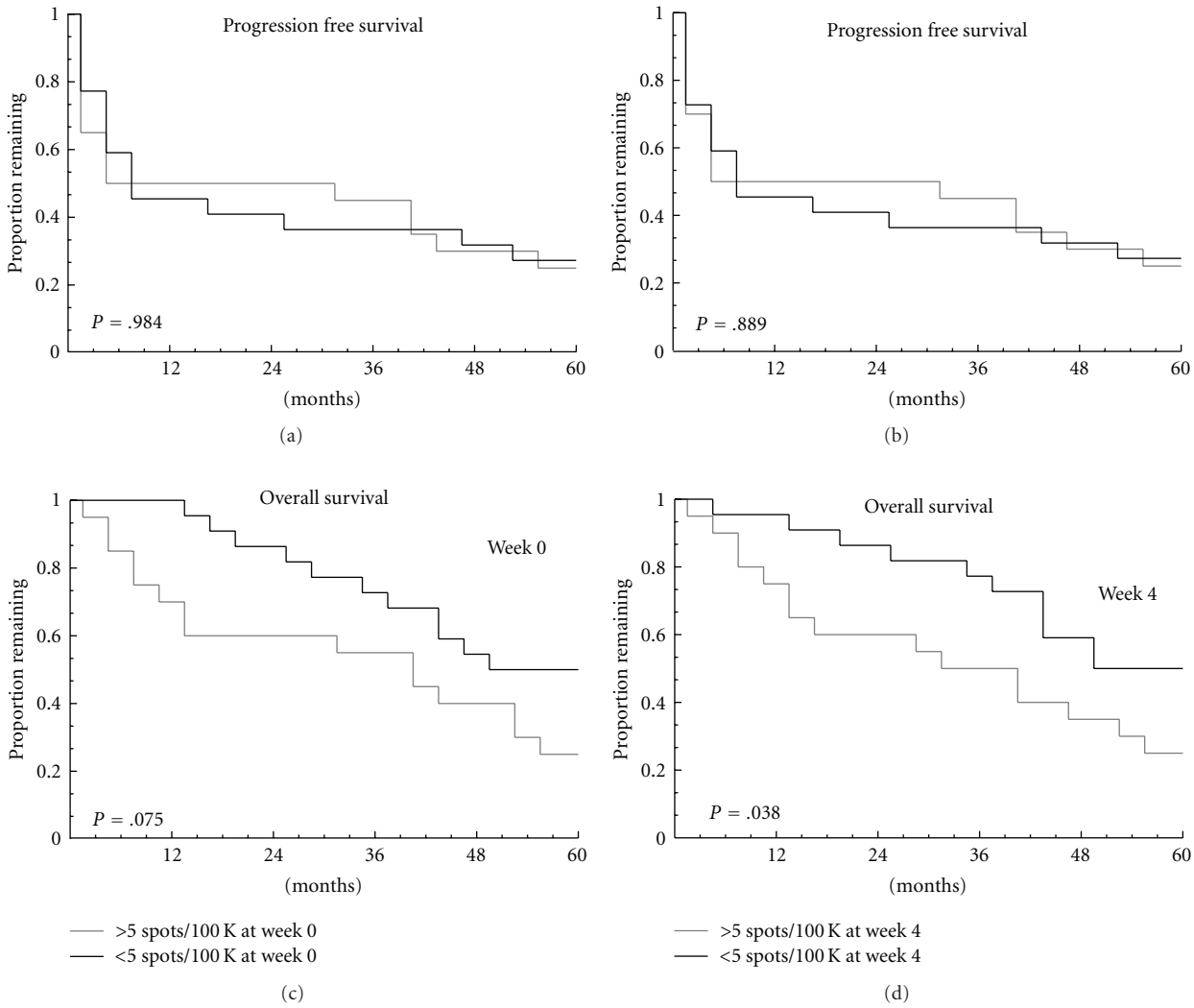

FIGURE 3: The induction of IFN-γ secreting lymphocytes in response to autologous tumor cells and correlation to either progression-free or overall survival at either week 0 or week 4. Patient cohorts were grouped according to whether they had an average of greater than (>) or less than (<) 5 spots/100 K PBMC per well observed at week 0 and week 4. Sample sizes: Week 0, $N = 20$ for >5 spots/well and 22 for <5 spots/well, and for week 4, $N = 20$ for >5 spots/well and 22 for <5 spots/well. Log-rank testing was used to determine P values.

immune monitoring was conducted was also a contributing factor in measurable responses. Patients with heavy disease burden were reported to be less responsive immunologically to therapy [17]. However, in our study it appeared that many of the patients who had a higher ECOG performance scores (ECOG 1 or 2) retained their ability to respond to their autologous tumors cells. It should be noted however that patients in this study group with ECOG scores of 1 or 2 had significantly lower progression-free and overall survival than those who had ECOG scores of 0 ($P = .002$ and .001, resp.). This contribution may explain why there appears to be a significant difference in overall survival in patients who have >5 spots/100 K PBMC as indicated in Figure 3.

However, the observation of the correlation of lack of response to autologous tumor cells and improved overall survival is counter intuitive. The reason for that observation remains unclear but a recent publication by our laboratory showed a relationship between the induction of apoptosis in response to interferon-gamma by tumor cells and poorer clinical performance [18]. The use of apoptotic cells in immunotherapy has been associated with the induction of tolerance in some cases [19]. It is possible that patients who responded to their autologous tumor cells by releasing interferon-gamma in the ELISPOT assay may also be inducing tolerance in situ (i.e., at the site of disease). Tumor infiltrating lymphocytes encountering tumor cells may be releasing interferon-gamma which in turn induces tolerance through apoptosis of tumor cells and phagocytosis of resident dendritic cells.

Overall, the results are in line with previous reports dealing with complex immune responses but further investigations will be necessary to determine what immune monitoring assay can be employed in this setting.

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Review Article

Immunotherapy for Lung Cancers

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Lung cancer is the leading cause of cancer-related deaths worldwide. Although treatment methods in surgery, irradiation, and chemotherapy have improved, prognosis remains unsatisfactory and developing new therapeutic strategies is still an urgent demand. Immunotherapy is a novel therapeutic approach wherein activated immune cells can specifically kill tumor cells by recognition of tumor-associated antigens without damage to normal cells. Several lung cancer vaccines have demonstrated prolonged survival time in phase II and phase III trials, and several clinical trials are under investigation. However, many clinical trials involving cancer vaccination with defined tumor antigens work in only a small number of patients. Cancer immunotherapy is not completely effective in eradicating tumor cells because tumor cells escape from host immune scrutiny. Understanding of the mechanism of immune evasion regulated by tumor cells is required for the development of more effective immunotherapeutic approaches against lung cancer. This paper discusses the identification of tumor antigens in lung cancer, tumor immune escape mechanisms, and clinical vaccine trials in lung cancer.

1. Introduction

Lung cancer is the most common cause of cancer death worldwide in both men and women, accounting for 1.2 million deaths per year. Despite recent advances in surgery, irradiation, and chemotherapy, the prognosis is poor [1–3]. Therefore, the development of new therapeutic strategies is essential. Immunotherapy is an attractive candidate because the generation of specific antitumor immune responses through the identification of tumor-specific antigens can promote tumor cell death with minimal impact on normal tissue [4]. However, immunotherapy is effective in only a limited subset of patients. Tumor escape mechanisms from host immune surveillance remain a major obstacle, and many tumor cells, including lung cancer, are able to promote immune tolerance and escape host immune surveillance, resulting in the inhibition of anti-tumor immunity [5, 6]. These include a decrease or loss of the expression of tumor antigen, downregulation or loss of expression of human leukocyte antigen (HLA) molecules, expression of immunosuppressive factors by cancer cells, regulatory T cells, and tolerant dendritic cells. Understanding of the immune-evasion mechanisms regulated by tumor cells is necessary in developing more effective immunotherapeutic approaches to lung cancer.

2. Immune Recognition of Cancer

Tumor regression *in vivo* is mediated by innate and adaptive immune responses involved with tumor-antigen presentation in the patient's lymphoid tissues. Innate mechanisms trigger inflammatory responses in the tumor microenvironment that presents sufficient local cytokines (i.e., IL-2, IL-12, IL-18, and IL-23) and stimulates antigen presenting cells (APCs) and dendritic cells (DCs) against tumor antigens [7, 8]. After DCs capture and digest tumor cells, tumor antigens associated with human leukocyte antigens (HLA I or HLA II) on the DC surface are presented to T-cell receptors (TCRs) of naive $CD4^+$ and $CD8^+$ T cells, resulting in the activation of naive T cells. Subsequently, costimulatory molecules (CD80, CD86) on DCs interact with CD28 on T cells for the full activation of T cells. After

activation and costimulation, $CD4^+$ and $CD8^+$ cells both produce a series of cytokines that differentiate T-Helper ($CD4^+$) lymphocytes into two subpopulations: Th 1 and Th 2 cells [9–11]. Th 1 cells produce IL-2, IFN-γ, TNF-α, and granulocyte macrophage colony stimulating factor (GM-CSF) that increase the activation of macrophages and upregulation of HLA I molecules on the surfaces of $CD8^+$ cells. Th 2 cells secrete IL-4, IL-5, IL-6, and IL-10 that induce naive B cells to produce specific antibodies.

The shifting towards Th2 pattern has recently been associated with increased tumor metastasis and decreased survival in many human and animal neoplasia. IL-4, IL-6 and IL-10 levels, but not IFN-γ and IL-2, were significantly higher in the serum, secreting supernatant or transcripts produced by PBMCs from lung cancer patients [12, 13]. IL-6 and IL-10 secretion derived from lung cancer cells is upregulated by tumor cell-derived prostaglandins and TGF-β. IL-6 induces directly STAT3 signaling of cancer cells to upregulate several genes, such as c-myc, bcl-2 and Mcl-1, resulting in induction of tumorigenesis [14]. IL-10 also possesses several properties that suppress the generation of anti-tumor immunity [12, 13]. IL-10 inhibits a broad array of immune parameters, including proinflammatory cytokine production by macrophages, antigen-presentation function, T lymphocyte proliferation, and Th1 cytokine production. Increased IL-4 by tumor cells repressed the secretion of Th1 cytokines has been found to have inhibitory effects on anti-tumor immune response. IL-4 directs the development of Th2 cells and downregulates IFN-γ production in Th1 cells, inhibits the production of IL-12 and IFN-γ by monocytes [12, 13]. Therefore IL-4 and IL-10 are key cytokines for the inhibition of Th1 cytokine response and the development of the Th2 cytokine response, which reduces the protective cellular immunity and induces tumor progression.

Cytotoxic T cell (CTL) is a major effector of tumor regression. When $CD8^+$ T cells bind to class I antigens on APCs, Th1 cytokines stimulate the generation of antigen-specific CTL, which expresses perforins, granzyme, and Fas ligand that directly eliminate neoplastic cells. CTLs also secrete specific cytokines (IFN-γ, TNF-α, and TNF-β) and activate macrophages against tumor cells directly [10, 11, 15]. Conversely, depending on the tumor microenvironment, these cytokines also stimulate tumor progression [16].

Natural killer (NK) and Natural Killer T (NKT) cells are innate immune cells critical for the first line of defense against tumorigenesis [17]. Different from T cells, NKs and NKT cells inhibit tumor growth in an MHC-nonrestricted manner [18–23]. Natural Killer (NK) cells are a type of cytotoxic lymphocyte that exhibit cytolytic activity against a variety of allogeneic targets in a nonspecific, contact-dependent, nonphagocytic process which does not require prior sensitization to an antigen [18–20]. NK cells share several properties with conventional cytotoxic T cells (CTL) and appear to possess similar mechanisms for cytolysis including secretion of perforin and granzyme. Their cytotoxic activity is positively regulated by IL-2 and IFN-r. Frequently, tumor cells (like stressed cells) express different glycoproteins (MICA and MICB) on their surfaces that function as ligands for NKG2D receptors on NK cells. Once activated, these receptors stimulate NK cell activity to lyse tumours through the perforin/granzyme pathway or apoptosis-inducing ligands such as tumour-necrosis factor (TNF-) related apoptosis-inducing ligand (TRAIL or FasL). NK cells secrete IFN-γ by IL-12, which inhibits tumour-cell proliferation, enhances tumour-cell apoptosis, improves tumour antigen presentation and inhibits angiogenesis [18–21].

NKT cells are a subset of T cells that coexpress an $\alpha\beta$ T-cell receptor (TCR), but also express a variety of molecular markers that are typically associated with NK cells, such as NK1.1 [22–24]. NKT cells are restricted by the nonpolymorphic CD1d molecule and are activated by lipid and glycolipid antigens presented by CD1d. NKT cells share other features with NK cells as well, such as CD16 and CD56 expression as well as cytolytic perforin and granzyme release. Although NKT cells possess NK-like cytolytic activity, their activation results in rapid production of IFN-γ and expression of CD40L, thus providing help for activation of CD40-expressing APCs and generation of cellular and humoral immune responses [17, 22–24]. Under the existence of tumor cells, NKTs cell recognition of glycolipid antigens of tumor cells presented by CD1d can either lyse tumour cells directly using the perforin/granzyme system or ligands (TRAIL or FasL) for death receptors or stimulate other cytotoxic cells such as NK and $CD8^+$ T cells through IFN-γ secretion [22–24]. NK and NKT cells both produce chemokines that are important for recruiting effector T cells, B cells, neutrophils, and other NK and NKT cells to the disease site. NK- and NKT-derived IFN-r by stimulation of IL-12 is able to up-regulate the expression of the chemokine receptor CXCR3, which mediates subsequent recruitment of $CXCR3^+$ T and NK cells to tumor-infiltrated tissues [17, 22–24].

3. Vaccine Strategies

The capture and presentation of tumor antigen by APCs are key steps for successful active immunotherapy [25, 26]. In comparison to restriction of class I or class II pathways and selective stimulation of either $CD4^+$ Th cell or $CD8^+$ cytotoxic T-cell effectors by peptides, whole recombinant proteins are processed into multiple peptides and presented by APCs via class I and class II pathways to $CD4^+$ and $CD8^+$ T cells, respectively, and have the potential for generating immune effectors and immune memory [25]. Tumor-derived antigen mixtures contain multiple dominant and minor antigenic determinants within whole proteins, permitting the host to select, process, and present on HLA, the most immunogenic epitopes relative to that individual [25].

The most commonly used multivalent formulations employ autologous or allogeneic tumor cells. Autologous tumor vaccine is produced by isolating adequate amounts of tumor cells from an individual and processing these tumor cells into a vaccine formulation in vitro; the vaccine is then administered to the individual from whom the tumor cells were isolated. Autologous tumor vaccines have been shown to have immunologic activity in a number of studies.

An autologous tumor vaccine usually combined with an adjuvant elicits effectively a specific CTL-mediated cytolytic response against tumor cells [25–28].

Allogeneic tumor vaccine composed of tumor cells isolated from the tumor of one patient, killed and processed, and administered to another patient in order to stimulate cytotoxic immune responses to a similar tumor cell type. The cells found in this type of whole-cell vaccine express many cell-surface tumor-associated antigens. This vaccine is frequently administered with an adjuvant immunostimulant. Using allogeneic antigens also generates a uniform preparation, which speeds up the immune assessment and comparability not offered by the use of autologous tumor antigen, thereby allogeneic approaches are attractive during therapeutic development and clinical testing [25–28].

Two additional allogeneic sources of antigen are synthetic peptide and recombinant protein. In contrast to allogeneic tumor, peptides and proteins are applied in monovalent formulations. In spite of being easily synthesized and uniform, providing the simplest and most reproducible immunologic measures of biological efficacy, peptides require patient selection based on HLA tissue typing and also have designated restriction to class I or class II pathways, selectively stimulating either $CD8^+$ cytotoxic T-cell effectors or $CD4^+$ Th cells, responsible for immune memory. By contrast, whole recombinant proteins are processed into multiple peptides and presented by APCs via class I and class II pathways to $CD4^+$ and $CD8^+$ T cells, respectively, and have the potential for generating responses of immune effectors and immune memory [25–28].

By identification of tumor-associated antigens, many tumor vaccines have been established by investigators and effective generate specific immunity against tumor cells and treatment in lung cancer patients. Cancer-associated mucins are a potential target for immunotherapy. These molecules facilitate adhesion of malignant cells to the endothelial cell surface and promote metastases. They are tumor-specific immunogens because they exhibit unique glycosylation patterns [29]. The BLP25 liposome vaccine (L-BLP-25) carries the mucin-1 (MUC-1) protein admixed with monophosphoryl lipid A as an immune adjuvant. Trials of the L-BLP-25 vaccine in stage III and IV NSCLC patients have demonstrated safety but not a statistically significant survival benefit. Nonetheless, a subset of patients ($n = 75$) with IIIB disease has shown a trend towards improved survival ($P = .09$). In 2007, Merck Serono sponsored a multicenter (international) phase III, randomized, double-blind, placebo-controlled trial where 1300 patients with unresectable stage III NSCLC responded to first-line, platinum-based chemoradiotherapy [30].

The C-T antigens (MAGE-1, MAGE-3, BAGE, BAGE, GAGE, KK-LC-1, and NY-ESO-1) are encoded by genes that are completely silent in most normal tissues but are activated in a wide variety of tumors. Although normal cells, placental trophoblasts, and male germ-line cells express C-T antigen, the cells lack HLA I molecules and cannot present the antigens to T cells [31, 32]. Therefore, tumor C-T antigens are considered to be highly promising targets for anticancer vaccine [33]. MAGE-3 is aberrantly expressed in a wide variety of tumors, including NSCLC. Several $CD8^+$ T-cell epitopes of MAGE-3 have been identified *in vitro*. GlaxoSmithKline produced a vaccine that carries recombinant MAGE-3 fusion protein (His-tagged/full-length MAGE-3 protein/influenza protein D) plus immune adjuvant AS02B (monophosphoryl lipid A and QS21) [34]. A recent randomized phase II trial conducted on 182 stage IB or II NSCLC MAGE-3 positive patients (122 vaccine and 60 placebo) has demonstrated a trend towards improved survival in stage II patients receiving the vaccine compared to placebo. The results are enough for a phase III investigation. The study plans to accrue 2270 MAGE-3-positive patients with completely resected stage IB, II, or IIIA NSCLC. Furthermore, epitopes from the CT antigens TTK protein kinase (TTK), lymphocyte antigen 6 complex locus K (LY6 K), and insulin-like growth factor (IGF)-II mRNA-binding protein 3 (IMP-3) have been demonstrated to elicit CD8 responses in 20%–70% of HNSCC patients tested [35], and 50% HNSCC (5/10) patients vaccinated against these peptides have resulted in clinical responses [36].

Epidermal growth factor (EGF), now a well-established target for biologic therapy, is also a potential tumor antigen. Preclinical studies have established the antigenicity and antitumor activity of EGF protein administered to animals [37]. In two randomized phase II studies, recombinant EGF conjugated to Neisseria meningitides P64K protein as carrier protein and emulsified with the adjuvant Monotanide ISA51 was administered to 40 advanced NSCLC patients. Anti-EGF antibody responses were identified with a significant increase in survival for patients who maintained antibody response (9.1 months versus 4.5 months). The same agent was tested in a larger randomized phase II clinical trial that vaccinated 100 patients with stage IIIB or IV NSCLC who had progressed through first-line chemotherapy, and 45% of vaccinated patients developed a strong anti-EGF antibody response and decreased serum EGF concentration. Compared to controls (best supportive care), those who received the treatment had significantly longer overall survival (8.5 versus 4.3 months) [38, 39].

Xenogeneic anti-idiotype antibodies are quite unique antigen-mimic preparations, generated as antibodies to tumor antigen-binding sites on other antibodies (that generates a template of the antigen). The xenogeneic nature of these preparations makes them inherently immunogenic, and the similarity of the antiidiotype antibody to the tumor antigen allows cross recognition of the parent/native protein. Antiidiotypic vaccines are used to elicit tumor-specific antibodies as the dominant effectors for therapeutic activity; these have been the most widely tested immunotherapy approaches in SCLC [25–28].

Tumor antigens like the ganglioside, GD-3, have been identified as targeted active immunotherapy strategies become more feasible. In SCLC patients after chemotherapy or combined chemotherapy and radiotherapy, vaccination with an anti-idiotype GD3 monoclonal antibody (BEC2) and BCG induces antiganglioside GD3 antibodies and prolong survival compared to control subjects. However, this agent provides no survival benefit in a large randomized international phase III trial by Merck. BEC plus BCG vaccine

induces humoral response in only one-third of 213 patients and the investigators suggest that a multivalent rather than a monovalent approach may be better in the treatment of lung cancer patients [40].

Other tumor-associated antigens, hyaluronic acid-mediated motility (RHAMM) and carboanhydrase IX (G250/CAIX), are overexpressed in HNSCC and served as immunogens *in vivo* in 4 of 8 HLA-A2+ patients, while 0.06%–0.13% of $CD8^+$ effector T cells recognized tetramers for RHAMM or G250 and secreted IFN-r and granzyme B in ELISPOT assays [41]. Otherwise, NKG2D ligands MHC class I-related chain molecules A (MICA) and UL16-binding proteins (ULBPs) are over-expressed in the primary HNSCC as compared to nontumor tissues of vocal cord polyps. The ligands reportedly activate NK cells and generate adaptive immunity through binding to NKG2D receptor. However, other studies demonstrate significant variability of expression [42, 43].

4. Promotion of Antigen Recognition

In order to initiate or promote antigen-specific responses, tumor antigens have to incorporate adjuvants that lead to increases in various arms of the immune cascade, antigen recognition, uptake, presentation, and/or antigen-specific cellular reactivity [25, 26]. Some biologic adjuvants [25, 26, 44] (bacillus Calmette-Guerin (BCG), diphtheria toxoid, and tetanus toxoid and chemical adjuvants (aluminum hydroxide, montanide ISA 51, and incomplete Freund's adjuvant) induce an inflammatory response at the site of delivery, which accelerates the migration of APCs to the site of delivery and enhance the capture and processing of tumor antigens by APCs in the inflammatory environment. Moreover, DC precursors are harvested from patients and cultured with antigen to activate DCs *ex vitro* [45]. The activate DCs are subsequently delivered back to the individual, where they expectedly migrate to the lymph node and come to the desired antigen-specific immune response.

Small molecules like Toll-like receptor-9 (TLR9) agonists [46, 47] can stimulate Toll-like receptors and initiate the innate and adaptive immune responses and have been under investigation for treating cancer. TLR9 is expressed in endosomes of dendritic cells, plasmocytoid dendritic cells, and T and B lymphocytes and regulates innate antigen-specific immunity via the recognition of pathogen-associated molecular pattern. Activation of TLR9 signalling pathway by TLR9 agonists leads to increased production of proinflammatory cytokines and chemokines and stimulation of an immune response with antitumor effects. Several new immunomodulatory oligonucleotides have been evaluated in models of human cancer [46, 47]. Among these, PF-3512676 (ProMune) is particularly promising. It contains unmethylated cytosine and guanine (CpG) motifs and a nuclease-resistant phosphorothioate backbone. The anticancer activity of PF-3512676 is related to direct and indirect immunomodulation of both innate and adaptive immune responses. Plasmocytoid dendritic cells stimulated by PF-3512676 express increased levels of MHC I and II and costimulatory molecules (leading to improved antigen presentation) secrete cytokines and chemokines that enhance natural killer (NK) cell activity directed toward tumor cells, present tumor-specific antigens and costimulatory molecules to B and T cells and generate long-living antigen specific cytotoxic T-lymphocytes, and antibody responses. A good indicator of activation and maturation of dendritic cells by PF-3512676 is the production of IFN-α and the subsequent induction of interferon-inducible protein 10 (IP-10), an antiangiogenic cytokine [46]. In NSCLC, a phase II study enrolling 112 chemonaive patients with NSCLC was conducted. The patients received PF3512676 in combination with platinum, and taxane doublet chemotherapy. Twenty-eight (37%) patients had a partial or complete response with the combination of chemotherapy and PF-3512676 and 7 (19%) with chemotherapy alone. Based on these preliminary data, two phase III trials were conducted to test the efficacy of PF-3512676 in combination with platinum based chemotherapy in advanced NSCLC patients [46, 47].

Cytokine can be used at the site of tumor or combined with exogenous tumor antigen to promote APC maturation and activation and HLA class I molecule expression on tumor cells, which generates effective CTL responses againt tumor cells [25, 48]. *In vivo* cytokine gene transfer can also target normal cells in the tumor environment, thereby achieving high local concentrations of cytokine that avoid toxicities associated with systemic administration. Gene therapy has been applied in clinical trials for over a decade. Gene transfer of cytokines or costimulatory molecules directly to tumor cells *ex vivo* and *in vivo* are attractive ways of making nonimmunogenic cells more immunostimulatory [25, 49]. The cytokine granulocyte-monocyte colony stimulating factor (GM-CSF), a significant mediator of proliferation, maturation, and migration of dendritic cells can enhance the generation of potent, durable anti-tumor immunity [50, 51]. GM-CSF and IL-2 combined with tumor antigen causes high local concentrations of stimulatory cytokines at the site of antigen delivery and stimulates APC and T cell activation. Fas ligand (FasL) and GM-CSF coexpressed in tumor cells administrate in to mice, which accelerate the recruitment of innate immune cells, activation of dendritic cells, and the generation of specific and memorial anti-tumor immunity against tumor cells *in vivo* [52]. The benefit of incorporating GM-CSF into anti-tumor vaccines is well established. In a multicenter phase I/II trial, Nemunaitis et al. produced a vaccine (GVAX) that contains autologous, irradiated lung tumor (NSCLC) cells engineered to secrete GM-CSF. Among 33 patients with advanced NSCLC, three (2 with bronchoalveolar carcinoma) achieved complete response and prolonged remission. Longer median survival was observed in patients whose vaccines secreted more GM-CSF (17 months versus 7 months), suggesting a cytokine dose-response relationship. Eight of ten patients with early-stage lung cancer remained disease-free with a medium followup of 12 months. However, establishing GVAX required much time. In the beginning, 83 tumors had to be harvested. Vaccines could not be successfully produced in 16 patients and 11 others died before vaccine was delivered. The medium generation time was 49 days. There were only 43 patients immunized with the vaccine [50, 51].

Most immunomodulatory drugs, including cyclooxygenase-2 (COX-2) inhibitors and thalidomide-like agents (Lenalidomide), have immunologic properties that promote a favorable immune environment [53, 54]. Furthermore, antisuppressive agents like cyclophosphamide and fludarabine abrogate the activity of immunosuppressive cells-regulatory T cells (T-reg). Therefore, these agents have therapeutic potential that can synergize with cancer vaccines and other active immunotherapy strategies [25, 55–60].

COX-2 is an enzyme that catalyzes the synthesis of prostaglandins (PGs), including prostaglandin E2 (PGE2) [61]. COX-2 and PGE2 overexpression are seen in many malignancies including lung cancer. In nonsmall cell lung cancer (NSCLC), COX-2 is overexpressed in most adenocarcinomas and squamous cell carcinomas. Elevated tumor COX-2 and PGE2 levels have been implicated in angiogenesis, tumor invasion, resistance to apoptosis, and suppression of antitumor immunity. PGE2 secretion mediated by COX-2 can negatively regulate T-lymphocyte proliferation and cytotoxicity, and mediate the imbalance between IL-10 and IL-12 in favor of IL-10 production. The tumor microenvironment is predominantly polarized toward Th2-like or immunosuppressive immune responses. The overexpression of phosphoglycerate kinase (PGK-1) [62, 63] or Interlukin-27 (IL-27) [64] in lung cancer cells both downregulate COX-2 and PGE2, which not only directly suppress tumorigenesis but also enhance the activation of immune cells and generation of specific Th1 anti-tumor immune response *in vivo*. Preclinical animal model studies show tumor reduction when animals are treated with either nonspecific or specific inhibitors of COX-2. Based on these observations, celecoxib, a selective COX-2 inhibitor, has been evaluated in combination with chemotherapy for the management of metastatic NSCLC in patients who have failed prior chemotherapy. Ongoing clinical trials are also evaluating the combination of celecoxib with chemotherapy (paclitaxel and carboplatin) and/or radiation or celecoxib in combination with epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI; gefitinib) of NSCLC [53, 61].

Lenalidomide [47, 54] was synthesized based on the structural backbone of thalidomide, by adding an amino group at position 4 of the phthaloyl ring and removal of the carbonyl group of the 4-amino-substituted phthaloyl ring. Such structural changes were designed to enhance its immunomodulatory and antitumor activity. Lenalidomide induces increase in IL-2 and IFNγ secretion and upregulation of CD40L expression on anti-CD3-stimulated T cells, resulting in activation of natural killer cells, and thus improving host immunity against tumor cells. Compared to thalidomide, lenalidomide is 50 to 2000 times more potent in stimulating T-cell proliferation and activation and 50–100 times more potent in augmenting IL-2 and IFNγ production. In addition, lenalidomide has been shown to inhibit endothelial cell migration and adhesion, perhaps by downregulating endothelial cell integrins. Lenalidomide is reported to downregulate key cytokines such as TNF-α, IL-6, IL-8, and VEGF, that is, cytokines which favour tumor cell survival, proliferation and possibly resistance to therapy, mainly by affecting the tumor vasculature. In solid tumors, lenalidomide proved to have a good safety profile both in monotherapy and in combination with chemotherapy showing results in terms of antitumor activity in several tumor types and also in NSCLC. In fact, Miller et al. tested the feasibility of lenalidomide at a dose escalated from 5 to 10 to 25 mg/day in 20 patients with solid tumors refractory to standard treatment. One partial response and three stable diseases were documented; of these patients, three had NSCLC diagnosis. This study recommended 25 mg/day, orally, of lenalidomide as single agent for 4 weeks followed by 2-week rest period. Similarly, Kalmadi et al. explored safety and tolerability of lenalidomide in association with docetaxel and carboplatin in 14 patients with advanced solid tumors.

5. Challenges in Immunotherapy: Tumor Escape Mechanism

By identifying tumor-specific antigens recognized by CTL, several clinical trials of therapeutic vaccine bearing with these antigens have promoted tumor-specific immunity. However, only 2%–4% of patients have observed tumor regression [65]. There is a number of escape mechanisms from the host's immunosurveillance regulated by cancer cells, including loss of tumor antigen, downregulation of HLA molecule expression, and secretion of immunosuppressive soluble factors ligands [65].

During tumor progression, tumor cells often display loss or down-regulation of HLA I antigen. In surgically resected specimens, 25%–94% of NSCLCs have down-regulated HLA I expression. Thereafter, one possible mechanism of the escape host immuno-surveillance immune escape is tumor cells with abnormal HLA I antigen expression, leading to develop clinical cancer [66]. A haplotype loss of HLA I antigen is a common cause of abnormal HLA expression in various types of tumors, as mentioned above [67–70]. Moreover, β2-microglobulin gene ($\beta2$-m) abnormality is common in abnormal expressions of HLA I [67]. Transduction of the wild-type $\beta2$-m gene renders them positive for HLA class I expression. An autologous CTL clone is induced by stimulating the wild-type $\beta2m$-transduced lung cancer cell line with the genetic abnormality of $\beta2m$. HLA class I-deficient cancer cells can escape from an attack by CTLs, and a reformation of HLA class I expression in cancer cells restores CTL recognition against cancer cells.

Cancer cells often secrete immuno-suppressive cytokines, including transforming growth factor-β (TGF-β), interleukin-10 (IL-10), and indoleamine 2,3-dioxygenase (IDO) [71, 72]. IDO is a tryptophancatabolism enzyme that is overexpressed in various tumors. It leads to T-cell dysfunction and apoptosis through the depletion of tryptophan. Arginase, an amino acid-catabolizing enzyme, is expressed in tumor cells to decrease CD3z expression of T-cell clones [73] and inhibit antigen-specific recognition. The infiltrating T cells in the patients possess a high level of arginase activity (arginase I) and decreased CD3ξ levels. Soluble MHC class I chain-related molecule A (MICA) derived from tumor cells is able to systemically downregulate NKG2D expression on the surface of CD8 T cells and natural killer (NK) cells [74], thereby impairing activity of effector

cells against tumor cells. Thus, tumor-derived soluble factors assist tumor cells in the evasion of immune attack, allowing tumor progression and metastasis.

Many cancers express immuno-suppressive costimulatory molecules such as programmed death ligand-1 (PD-L1) [75, 76] PD-L1 has been shown to suppress immune responses through PD-1 receptor on activated T cells and B cells, which decreases immune responses. PD-L1 on lung cancer cells demonstrates that it is able to increase apoptosis of antigen-specific T cells and to inhibit CD4 and CD8 T cell activation, resulting in reduced anti-tumor immunity and evasion of host immune surveillance [75, 76]. Fas system is one of the killing pathways by CTLs and NK cells to tumor cells in human body. However, reducing Fas expression and the over-expression of Fas ligands are observed in lung cancer, contributing to tumor immune privilege by inducing FasL-mediated apoptosis of host CTL and NK cells and destructing infiltrating Fas-bearing lymphocytes [77].

6. Immunosuppressive Immune Cells (MDSC, TAM, Treg)

Solid tumors consist of both malignant cells and a number of nonmalignant stromal cell types, including endothelial cells, fibroblasts, and various immune cells. Complex interactions occur between these within the tumor microenvironment and impact on immunosurveillence and tumor progression [78]. It has been reported that anti-tumor immune responses are downregulated by immuno-suppressive immune cells, which include myeloid-derived suppressor cells (MDSCs), M2 macrophages, and regulatory T cells (Tregs). VEGF, GM-CSF, M-CSF, IL-6, and IL-10 secreted by growing tumors and stromal cells cause abnormal myelopoiesis that ultimately leads to the suppression of immune responses. The success of immune therapy for cancer will depend on integrating strategies that down-regulate immune suppression [79, 80].

Studies provide evidence that MDSCs are directly involved in the suppression of immune responses in cancer. An increase in the number of MDSCs has strong natural suppressive activity in cancer patients or tumor-bearing mice [81, 82]. In murine tumor models, the number of MDSCs in spleen increase by 5- to 20-fold, depending on the tumor model, and is easily detected in the lymph node and tumor site. Recent findings demonstrate that ROS and peroxynitrite derived from MDSCs can induce antigen-specific $CD8^+$ T cell tolerance through a posttranscription mechanism that involves the modification of CD8 and TCR itself on the T cell surface [83–85]. $CD8^+$ T cells from MDSC-treated mice are unable to produce IFN-r and interleukin-2 in response to specific peptides and do not kill peptide-load target cells. MDSCs, in addition to inducing tumor-specific T-cell tolerance, also cause the development of Tregs. MDSCs in tumor-bearing hosts also reduce the number and activation of T-cells through the production of nitric oxide (NO) and arginase-1 [86, 87]. NO inhibits T cells through the blockade of activity in the JAK3 and STAT5, inhibition of HLA II gene expression, and induction of T cell apoptosis, while arginase 1 causes the depletion of arginine and translational blockade of the ξ-chain of CD3. Combination of high arginase activity and increased NO production by MDSCs also leads to increased ROS production. This increase is able to suppress T cells by cell-to-cell contact. Depleting of MDSCs by using anti-Gr1 antibodies has been shown to significantly improve $CD8^+$ T cell immune response and allow for eradication of the variant tumor cell lines [81, 82]. In addition, elimination of MDSCs with All *trans*retinoic acid (ATRA) has also been found to promote CD4- and CD8-mediated tumor-specific immune responses, and may open an opportunity to improve the effect of cancer vaccine [81, 82].

In some cases, macrophages can represent 50% of the cellularity within a tumor. The increased number of M2 macrophages in the tumor stroma is associated with poor prognosis in NSCLC [88–92]. M2 macrophages are derived from circulating monocytes that are recruited to tumors by chemotactic factors such as CCL2, VEGF and M-CSF [88–90]. M2 macrophages are able to secrete IL-10 and TGF-β and inhibit Th1 immune response, leading to enhanced wound healing and tissue remodeling as well as promotion of tumor formation. Differentiation of M2 macrophages is induced by IL-4, IL-10, IL-13, IL-21, activin A, immune complexes, and glucocorticoids. M2 macrophages also express high levels of IL-1 receptor antagonist, CC ligand 22 (CCL22), scavenger, mannose receptor, galactose receptor, arginase I, and CD163 antigen. In tumor angiogenesis, M2 macrophages play an important role of secreting proangiogenic factors and enzymes, including vascular endothelial growth factor (VEGF) and matrix metalloproteinase 9 (MMP9) [91, 92]. Several studies have shown that the activation of TLRs, such as TLR9, decreases the development and activity of M2 macrophage [88–90, 93], and activation of TLR9 by synthetic CpG oligodendronucleotides demonstrated anti-tumor effects and survival increased significantly in many preclinical models. Knock-down of a crucial phosphatase, SHIP1, has been showed to suppress development of M2 macrophages in mice, and thus, pharmacological modulators of this phosphatase are under investigation currently [88–90, 93].

The accumulation of regulatory T cells (Tregs) in tumor is reportedly associated with unfavorable prognosis in NSCLC patients [94]. The number of Tregs exist in high proportions in the TIL of patients with lung cancer and play a role in suppressing anti-tumor immune responses. Tregs can be recruited to tumor sites by secretion of CCL22 derived from tumor cells and TAMs [95]. Tregs isolated from tumors mediate the potent inhibition of proliferation of autologous peripheral blood T cells stimulated by anti-CD3 or anti-CD3/anti-CD28 [96]. These Tregs play a role in inducing or maintaining tolerance to tumor in patients with lung cancer. Tregs are known to suppress DC function via TGF-β and IL-10 [97]. Recent clinical studies indicate that high levels of tumor infiltration by activated $CD8^+$ T cells combined with a low number of Tregs is a significant positive prognostic factor for survival in cancer patients [98, 99]. Thus, reducing the number or activity of Tregs in tumor-bearing hosts may induce effective tumor immunity by activating tumor-specific as well as nonspecific effector cells. Removal of Tregs by anti-CD25 antibody can augment effector T cell-mediated

tumor immunity that strongly inhibits tumor growth in cancer patients [100, 101]. Activation of GITR signaling by agonist anti-GITR antibody or GITR ligand can inhibit the suppressive activity of Tregs and enhance tumor-specific $CD4^+$ and $CD8^+$ T cell responses. CTLA-4 blockade by anti-CTLA-antibody also augments tumor inhibition by attenuating Treg suppression and augmenting effector T-cell activity. The combination therapy of anti-CTLA-4-blocking antibody and anti-GITR agonist antibody has demonstrated that there have synergistic antitumor effects causing rejection of advanced stage tumors compared with either antibody therapy alone [100, 101].

7. Conclusion

Immunotherapy for lung cancer is potentially effective treatment in terms of high specificity, low toxicity, and prolonged activity. Nonetheless, it is necessary to integrate novel approaches with traditional therapeutic methods to offer more appropriate therapy, including representation of antigen epitopes, restoration of APC immune-stimulating activity, expansion of tumor-reactive T cells, and down-regulation of suppressor pathways. In the future, using combinations of multiple immunologically active agents, conventional treatment modalities, and novel targeted therapies will overcome limitations of any single approach and lead to significant improvements in therapeutic outcomes of lung cancer.

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Review Article

The Consequence of Immune Suppressive Cells in the Use of Therapeutic Cancer Vaccines and Their Importance in Immune Monitoring

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Evaluating the number, phenotypic characteristics, and function of immunosuppressive cells in the tumor microenvironment and peripheral blood could elucidate the antitumor immune response and provide information to evaluate the efficacy of cancer vaccines. Further studies are needed to evaluate the correlation between changes in immunosuppressive cells and clinical outcomes of patients in cancer vaccine clinical trials. This paper focuses on the role of T-regulatory cells, myeloid-derived suppressor cells, and tumor-associated macrophages in cancer and cancer immunotherapy and their role in immune monitoring.

1. Introduction

In April 2010, the U.S. Food and Drug Administration (FDA) approved sipuleucel-T (Provenge, Dendreon Corp., Seattle, WA) for the treatment of asymptomatic or minimally symptomatic metastatic castration-resistant prostate cancer (mCRPC). Provenge has been shown to improve overall survival in mCRPC patients by 4.1 months compared to placebo (25.8 months versus 21.7 months, resp.; $P = .032$; HR: 0.775; 95% CI: 0.614, 0.979) [1]. Approval of the first therapeutic cancer vaccine is a milestone in cancer immunotherapy; nevertheless, the question still remains: how do therapeutic cancer vaccines work? Immune response to a pathogen (i.e., virus, bacterium, yeast) or to tumor cells is a complex, incompletely understood process involving multiple factors.

The rationale for therapeutic cancer vaccines (as opposed to preventive cancer vaccines, which are not the subject of this paper) arose from the hypothesis that the cancer cells are under surveillance of a healthy immune system, and that cancer spreads when the host immune system fails to control the growth of tumor cells. The specific reasons for this failure of the immune system are not well known. In the last decades, much research in cancer immunology has focused on defining tumor-specific antigens or tumor-associated antigens (TAAs) capable of inducing an immune response against tumors. Various vaccine strategies and modalities have also been tested in an effort to achieve this goal [2, 3]. To date, this pursuit has encountered some major obstacles. The lack of a strong antitumor response might be related to the intrinsic nature of the tumor antigen itself which, unlike a viral or bacterial invader, is usually a self-antigen. Moreover, a weak immune response is frequently associated to the treatment (i.e., chemotherapy or radiotherapy) the cancer patient has previously received. This should thus be taken into account when designing clinical trials employing a combination of cancer vaccines and standard therapies. Particular emphasis should also be placed on the optimal schedule for the various treatments because, while chemotherapy and radiotherapy can have an immunosuppressive effect, studies have shown that they may also increase the expression of several TAAs on tumor cells, or cause a "rebound effect" on immune cells that can be used to enhance the antitumor response (see [3] for review). In this scenario, the analysis of the immunological effects of targeted therapies that use antibodies and small

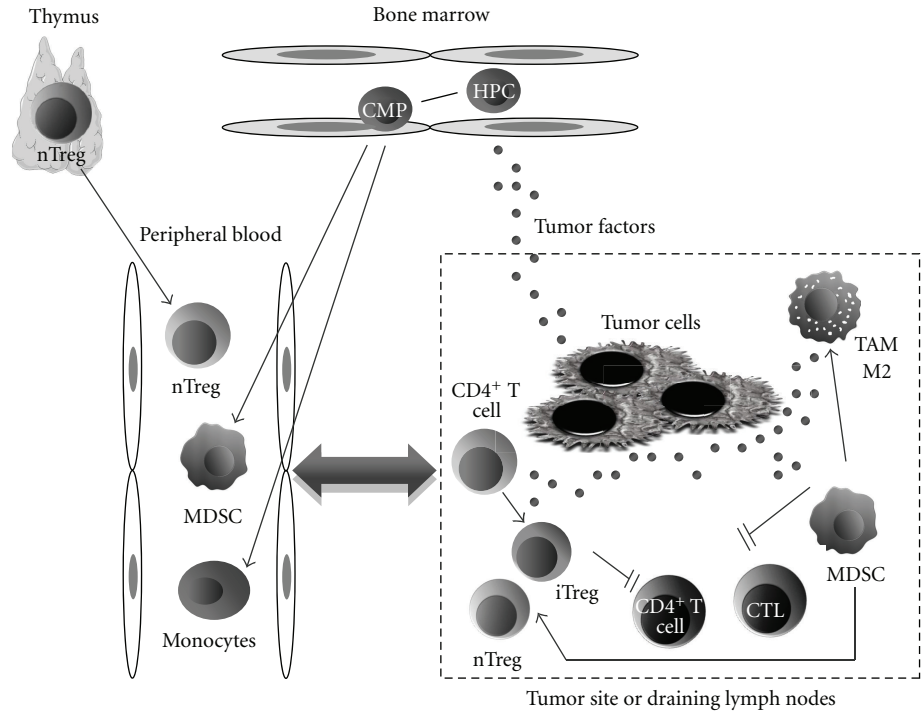

Figure 1: Schematic representation of the potential role of the immune suppressive cells (ISCs) in cancer. The release into the circulation of ISCs derived from thymus (nTregs) or from bone marrow (MDSCs) is partially driven by factors secreted by the tumor. Successively, ISCs can migrate into the tumor site (or a draining lymph node). Moreover, several factors produced in the tumor microenvironment may induce the conversion of $CD4^+$ T cells into iTregs or drive the polarization of monocytes towards a M2-phenotype. All these phenomena lead to an inhibition of $CD4^+$ T-helper cells and cytotoxic T lymphocytes (effector cells), resulting in a general decrease of the antitumor immune response. Theoretically, the release of ISCs from the thymus, the bone marrow, and the tumor site to the peripheral blood could reflect the immunosuppressive status of the antitumor immune response and could be of use in the real time immune monitoring of patients enrolled in therapeutic vaccine clinical trials. HPC: hematopoietic progenitor cells; CMP: committed myeloid progenitors; nTreg: thymic-derived, naturally occurring regulatory T cells; iTreg: induced or adaptive regulatory T cells; MDSC: myeloid-derived suppressor cells; TAM-M2: tumor-associated macrophages characterized by M2-polarization; CTL: cytotoxic T lymphocytes.

molecules to inhibit specific molecular pathways is also being evaluated.

Although preclinical data have shown that it is possible to break tolerance to a specific self-antigen, most clinical trials employing cancer vaccines have mostly failed to demonstrate a real advantage in terms of long-lasting clinical responses or prolonged overall survival. In an immunocompetent cancer patient, the immune system actually suppresses attacks against self-antigens, including TAAs, particularly in the tumor microenvironment. Recently, research has focused more on the suppressive component of the immune response in "breaking tolerance" and in steering the immune system toward "autoimmunity."

The major components of the suppressive compartment of the immune system are a group of heterogeneous immune cells. One of the major problems in characterizing these cells is their extreme plasticity. Cells normally committed to activating an immune response can transiently acquire suppressive characteristics. This is no doubt an important mechanism by which the immune system fine-tunes a specific immune response, balancing the number and function of immune cells involved in the process. Moreover, the heterogeneity and plasticity of this compartment of the immune system makes it difficult to define by cellular markers. A schematic representation of the specialized immune suppressive cells involved in the antitumor immune response is shown in Figure 1. A better understanding of the mechanisms that regulate the homeostasis of these suppressive cells could lead to development of more effective cancer immunotherapies and better immune monitoring of patients receiving cancer vaccines, and ultimately help to answer the question: how do cancer vaccines work? This paper will focus on the role of T-regulatory cells, myeloid-derived suppressor cells, and tumor-associated macrophages in cancer immunotherapy and immune monitoring.

2. Regulatory T Cells

Regulatory T cells (Tregs) are a specialized subpopulation of T cells characterized by their ability to directly or indirectly suppress T-cell activation. Since their discovery in the early 1970s [4–6], the definition of Tregs has continually changed due to their extreme heterogeneity and lack of specific markers. In mice, Tregs are universally characterized by concurrent expression of CD4, CD25, and FoxP3. Although FoxP3 expression is an essential identifier

of this population in humans, it is not sufficient, since most activated $CD4^+$ T cells can transiently express FoxP3. For this reason, several different markers have been proposed to further define the phenotype of Tregs, including CD127, cytotoxic T-lymphocyte antigen 4 (CTLA-4), HLA-DR, glucocorticoid-induced TNFR-related protein (GITR), lymphocyte-activation gene 3 (LAG-3), CD45RA, and CD39. Unfortunately, since none of these has been demonstrated to be Treg specific [7], there is still a lack of clearly defined markers for human Tregs.

To date, there are at least 2 recognized populations of Tregs characterized by the expression of FoxP3: (1) thymic-derived, naturally occurring Tregs (nTregs) that constitutively express FoxP3, and (2) induced or adaptive Tregs (iTregs), such as $CD4^+CD25^-$ T cells that peripherally acquire the immunosuppressive characteristics of Tregs. Recently, it was demonstrated that these 2 lineages can be distinguished by analysis of demethylation within the FoxP3 locus [8]. To further complicate matters, at least 2 other populations of $CD4^+$ T cells are characterized by immunosuppressive activities: Tr1 and Th3. Unlike Tregs that require cell-to-cell contact to exert their suppressive activity, Tr1 and Th3 cells suppress mainly by the release of IL-10 and TGF-β, respectively.

The role of CTLA-4 expression on human Tregs has been investigated for several years. CTLA-4 is expressed both within and on the surface of $CD4^+$ T cells and Tregs, and is a negative regulator of T-cell activation. CTLA-4 counteracts the function of the costimulatory protein CD28 during antigen presentation. In fact, both molecules bind to CD80 and CD86 on antigen-presenting cells (APCs), but while CD28 transmits a stimulatory signal to T cells, CTLA-4 transmits an inhibitory signal, resulting in impaired T-cell activation. Expression of CTLA-4 on the surface of $CD4^+$ T cells is induced in response to TCR ligation, and evidently represents a mechanism of autoregulation of the immune response. In contrast, CTLA-4 is constitutively expressed on the surface of Tregs, suggesting a possible core contribution of CTLA-4 in Treg-mediated suppression [9]. Recent findings have shown that Treg-specific CTLA-4 deficiency in conditional knockout mice is associated with a profound reduction in immune suppressive capacity [10]. In addition, blockade of CTLA-4 by specific antibodies has been shown to enhance immune responses against cancer in several clinical trials [11–14]. It is unclear, however, whether the primary target of these blocking antibodies is the effector T cells or the regulatory T-cell compartment. Studies in mice have shown that anti-CTLA4 monoclonal antibody (MAb) can enhance the avidity of effector T cells [15, 16]. Findings in mice expressing a chimeric CTLA-4 composed of the human extracellular domain have shown that a concomitant blockade of both compartments leads to a synergistic effect and maximal antitumor activity [17]. These data indirectly indicate the importance of balance between the stimulatory and inhibitory compartments generated during an antitumor immune response. A better understanding of these mechanisms could aid the development of novel immunotherapeutic strategies in the treatment of cancer.

3. Tregs in Cancer Immunotherapy and Immune Monitoring

Increased numbers and/or enhanced functionality of Tregs have been detected in the peripheral blood mononuclear cells (PBMCs), the tumor microenvironment, and in draining lymph nodes of patients with hematologic malignancies [18–20] and various types of solid tumors [21–33]. Several studies have demonstrated that Treg depletion can be used efficiently to enhance vaccine-mediated antitumor immunity in cancer patients [34–36]. A direct correlation has also been demonstrated between the frequency and function of Tregs and overall survival [35, 37–39].

A randomized placebo-controlled 43-center Phase II trial in patients ($n = 125$) with mCRPC employing a poxviral-based vaccine containing the transgenes for PSA and 3 costimulatory molecules (PSA-TRICOM) demonstrated a statistically significant ($P = .0061$) survival advantage in the vaccine arm. In a recent study at the National Cancer Institute (NCI) employing the same vaccine, we investigated the number, phenotype, and functionality of Tregs in 32 patients with mCRPC. The median overall survival for these patients was similar to that observed in the multicenter trial of 26.6 months, with a median followup of 44.6 months; this was an improvement of 9.2 months over the median predicted survival of 17.4 months in a comparable patient population, as calculated by the Halabi nomogram [40]. Interestingly, the subpopulation of patients with a Halabi-predicted survival (HPS) >18 months (i.e., patients with low tumor burden) seemed to benefit most from PSA-TRICOM vaccination, with an actual overall survival of ≥37.3 months (median not reached) compared to an HPS of 20.9 months ($P = .035$) [41]. In evaluation of PBMC of these patients, we found a significant correlation between overall survival and Treg suppressive function after 3 monthly vaccinations versus prevaccination ($P = .029$). Of patients with overall survival > HPS, 80% had decreased Treg function after 3 monthly vaccinations. On the other hand, 75% of patients with overall survival < HPS showed increased Treg suppressive activity. We also investigated whether these changes in terms of Treg functionality could be related to phenotypic modifications on the surface of these cells. Based on previously published research [9], we looked at the expression of CTLA-4 as a potential marker of Treg-mediated suppression and found a significant correlation between the ratio of $CD4^+CD25^-$ (effector) cells to CTLA-4^+ Tregs and the overall survival of these patients. In particular, we found that the ratio increased after 3 monthly vaccinations in the subgroup of patients with overall survival > HPS ($P = .029$) and decreased after vaccination in the subgroup with overall survival < HPS ($P = .027$) [42].

Altogether, these data suggest an association between changes in Treg function after vaccination and clinical outcomes, leading to either or both of the following hypotheses: (a) these changes are a direct consequence of the postvaccination immune response, and/or (b) they reflect tumor burden and tumor escape mechanisms. Further studies are needed to address these questions. Potentially, Treg function and/or phenotype and the ratio of effector : CTLA-4^+ Tregs could

potentially be used to monitor immune function (the balance between immunostimulatory and immunosuppressive factors) in patients enrolled in clinical trials of therapeutic cancer vaccines. Analysis of Tregs in real time as part of the immune monitoring of patients could also help in identifying the subpopulation of patients who would most likely benefit from vaccine therapy versus those who would not.

4. Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous cell population composed mainly of myeloid progenitor cells that do not completely differentiate into mature macrophages, dendritic cells, or granulocytes. Immature bone-marrow-derived myeloid cells (IMCs) represent less than 1% of PBMCs in healthy individuals; characteristically, they retain the ability to terminally mature. In various diseases, including cancer, this subset of cells can be increased 4- to 10-fold, which is associated with partially blocked differentiation and acquisition of suppressive activity [43]. Thus, MDSCs represent critical regulators of antitumor immunity, since they can potentially inhibit both tumor-specific and nonspecific T-cell responses. MDSCs have also been shown to regulate immune responses during bacterial and parasitic infections and inflammation as well as autoimmunity [44–47]. Interestingly, a transient expansion of MDSCs has been observed after immunization with various antigens, as well as recombinant vaccinia virus expressing IL-2 [48–50].

Mouse MDSCs characteristically express markers of myeloid lineage, such as myeloid differentiation antigen (Gr1) and integrin alpha M (CD11b, also called macrophage-1 [Mac-1] antigen), but they typically lack myeloid cell maturation markers. In humans, the absence of a Gr1 gene homolog has made the definition of MDSCs more challenging, and has led to the use of combinations of several different phenotypic markers, such as CD11b, CD34, CD33, CD15, CD13, CD14, IL-4Rα, and HLA-DR [48]. MDSCs can be roughly divided into 2 major subpopulations: granulocytic (PMN-MDSCs) and monocytic (MO-MDSCs). While both of these subpopulations are characterized in humans by expression of CD33, CD11b, and IL-4Rα, they differ in terms of CD14 and CD15 levels ($CD14^{-}CD15^{+}$ for PMN-MDSCs; $CD14^{+}CD15^{-}$ for MO-MDSCs).

Because of the vast heterogeneity of MDSCs, several different mechanisms of suppression have been proposed for various subpopulations of these cells [49]. A major fraction of MDSCs express high levels of intracellular arginase, an enzyme responsible for the catabolism of L-arginine, a nonessential amino acid required by many cells, including T cells, for protein synthesis. Uptake of L-arginine by MDSCs can rapidly lead to depletion of this amino acid, resulting in a T-cell arrest in the G_0-G_1 phase [50]. Moreover, PMN-MDSCs can also suppress T cells by producing reactive species of oxygen, while the suppressive function of MO-MDSCs is generally mediated by inducible nitric oxide synthase (iNOS). A third potential mechanism of suppression may be related to the ability of $CD14^{+}HLA\text{-}DR^{-/lo}$ to induce Tregs and inhibit natural killer cells [51, 52]. Other mechanisms have also been proposed, such as sequestration of cystine (the main form of cysteine in the oxidizing extracellular environment) by MDSCs. In fact, the only form of cysteine available to T cells comes mainly from APCs during antigen processing and presentation, since lymphocytes lack both the enzyme (cystathionase) responsible for synthesis of this amino acid and the xCT chain of the x_c^{-} cystine transporter. MDSCs have been shown to express high levels of the xCT chain needed to import cystine, but they lack the alanine-serine-cysteine transporters needed to export cysteine. This results in sequestration of cystine from the extracellular space that ultimately leads to lower levels of cysteine available for T-cell activation. MDSCs could also act indirectly by inducing Tregs in the tumor microenvironment in the presence of IL-10 and IFN-γ, or promoting Treg expansion by acting as tolerogenic APCs [53, 54]. Finally, MDSCs could impair the homing of naïve T cells to draining lymph nodes by shedding L-selectin (CD62-L), operated by the ADAM metallopeptidase domain 17 (ADAM17, also called TACE, for tumor necrosis factor-α-converting enzyme), a transmembrane glycoprotein highly and constitutively expressed on the surface of MDSCs [49, 55]. Since ADAM17/TACE has also been related to the shedding of other important proteins involved in tumor growth and tumor escape mechanisms, such as mucin 1 and the major histocompatibility complex class I chain-related gene-A (MICA), the constitutive expression of this "sheddase" on the surface of MDSCs may cast new light on the basic mechanisms of cancer progression, in which the accumulation of MDSCs in the tumor site could play an important role.

5. MDSCs in Cancer Immunotherapy and Immune Monitoring

The characteristic heterogeneity of MDSCs probably reflects the plasticity of this cell population in response to different signals received from the tumor microenvironment. In fact, each particular tumor microenvironment seems to have a unique effect on the composition of cancer-induced MDSCs, through the release of various tumor-derived factors involved in the expansion and activation of MDSCs. Cyclooxygenase 2, prostaglandins, granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage CSF (M-CSF), IL-6, IL-10, vascular endothelial growth factor (VEGF), stem-cell factor, IL-3, FMS-related tyrosine kinase 3 (FLT3), and cell-expressed molecules (such as Notch) have been implicated in the expansion of MDSCs. Most of these factors trigger signaling pathways involving Janus kinase (JAK) protein family members and signal transducer and activator of transcription 3 (STAT3). IFN-γ, ligands for Toll-like receptors, IL-4, IL-13, and TGF-β seem to be involved in the activation of MDSCs by STAT6, STAT1, and nuclear factor-κB [56].

Recently, a subpopulation of MO-MDSCs phenotypically defined as $CD14^{+}HLA\text{-}DR^{-/lo}$ was shown to be significantly expanded in patients with metastatic melanoma, hepatocellular carcinoma, glioblastoma, and prostate cancer [51, 57–59]; increased circulating MDSCs have been correlated with

tumor stage and metastatic spread in different types of tumors [54, 60]. Moreover, it has been demonstrated that MDSCs can be differently affected by some standard of care therapies such as sunitinib, doxorubicin-cyclophosphamide, and docetaxel and by some immunotherapies [57, 60–62]. These findings suggest a potential use for these cells in immune monitoring of cancer patients.

We have recently investigated the frequency of $CD14^+HLA\text{-}DR^{-/lo}$ or $CD11b^+CD33^+$ cells in the PBMCs of patients with mCRPC before and after vaccination with PSA-TRICOM. Our preliminary results show that the percentage of these populations of MDSCs was significantly higher than in age-matched healthy controls. Moreover, 7 out of 10 patients with overall survival > HPS showed a decreased frequency of MDSCs after vaccination. Further studies involving a range of human malignancies are obviously warranted to validate and/or expand these findings.

6. Tumor-Associated Macrophages

Up to 50% of a malignant tumor mass can be composed of M2-polarized tumor-associated macrophages (TAMs). Similar to Th1/Th2 polarization, monocytes in circulating blood can peripherally acquire different characteristics in response to environmental changes, assuming distinctive M1 (classical activation) or M2 (alternative activation) features. Exposure to microbial products, such as lipopolysaccharides, or IFN-γ determines the acquisition of M1 polarization and cytotoxic functions. M1-macrophages have the ability to present antigens and activate T cells. They produce high levels of IL-12, IL-23, and toxic intermediates such as nitric oxide and reactive oxygen intermediates. Altogether, this activation leads to a proinflammatory response aimed at killing microorganisms and tumor cells. On the other hand, the presence of Th2-related cytokines (such as IL-4, IL-10, and IL-13) or glucocorticoids can drive the differentiation of peripheral monocytes toward an M2 phenotype, characterized by production of low levels of inflammatory cytokines and high amounts of TGF-β. M2-macrophages mainly function as scavengers, expressing surface markers such as CD206 (mannose receptor) and CD204 (scavenger receptor A) and can promote angiogenesis (they are physiologically involved in repairing and remodeling wounded/damaged tissues). It has been shown that TAMs are primarily characterized by M2-polarization and are capable of promoting tumor growth, neoangiogenesis, invasion, and metastasis by several mechanisms [63, 64].

7. TAMs in Cancer Immunotherapy and Immune Monitoring

Clinical studies have demonstrated a correlation between increased numbers of TAMs and poor prognosis for esophageal, bladder, prostate, endometrial, breast, and lung cancers [65–70]. In addition, TAMs have recently been associated with expression of VEGF and epidermal growth factor receptor in breast tumor cells [71], and have been shown to correlate with vessel density in several malignancies [72–74]. The aminobisphosphonate zoledronic acid, routinely used to prevent skeletal-related events in patients with bone metastases, has recently been shown to delay disease progression and improve survival in patients with different types of advanced cancers [75, 76]. One of the mechanisms by which bisphosphonates prevent disease progression could be related to the inhibition of myeloid differentiation, leading to a decrease in TAMs and a shift from M2- to M1-macrophages [77].

A speculative analysis of these data could suggest a possible use of TAMs in the immune monitoring of cancer patients enrolled in clinical trials employing therapeutic vaccines. Theoretically, an efficient cancer vaccine should be able to shift an immune response against tumor toward a Th1/M1 polarization. This phenomenon could alter the frequency of TAMs in the tumor microenvironment and, consequently, in the peripheral blood of cancer patients. Thus, like Tregs and MDSCs, TAMs could likely be another suppressive cell population useful for monitoring patients in the early stage of cancer vaccine therapy.

8. Conclusions

Our understanding of the mechanisms that regulate suppression of immune responses has rapidly increased in recent years. In particular, the immunosuppressive role played by specific immune cells has raised questions about the importance of the balance between immunostimulation and immunosuppression in cancer immunotherapy. In many phase II/III clinical trials, boosting an antitumor immune response without counteracting the resulting immunosuppression has been shown to be only partially effective in achieving objective responses and/or prolonged overall survival [78]. Accumulating evidence suggests the potential of vaccine therapy in combination with treatments specifically aimed at depressing the number and function of immunosuppressive cells. A recent phase III trial employing ipilimumab, a monoclonal antibody that targets CTLA-4, showed an improvement in overall survival of 3.7 months in patients with advanced melanoma [79]. In addition, the receptor tyrosine kinase inhibitor sunitinib has been demonstrated to enhance antitumor immunity by reversing MDSC-mediated tumor-induced immunosuppression, and consequently improving type 1 T-cell function in renal cell carcinoma patients [61].

Evaluation of the balance between the immunostimulatory and immunosuppressive compartments of the immune system could result in an earlier and better understanding of how a specific vaccine is working (or not) in a particular patient. The current assays used to monitor immune responses in cancer immunotherapy trials (such as enzyme-linked immunospot assays, tetramer-based assays, intracellular cytokine flow cytometry, antibody tests, proliferation assays, and reverse transcription polymerase chain reaction) have shown only some usefulness as surrogate markers for clinical efficacy [80]. An assay or assays that measure the balance between immunosuppression and immunostimulation before versus after vaccination may thus fill a pressing need.

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Research Article

Development of a Serum Biomarker Assay That Differentiates Tumor-Associated MUC5AC (NPC-1C ANTIGEN) from Normal MUC5AC

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A serum ELISA using a monoclonal antibody that detects a MUC5AC-related antigen (NPC-1C antigen) expressed by pancreatic and colorectal cancer was developed. The NPC-1C antibody reacts with specific epitopes expressed by tumor-associated MUC5AC that does not appear on MUC5AC from normal tissues. Based on observations of a highly specific antibody, we tested the ELISA to differentiate serum from healthy blood donors compared to serum from patients with colorectal or pancreatic cancer. Additionally, patient tumor tissue was stained to examine the expression pattern of MUC5AC-related antigen in pancreatic and colorectal cancers. The results indicate the NPC-1C antibody ELISA distinguished serum of cancer patients from normal donors with very good sensitivity and specificity. Most patient's tumor biopsy exhibited NPC-1C antibody reactivity, indicating that tumor-associated MUC5AC antigen from tumor is shed into blood, where it can be detected by the NPC-1C antibody ELISA. This serum test provides a new tool to aid in the diagnosis of these cancers and immune monitoring of cancer treatment regimens.

1. Introduction

The early diagnosis of colorectal and pancreatic cancers remains an area of high unmet medical need, as underscored by the U S estimated combined, annual death rate of >89,000 [1]. Although the serum marker CA19-9 is elevated in the majority of pancreatic cancer patients, the specificity of CA19-9 is limited. CA19-9 is frequently elevated in patients with various benign pancreaticobiliary disorders [2–4]. As a result of all of these issues, CA19-9 is not recommended as a screening test for pancreatic cancer [5]. The American College of Gastroenterology (ACG) recommends colonoscopy as the preferred screening/prevention test for colorectal cancer. Noninvasive fecal immunochemical tests are only recommended for patients who decline cancer prevention tests [6]. Currently, there is no consensus for screening for the early detection of pancreatic cancer. Unlike colorectal cancer, the majority cases of pancreatic cancer are detected when a patient is symptomatic which often times represents late stage cancer, resulting in an overall 5 year survival of less than 5% [1]. The majority of colorectal and pancreatic cancer patients are diagnosed utilizing invasive procedures that are expensive, and usually reveal the diagnosis later in the disease process. Newer approaches are being investigated that could allow for earlier detection of disease, in a cost-effective manner, that furthermore could result in better outcomes for patients with these diseases.

As an alternative diagnostic approach, we developed an ELISA using a promising novel tumor-specific monoclonal antibody generated against a clinically tested human colon cancer vaccine. NPC-1 is a monoclonal antibody that was derived from a Tumor Associated Antigen- (TAA-) based vaccine that was previously tested in Phase I-II clinical trials performed in the United States [7–9]. The TAA utilized in these studies was derived from pooled allogeneic colon cancer specimens from multiple patients, which was obtained postoperatively. Cell membranes were isolated from the tumor, and proteins from solubilized membranes were prepared by sonication and Sephadex G-200 chromatography. Semipurified TAAs were identified by *in vitro* and *in vivo* testing in colon cancer patients and healthy volunteers for cell-mediated immunoreactivities. The colon TAA was

detected in fetal intestine and cell membranes, and was localized on tumor cell membranes. Using discontinuous, gradient gel electrophoresis, both colon TAA and CEA were separated and cross-compared. The TAA was shown to be distinct from CEA [8]. The cDNA encoding the NPC-1 antibody was cloned from hybridoma cells, chimerized by genetic engineering, and expressed in a heterologous expression system (Chinese hamster ovary cells). The purified recombinant chimeric antibody is denoted NPC-1C.

The NPC-1C antibody binds to a protein antigen biomarker expressed by human colorectal and pancreatic tumors. In immunohistochemical testing, NPC-1C did not react significantly with tissues from healthy donors or other types of cancer. Furthermore, as discussed below, the NPC-1C antibody ELISA developed can distinguish serum of patients with colorectal or pancreatic cancer from healthy volunteers, thereby providing the rationale for accelerated development and testing of the variant MUC5AC (NPC-1C antigen) detection assay. The test may have application in diagnosis and treatment monitoring of patients with pancreatic or colorectal cancers.

2. Materials and Methods

2.1. ELISA Test. A sandwich ELISA was developed using NPC-1C antibody as the capture reagent. Biotin-labeled NPC-1C was used as the detection antibody. This homologous antibody format was possible due to the discovery of multiple NPC-1C antigen-binding sites expressed by the cancer-associated MUC5AC-related (NPC-1C) antigen. Serum samples were procured from various commercial and private sources under appropriate IRB-reviewed protocols. The assay developed here used serum from colorectal and pancreatic cancer patients, and serum from healthy blood donors.

Microtiter plates (96-well Nunc Maxisorp) were coated with purified unlabeled NPC-1C antibody at 10 μg/mL in 0.5 M sodium carbonate pH 9.5 overnight at 25°C. Plates were then blocked with 1% skim milk made in Tris-Buffered Saline (TBS) containing 5 mM EDTA and 1% sucrose for 4 hours at 25°C. Plates prepared in this manner could be stored dried and sealed for at least 12 months. All dilutions were made in ImmunoBooster buffers (Bioworld Consulting Laboratories, LLC) supplemented with 20 mM EDTA. Wash buffer was TBS containing 0.05% Tween-20 nonionic detergent. A detergent extract of cultured human LS174T colorectal tumor cells was used as a source of NPC-1C antigen to derive a standard curve. Units were in cells/well. Extracts derived from human pancreatic CFPAC-1 tumor cells or human lung A549 tumor cells were generated similarly. All tumor cell lines were purchased from American Type Culture Collection (Manassas, VA) and grown in RPMI medium containing 10% FBS (heat-inactivated) with 8 mM glutamine. To measure direct binding of NPC-1C antibody to the variant MUC5AC (NPC-1C) antigen, CFPAC-1 cells were grown in serum-free medium for 5 days and the conditioned medium was filtered and stored in one large lot at 4°C.

The sandwich ELISAs were performed by diluting the cell extract standard on each plate, next to patient or normal serum samples diluted 1 : 24 in the diluent. All incubations were performed at 25°C and all volumes were 100 uL per well. The plates were incubated for 15 minutes and washed three times with wash buffer. The biotin-labeled NPC-1C antibody was then added to the wells at 1 μg/mL, incubated for 15 minutes, and plates were washed three times. Peroxidase-conjugated streptavidin (1 : 5,000 dilution) was added to the plates for 15 minutes, and plates were washed three times with wash buffer and two times with TBS. The assay was developed by the addition of TMB substrate (BioFX Laboratories Inc.) to the plates, incubation for 15 minutes, then the color reaction was stopped with the addition of 0.5 M sulfuric acid. The data was acquired by measuring absorbance at 450 nm. The data was analyzed using GraphPad Prism or Microsoft Excel software programs.

NPC-1C antibody-binding competition assays were performed by coating microtiter plates with serum-free conditioned medium from CFPAC-1 tumor cells shown to secrete the variant MUC5AC antigen into the culture medium. Following a blocking step as described above, a solution of 1 μg/mL NPC-1C antibody was mixed with serial dilutions of conditioned medium from CFPAC-1, LS174T, and A549 tumor cells. The ELISA was developed using anti-human IgG peroxidase-conjugated antibody followed by TMB substrate incubation as described above. Inhibition curves were plotted using Microsoft Excel.

2.2. Immunohistochemistry. Tumor biopsy specimens from colorectal, pancreatic, or lung cancer patients were deparaffinized at 60°C for 30 minutes prior to staining with NPC-1C antibody. Subsequently, all staining steps were carried out at 25°C. Slides (4 microns thick) were blocked with Peroxo-Bloc inhibitor (Zymed Laboratories) for 2 minutes, rinsed with phosphate-buffered saline (PBS), and blocked with CAS (Zymed Laboratories) for an additional 10 minutes. Slides were stained with 10 μg/mL of biotin-labeled NPC-1C antibody for 1 hour, and washed three times with PBS containing 0.05% Tween-20 nonionic detergent. Previous titration of biotinylated-NPC-1C antibody demonstrated 10 μg/mL to be an optimal concentration for immunohistochemical detection of the variant MUC5AC antigen. A 1 : 400 dilution of peroxidase-conjugated streptavidin (Dako North America, Inc.) was then applied to the slides for 30 minutes and slides were washed 3 times. A solution of DAB (Zymed Laboratories) was applied for 3 minutes then rinsed with PBS. A solution of hematoxylin was then applied for 3 minutes and rinsed with tap water until clear. The slides were dehydrated with xylene and a coverslip was added using Permount mounting medium. Additional consecutive slides were stained with human cytokeratin AE1/AE3 (Abcam plc) as a positive control, and human IgG1 isotype as a negative control (AXXORA, LLC). The anti-MUC5AC antibody (clone 45M1) used to stain lung tumor tissue (10 μg/mL) was purchased from Abcam plc.

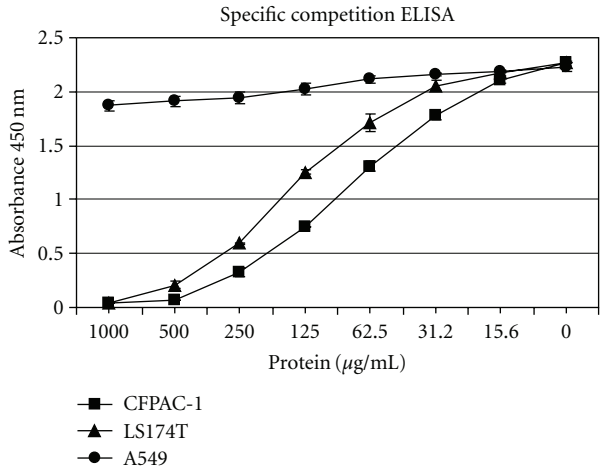

Figure 1: NPC-1C antibody reacts with a colorectal and pancreatic cancer-specific antigen. NPC-1C antigen secreted by serum-starved CFPAC-1 cells was used to coat a 96-well microtiter plate. Following a blocking step, 1 μg/mL of NPC-1C antibody was added to the wells in the absence or presence of increasing amounts of conditioned medium from CFPAC-1 (pancreatic), LS174T (colorectal), or A549 (lung) cells to compete for NPC-1C binding to the coated antigen. The ELISA was developed by adding anti-human IgG-conjugated peroxidase reagent followed by TMB substrate.

3. Results

The NPC-1 antibody was generated in mice immunized with a preparation of pooled human colon tumor tissue extract. Hundreds of hybridomas were screened for cancer-specific characteristics such as binding to tumor tissues and cell lines, with no cross-reactivity to normal human tissues. The original murine IgG, NPC-1, was cloned and chimerized by genetically engineering the mouse variable regions of heavy and light chains with human IgG1 constant regions of the heavy and light chains. The resulting chimeric antibody, NPC-1C, was then expressed in a recombinant Chinese hamster ovary cell clone for further preclinical and clinical development.

The NPC-1C antibody was used as an affinity chromatography tool to isolate and identify the target antigen expressed by colorectal and pancreatic tumor cells. It was shown early that although the murine NPC-1 antibody was generated against a preparation of colorectal tumor-associated antigens, the NPC-1 antibody cross-reacted with pancreatic cancer tissues and cell lines. The target antigen recognized by NPC-1C was determined to be related to the mucin 5ac (MUC5AC) protein, a member of the secreted mucin family of glycoproteins (reviewed in [10]). We rapidly determined that the variant MUC5AC antigen was shed into the supernatant of many cultured human colorectal and pancreatic tumor cells, but not by other tumor cell lines such as prostate or lung tumor cells. We therefore examined whether the NPC-1C antibody could be useful to detect the target MUC5AC-related antigen in humans afflicted with colorectal or pancreatic cancer.

To demonstrate by example that the secreted variant MUC5AC antigen recognized by NPC-1C antibody was expressed specifically by colorectal and pancreatic tumor cells, supernates from LS174T, and CFPAC-1 were admixed with NPC-1C in a competitive ELISA format. Figure 1 shows that the soluble variant MUC5AC antigen secreted by LS174T and CFPAC-1 tumor cells could compete effectively with binding to NPC-1C antibody when the variant MUC5AC antigen was coated on microtiter plates. In contrast, supernates from human lung A549 tumor cells that are known to secrete normal MUC5AC did not compete for binding to NPC-1C antibody. Similar competition curves were shown with other colorectal and pancreatic tumor cell lines, but not with another squamous tumor cell line (data not shown). Together the results demonstrate that NPC-1C binds specifically to a variant MUC5AC antigen expressed by colorectal and pancreatic tumor cells, but not MUC5AC secreted by other tumor cell types.

Chemical and enzymatic digestion of the NPC-1C antibody purified variant MUC5AC antigen revealed that each molecule of the NPC-1C target contained multiple epitopes for NPC-1C binding (data not shown). Therefore, we reasoned that it would be possible to use NPC-1C as a specific reagent for both the capture and detection antibody in a homologous format ELISA. Biotin-labeled NPC-1C antibody was prepared as the detection reagent and tested with variant MUC5AC antigen expressed by LS174T cells. Figure 2 shows that NPC-1C antibody was capable of measuring the cognate antigen expressed by LS174T in this ELISA format. In contrast, MUC5AC expressed by lung A549 tumor cells was not detected in the ELISA. Thus, a surrogate standard MUC5AC-related antigen reagent was generated, frozen, and used for all subsequent ELISA tests. The NPC-1C antibody immunoreactive antigen is reported here in units of LS174T cells/well equivalent.

The proof-of-concept to demonstrate that the NPC-1C antibody ELISA test could be utilized to detect the variant MUC5AC antigen in human serum specimens was demonstrated with a small number of serum samples from colorectal cancer patients. Figure 3 shows the results from five serum specimens collected from colorectal cancer patients compared to pooled AB serum from healthy donors. The results demonstrate a range of variant MUC5AC antigen shed into the blood of these colorectal cancer patients. In contrast, pooled AB serum from healthy donors did not yield a significant signal and was similar to the background levels for the ELISA. Following this, and other preliminary tests, an optimum serum dilution of 1 : 24 was routinely used in subsequent testing.

A larger number of serum samples were procured to test the utility of the serum-based ELISA in detecting the variant MUC5AC antigen. A sampling of 41 colorectal or pancreatic cancer patient sera was compared with sera collected from 28 normal healthy blood donors. In this population of cancer patients, blood was collected serially during an approximately 3-month period for several of the patients while they were undergoing various treatment regimens with a medical oncologist. For multiple reasons, blood was not collected from all patients at all three timepoints. Thus,

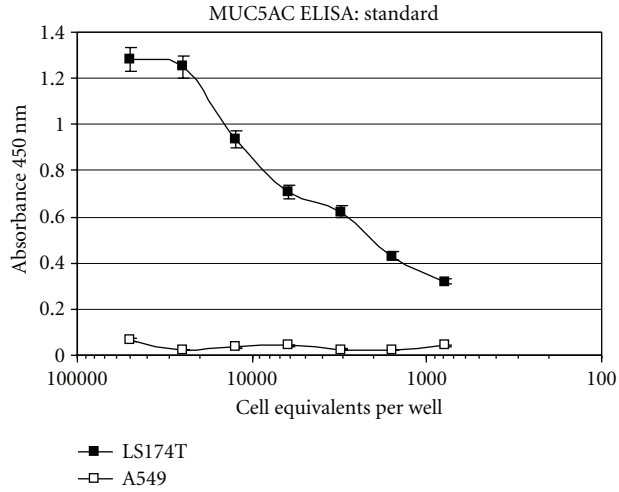

Figure 2: Development of a surrogate MUC5AC-related antigen standard. Protein extracted from human LS174T colorectal tumor cells was prepared to generate a standard curve in the NPC-1C antigen-capture ELISA. Unlabeled NPC-1C was used to coat a 96-well microtiter plate. Following a blocking step, a detergent extract made from human LS174T colorectal tumor cells or human A549 lung tumor cells were incubated on the plates. Biotin-labeled NPC-1C was then applied to the plates to detect the bound antigen, followed by development with streptavidin-peroxidase and TMB incubation steps.

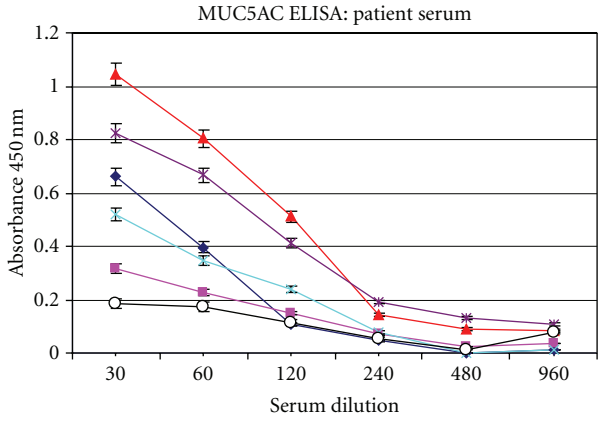

Figure 3: NPC-1C antigen detection in colorectal cancer patient serum. The NPC-1C antigen sandwich ELISA was used to test control serum pooled from normal donors (AB serum, shown in open circles with a thick connecting line), in comparison with serum from five individuals diagnosed with colorectal cancer (shown in various symbols and colors). Serum specimens were tested at the dilutions indicated on the x-axis.

there were 41 patients that donated blood at their first evaluation by the medical oncologist, followed by 33 patients that donated their blood at the second visit, and 25 patients who completed all three blood donations at the third visit. The majority of specimens tested in this preliminary study were from patients diagnosed with Stage III or IV disease. Figure 4 shows the results of testing this larger panel of colorectal and pancreatic cancer patient serum specimens,

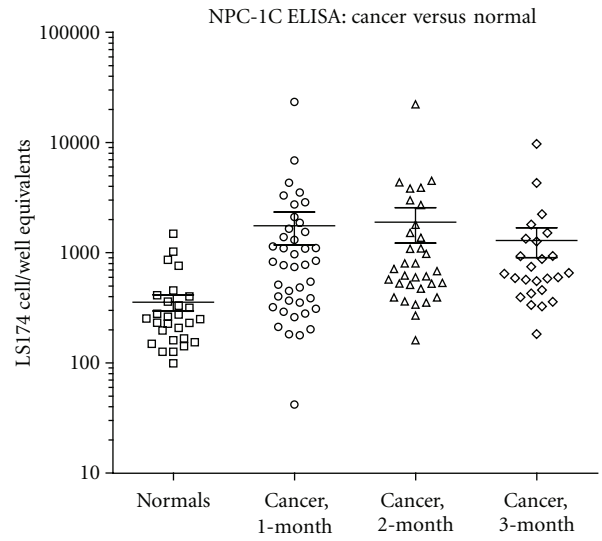

Figure 4: NPC-1C antigen detection in patients undergoing treatment. Serial blood draws of cancer patients over an approximate 3-month period were tested. The NPC-1C sandwich ELISA was performed at a 1 : 24 serum dilution as described in Section 2. Results are presented as a scatter plot of each experimental group, with the mean and standard error of the mean. There were 28 normal sera, 41 colorectal/pancreatic cancer sera at 1-month, 33 colorectal/pancreatic cancer sera at 2-month, and 25 colorectal/pancreatic cancer sera at 3-month.

compared to a group of normal healthy blood donors. Analysis of the results demonstrated approximately a 0.7 log difference between the cancer patients and the healthy donors at each of the three blood draws. The mean and standard error of the mean for each control group for the assays are Normals (355 ± 60), Col/Pan Ca: 1-month (1,757 ± 580), Col/Pan Ca: 2-month (1,894 ± 671), Col/Pan Ca: 3-month (1,293 ± 390). Using the unpaired t-test (2-tailed) method to evaluate the difference between the Normal sera group and the cancer sera groups, the differences for each comparison were Normal versus 1-month: $P = .0511$; Normal versus 2-month: $P = .0397$; Normal versus 3-month: $P = .0153$. Furthermore, using a cut-off value of 355 cells/well derived from the Normal sera average, 73% of Col/Pan Ca, 1-month sera were above the cutoff (30 of 41 samples), and 88% were above the cutoff in each of the 2-month (29 of 33 samples), and 3-month (22 of 25 samples) in those groups. Overall, the samples represent an average of 82% positive above the cutoff established for the assay. These results show that the NPC-1C antibody ELISA can distinguish differences between serum from normal donors and colorectal or pancreatic cancer patients, with a promising level of confidence.

The cancer patient population tested in this study was further stratified by disease type. Figure 5 shows that there was no difference distinguished by the mean NPC-1C antibody ELISA results among those patients diagnosed with colorectal cancer ($n = 36$) from those patients diagnosed with pancreatic cancer ($n = 5$). Both groups separately

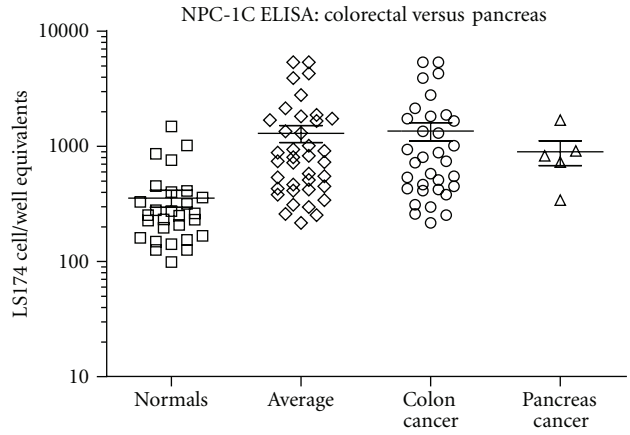

Figure 5: Colorectal and pancreatic cancer sera are detected similarly by NPC-1C. Serum specimens were sorted according to patients diagnosed with either colorectal ($n = 36$) or pancreatic cancer ($n = 5$). These were compared to the average of all cancer specimens and the normal serum specimens.

demonstrated approximately 0.7 log units higher variant MUC5AC antigen expression levels compared to the group of healthy donors.

From patients that donated multiple serum samples, the amount of variant MUC5AC biomarker detected in the assay was plotted versus the time of the blood draw. As shown in Figure 6, some patients appeared to express similar amounts of the variant MUC5AC antigen during the 2- or 3-month period when blood was drawn (subjects 5, 14, 15, 19, 25, 28, 29), whereas some patients appeared to experience an increase in this tumor-specific antigen expression (subjects 1, 2, 7, 33, 39) or a decrease in the biomarker expression (subjects 18, 22, 23, 28, 34, 36, 40). The significance of these shifts over time are presently unclear, but may be related to the tumor burden of the patient at the time the blood was drawn, which may be directly related to the specific treatment regimen of individual patients. Thus, the NPC-1C antibody ELISA may be a useful tool as a treatment monitoring biomarker assay. It was not possible within the 3-month time period of the blood collections to determine whether a correlation exists between the rate of variant MUC5AC increase or decrease and the outcome of the disease in these patients. This will be the subject of a future study.

4. Immunohistochemical Analysis

A number of tumor tissue specimens were procured to examine the level and incidence of variant MUC5AC antigen expression in colorectal and pancreatic cancer patients. Biotin-labeled NPC-1C antibody was used at 10 μg/mL, detected with streptavidin-horseradish peroxidase conjugate, and mounted on glass slides. A positive staining scale ranging from +1 to +5 was applied to the staining results, evaluated by light microscopy. Representative examples of the staining results to detect the variant MUC5AC antigen are shown in Figure 7. Tissues from normal pancreas and colon showed no cross-reactive staining with NPC-1C antibody (panels (a) and (c) resp.). In contrast, tissues from pancreatic and colorectal tumor biopsies demonstrated specific staining of both cytoplasmic and membrane associated antigen (panels (b) and (d) resp.). In the colorectal cancer tissues, frequent staining of secreted variant MUC5AC antigen was observed in the luminal spaces of the tissues (panel (d)). The tissue specificity of NPC-1C binding was demonstrated by the lack of positive staining of lung adenocarcinoma tissue (panel (f)), whereas a commercially available anti-MUC5AC antibody shows that the lung tumor tissue expresses MUC5AC (panel (e)). Thus, while MUC5AC is known to be expressed by lung cells, the NPC-1C antibody does not react with the MUC5AC expressed by lung tumor tissue.

Tissues stained with NPC-1C antibody were considered positive (+1 to +5) for 79% of the tumor samples procured and stained (30 of 38). These staining results are similar to results from several other studies completed with NPC-1C antibody using tissue array slides, and both frozen and paraffin-embedded surgical specimens.

5. Discussion

The proof of concept has been established for the value of the NPC-1C antibody in the detection of the tumor-associated MUC5AC antigen recognized by NPC-1C antibody. This development suggests a new effective, scalable serum biomarker ELISA for the potential diagnosis and immunoregulatory monitoring of patients with colorectal and pancreatic cancer. These results also support further development and large-scale early Noninvasive diagnostic screening of healthy populations for colorectal and pancreatic cancer.

The preliminary results described here demonstrate that the NPC-1C antibody can distinguish normal/healthy serum from serum derived from patients with colorectal or pancreatic cancer. A better defined cohort of healthy serum donors may permit improved comparisons regarding assay specificity and sensitivity. Interestingly, should the assays be predictive in diagnosing colorectal and/or pancreatic cancer, some of the "normal" donors tested in the assays described here may be predisposed to developing cancer, and the potential utility of these ELISAs may be underestimated, if they could detect cancer in asymptomatic persons. Indeed, while the patient population studied in this report was predominantly from Stage III and IV cancer patients, we are currently procuring serum specimens from earlier stage colorectal and pancreatic cancer patients (Stage I and II) as well as serum from asymptomatic persons at risk for developing these types of cancer.

Several research laboratories have demonstrated the association of aberrantly expressed MUC5AC in colorectal and pancreatic cancers [11, 12]. Many monoclonal antibodies that target MUC5AC have been generated [13–17]. However, none of these appear as specific as the NPC-1C antibody in defining the variant MUC5AC antigen expressed in colorectal and pancreatic patient serum as compared with

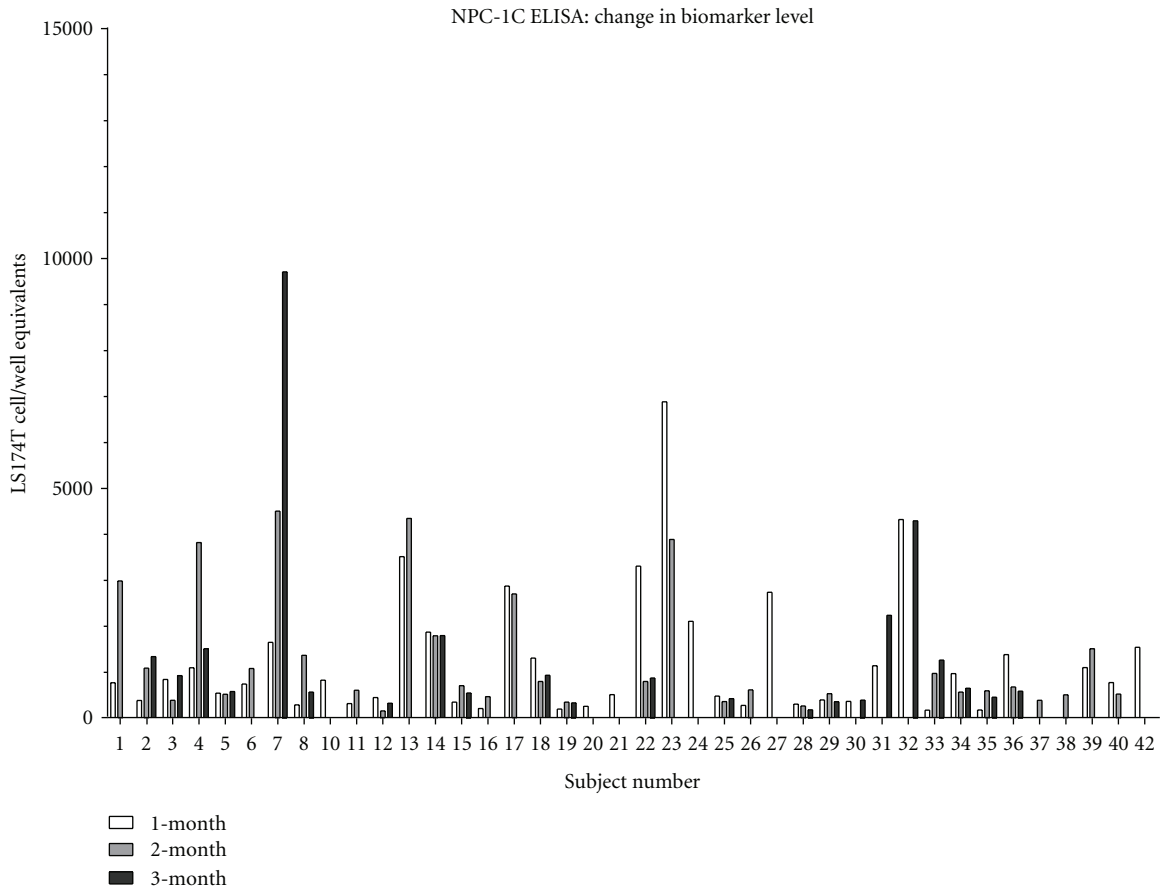

Figure 6: NPC-1C antigen detection in serum during treatment over 3 months. Serum was tested in the NPC-1C sandwich ELISA monthly from colorectal and pancreatic cancer patients over approximately 3 months during which they were being treated for their disease. Trends of increases, decreases, and stable biomarker levels were evaluated in each patient.

normal healthy donors, or as compared with MUC5AC antigen expressed by other tumor types. The reason for this difference may be due, in part, to the specific epitope that the NPC-1C antibody recognizes, which is currently an area of active investigation.

The assay specificity using the normal serum samples presented in this interim report are 71% (8/28 normal samples above the mean cutoff). The sensitivity for the NPC-1C antibody ELISA was 82% (18/99 cancer samples below the mean normal cutoff). Future testing and data analysis with new serum from healthy donors should increase the specificities of both serum assays. Importantly, testing earlier stage cancer patient (Stage I-II) will shed light on the application of this ELISA to detect cancers earlier, which will allow earlier interventions and improve treatment outcomes.

Reviewing the IHC results for each antibody, the tumor biopsy specimens were collected from patients diagnosed pathologically with Stage III and IV colorectal and pancreatic cancer. The NPC-1C antibody had a sensitivity of 79% (30 of 38 positive). The chimeric NPC-1C therapeutic antibody (Ensituximab) is currently being tested in a Phase I dose escalation clinical trial. As part of the eligibility criteria for the study, patients with advanced pancreatic or colorectal cancers must have their tumor biopsy stain positive for the NPC-1C antigen to be considered eligible for treatment. The preliminary immunohistochemical data presented here suggests that approximately 80% of patients may be treated with the therapeutic biological NPC-1C. This truly represents the new frontier of theranostics, where a biomarker can act as a companion to a specific therapeutic product.

The serum ELISA described here may have utility in monitoring colorectal or pancreatic cancer patients during the course of a treatment regimen. Patients with pancreatic cancer are typically treated with gemcitabine, whereas the treatment options for colorectal cancer patients can include chemotherapies (5-fluorouracil, FOLFOX, or FOLFIRI) or biologics such as cetuximab and bevacizumab. The NPC-1C antibody ELISA may be useful to aid in monitoring the patient responses to such therapies. The results shown in Figure 6 demonstrate trends for certain patients that may reflect cancer regression, progression, or stable disease. Once these data are coupled with the disease status in patients, the correlation may become apparent. Serum-based detection of colorectal and pancreatic cancer biomarkers will improve the chances for early detection

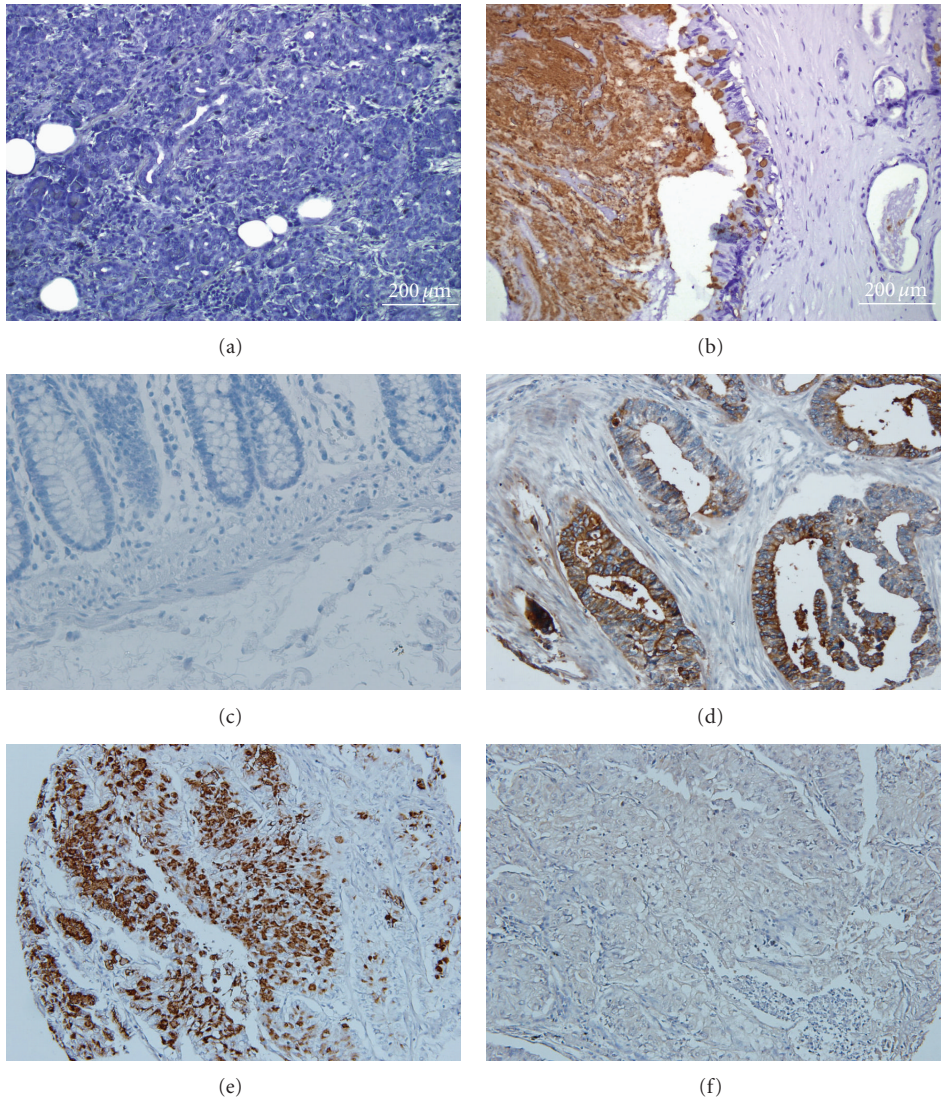

Figure 7: Immunohistochemical staining using NPC-1C. NPC-1C staining of normal human pancreatic (a), normal human colorectal (c), pancreatic cancer (b), and colorectal cancer (d). The brown stained areas indicate immunoreactivity of NPC-1C with the cancer-specific MUC5AC antigen. Lung adenocarcinoma stained with a commercially available anti-human MUC5AC antibody (e) or with NPC-1C (f), demonstrating that the NPC-1C antibody does not recognize the lung adenocarcinoma-associated MUC5AC.

of these deadly diseases. Current diagnostic methods are invasive, expensive, and often inconclusive. The preliminary results with the NPC-1C ELISA to specifically detect tumor-associated MUC5AC may improve the diagnosis of these solid tumors as well as aid in the immune monitoring and prognosis of patients undergoing treatment of their disease.

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Research Article

IgG Responses to Tissue-Associated Antigens as Biomarkers of Immunological Treatment Efficacy

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We previously demonstrated that IgG responses to a panel of 126 prostate tissue-associated antigens are common in patients with prostate cancer. In the current report we questioned whether changes in IgG responses to this panel might be used as a measure of immune response, and potentially antigen spread, following prostate cancer-directed immune-active therapies. Sera were obtained from prostate cancer patients prior to and three months following treatment with androgen deprivation therapy ($n = 34$), a poxviral vaccine ($n = 31$), and a DNA vaccine ($n = 21$). Changes in IgG responses to individual antigens were identified by phage immunoblot. Patterns of IgG recognition following three months of treatment were evaluated using a machine-learned Bayesian Belief Network (ML-BBN). We found that different antigens were recognized following androgen deprivation compared with vaccine therapies. While the number of clinical responders was low in the vaccine-treated populations, we demonstrate that ML-BBN can be used to develop potentially predictive models.

1. Introduction

Over the last two decades many new immunotherapy approaches to the treatment of cancer have entered clinical development due to the increased understanding of the mechanisms of antigen presentation, lymphocyte recognition, functions of the innate immune system, and the means of regulation of these responses and the means by which tumors can circumvent these responses. Many of these investigations have led to agents approved for standard clinical use, including infusional cytokine therapies for melanoma or renal cell cancer, intravesical BCG therapy for bladder cancer, and most recently an active cellular therapy targeting prostatic acid phosphatase (PAP, sipuleucel-T, Provenge, Dendreon) for patients with advanced metastatic prostate cancer. Many other agents have demonstrated benefit in large clinical trials, and approval is anticipated in the case of a monoclonal antibody targeting a T-cell checkpoint inhibitor targeting CTLA-4 (ipilimumab, Bristol-Myers Squibb) for advanced melanoma.

Ultimately, for these agents to be clinically approved there needs to be a demonstration that these treatments are relatively safe and patient care and outcome are positively affected. However, there is also an increasing recognition that some of these agents, while likely safe, may best be used in combination with other immune-activating or conventional therapies. This has presented challenges for evaluating these agents using traditional paradigms for clinical development. Consequently there is a need to identify markers of biological response, ideally associated with clinical outcome, but permitting an evaluation of biological effect of these agents used in combination. In the case of antigen-specific vaccines,

it has been relatively straightforward to evaluate immune responses to the target antigen as a "biomarker" of immunological efficacy. Unfortunately, there are few instances in which target antigen immune response has been associated with clinical benefit. The situation is more difficult for broadly active immune modulating agents such as T-cell checkpoint inhibitors, including antibodies targeting CTLA-4 or PD-1, or TLR agonists, in which appropriate biomarkers of response have been more elusive. Studies with anti-CTLA-4 monoclonal antibodies, in particular, have sought to identify whether amplification of other T-cell costimulatory molecules [1], or antibodies to defined antigenic tumor-associated proteins [2, 3], might be useful as biomarkers. For whole cell tumor vaccines where there is not a specific, defined antigen being targeted, surrogate antigens known to be expressed by the tumor vaccine have been used as a means of monitoring immune responses from the vaccine [4]. The use of immunologically recognized surrogate antigens, including HER-2/neu, MUC1, and p53, has been possible in the case of breast cancer where T-cell and IgG responses to these antigens have been identified. However it is unknown whether responses to these antigens can be useful to study agents in combination or whether changes in responses to these antigens are associated with clinical outcome.

Over the last several years we have used SEREX- (serological analysis of recombinant cDNA expression libraries) based studies to identify immunologically recognized proteins expressed by normal and malignant prostate tissue that might serve as targets for anti-tumor vaccines [7]. In particular, we have evaluated the targets of IgG responses in patients with chronic prostatitis or autoimmune disorders [8, 9], patients with prostate cancer treated with immune-modulating therapies [10], and IgG responses to cancer-testis antigens in patients with prostate cancer [11, 12]. Over the course of these studies we have effectively identified hundreds of immunologically recognized proteins associated with prostate tissue and/or recognized by patients with prostate cancer. While the identification of hundreds of proteins presents challenges in prioritization for the development of antigen-specific vaccines, we previously questioned whether these antigens might also have diagnostic value with IgG responses being able to distinguish individuals with prostate cancer (or other inflammatory conditions of the prostate) from men without prostate disease. Other groups have similarly reported that IgG responses to tissue-associated antigens might have diagnostic value in identifying patients with prostate cancer [13] or nonsmall cell lung cancer [14]. We have previously reported that a subset of 23 of these antigens were recognized in patients with prostate cancer as well as individuals with symptomatic prostatitis, suggesting that such autoantibody signatures might be useful to identify inflammatory conditions of the prostate, and potentially in a premalignant setting [15].

In the current report, we hypothesized that this same panel of previously identified prostate-associated antigens might be used as a monitoring tool to assess immune responses elicited following immune-modulating therapy. While B-cells or IgG production might not be an intended target of a particular therapeutic approach, IgG responses are often elicited with concurrent T-cell activation. We reasoned that IgG responses are easier to measure compared with antigen-specific T cells, and might be more stable over time in the peripheral blood compared with T-cell frequencies. Moreover, the identification of "off-target" IgG immune responses might further serve as an indication of "antigen spread" with secondary antigens recognized following immunological targeting and thus be more relevant to developing biomarkers associated with favorable clinical responses. To detect antibody responses to previously defined antigens, we applied a similar phage immunoblot approach evaluating IgG responses to multiple antigens simultaneously [15]. These types of complex biomarker data sets are historically very difficult to work with for two reasons: first is the complexity associated with biological networks; second is the challenge of infrequent observation of immune biomarkers in a complex system. As such, the identification of useful biomarkers in data sets such as this study can be very challenging. In this paper, we sought to evaluate the use of machine-learned Bayesian Belief Networks (ML-BBNs) as a method for identifying potentially promising biomarkers and potential biomarkers networks [16, 17]. We sought to train several ML-BBNs to identify promising biomarkers and then use these networks to select a subset of features to train a network of immune biomarkers as they related to observed declines in serum prostate-specific antigen (PSA). Our objective was to demonstrate the feasibility of this method to identify promising early biomarkers of immune response to vaccine therapies in our data.

For the current studies, sera samples were collected prior to treatment and after three months of treatment from three separate trials, one in which patients (n = 34) were treated with androgen deprivation (ADT) therapy only, a standard therapy known to elicit prostate-associated immune responses [18–20], a trial in which patients with castrate-resistant prostate cancer (n = 31) were treated with a viral vaccine encoding PSA (PSAV) [5], and one in which patients with early recurrent prostate cancer (n = 21) were treated with a plasmid DNA vaccine encoding PAP (PAPV) [6]. Patients treated with vaccines were subclassified as immunologic or clinical "responders" based on previously reported criteria to distinguish these groups. We report here that IgG immune responses could be detected to individual antigens, and as long as one year after therapy the recognition of specific antigens was associated with individual treatments. The evaluation of IgG responses to groups of antigens at three months suggests that predictive models might be developed with diagnostic potential. These findings support the concept of using measures of "antigen spread" as biomarkers of immunological efficacy for immune-active therapies, and IgG responses to panels of tissue-associated antigens as measures of this antigen spread.

2. Materials and Methods

2.1. Patient Populations. Sera used for the studies had been previously collected with IRB-approved, written consent as part of three separate clinical trials (Figure 1). All samples

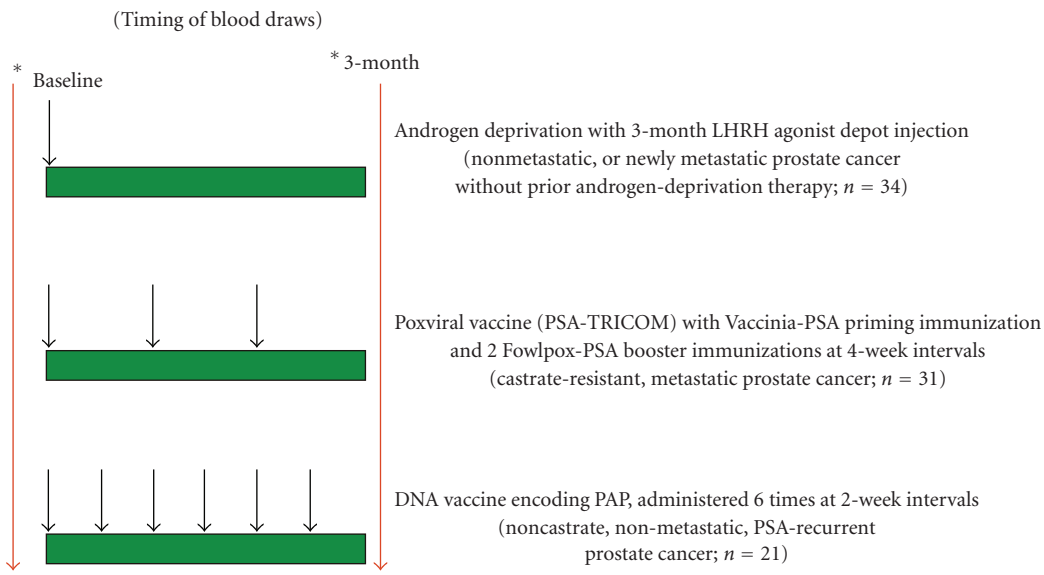

Figure 1: Schema for sample collection. Sera were collected from men with prostate cancer undergoing treatment on three separate clinical trials. Shown are the timing of blood collection and basic schema for these studies. In one study, blood was collected immediately preceding, and at three months following, standard androgen deprivation therapy with a 3-month depot injection of an LHRH agonist. Patients were men ($n = 34$) with prostate cancer who had not previously received androgen depriving therapy, and had PSA-recurrent and/or metastatic prostate cancer. In the second study, blood was collected immediately preceding and three months following initiation of treatment with a poxviral vaccine encoding PSA (PSA-TRICOM) [5]. Patients were men ($n = 31$) with castrate-resistant metastatic prostate cancer. In the third study, blood was collected immediately preceding, and at three months following, biweekly treatment with a DNA vaccine encoding prostatic acid phosphatase (PAP) [6]. Patients were men ($n = 21$) with non-castrate, PSA-recurrent prostate cancer without evidence of metastatic disease.

were stored at −80°C until used for analysis. These samples were all obtained prior to study treatment and after 3 months of treatment in the following settings: (1) a trial (ADT) in which patients ($n = 34$) with PSA-recurrent, or newly metastatic prostate cancer, who had never previously received androgen deprivation, received 22.5 mg leuprolide by intramuscular injection with or without daily oral bicalutamide; (2) a trial (PSAV) in which patients ($n = 31$) with castrate-resistant, metastatic prostate cancer were vaccinated at 2-week intervals with a poxviral vaccine (Prostvac, vaccinia virus encoding PSA priming immunization followed by fowlpox virus encoding PSA booster immunizations [5]; (3) a trial (PAPV) in which patients ($n = 21$) with PSA-recurrent nonmetastatic prostate cancer, not receiving androgen deprivation, were vaccinated at 2-week intervals with a plasmid DNA vaccine encoding PAP [6]. From the majority of patients treated with ADT ($n = 24$ of 34) or PAPV ($n = 19$ of 21), serum samples were also available 12 months after the baseline sample. From the vaccine studies, patients were grouped as clinical "responders" or "non-responders" as simply defined by a decrease in serum PSA level at the 3-month time point relative to the baseline value.

2.2. High Throughput Immunoblot (HTI). Phage immunoblot was performed as we have previously described [15]. In brief, 100,000 pfu lambda phage encoding 126 unique antigens were spotted manually in triplicate in a 16×24 array onto XL-1 blue *E. coli.* bacterial lawns in OmniTray plates using a Biomek FX liquid handling robot. These individual antigens included 29 cancer-testis antigens [21], 40 proteins identified in patients with chronic prostatitis [8], and 57 antigens identified in individual patients, some of whom were treated with androgen deprivation or other immunomodulatory therapies [9, 10, 20]. A listing of antigens and their GenBank Accession numbers is included in Supplemental Table 1 in supplementary material available online at doi:10.1155/2011/454861. Plates were allowed to air-dry after which 10-mM isopropyl ß-D-thiogalactopyranoside-(IPTG) suffused nitrocellulose membranes were overlain, and plates incubated at 37°C overnight to allow recombinant protein expression. Membranes were then washed, blocked, and probed with sera from patients pre- or post-treatment, diluted 1 : 100 in isotonic buffer. Human IgG was then detected with an IgG-specific secondary antibody conjugated to alkaline phosphatase and immunoreactivity detected by development with 0.3 mg/mL nitro blue tertazolium chloride (NBT) (Fisher Biotech) and 0.15 mg/mL 5-bromo 4-chloro 3-indoylphosphate (BCIP) (Fisher Biotech). Membranes were scanned and the digital format was assessed visually, with individual plaques scored positive or not by four independent observers, blinded to the treatment, timing of sample acquisition and membrane layout, as previously reported [8, 15]. All of the membranes for the entire study were evaluated by the same observers at the same time. Triplicate samples were evaluated for each antigen, and immunoreactivity to individual antigens was scored positive if there was concordance among 3 of 4 observers, and if immunoreactivity was scored positive in at least two of

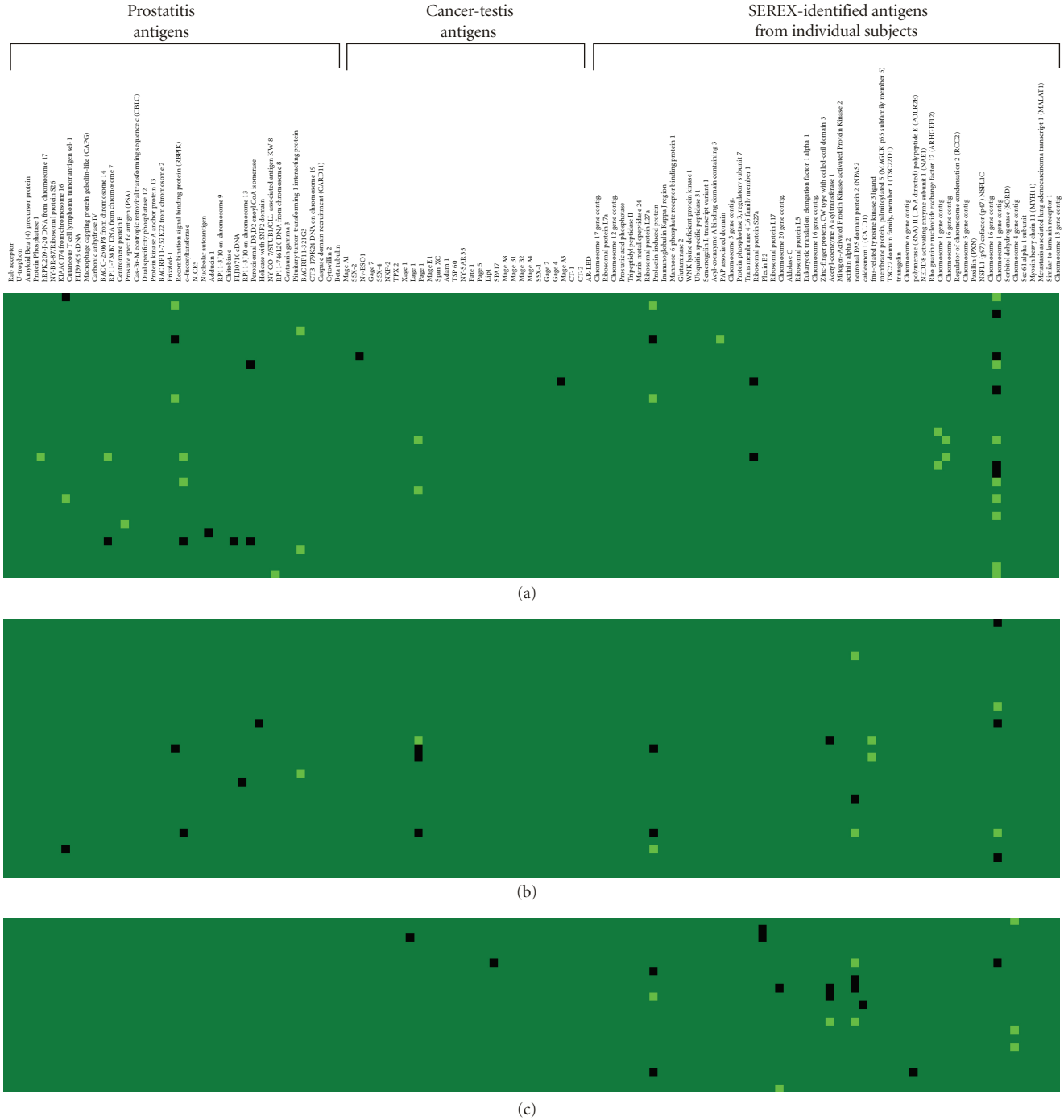

Figure 2: IgG responses to prostate-associated antigens are elicited following prostate cancer-directed therapies. Sera from patients pretreatment and three months following treatment with androgen deprivation ((a), $n = 34$), a PSA-targeted viral vaccine ((b), $n = 31$), or a PAP-targeted DNA vaccine ((c), $n = 21$) were evaluated for IgG responses to 126 prostate-associated antigens. Antigens (detailed in Supplemental table 1) are grouped according to the original studies from which they were derived (prostatitis antigens, cancer-testis antigens, or antigens detected by SEREX from individual subjects), and IgG responses were scored as previously described [15]. Shown is a heatmap representing gain of response pretreatment to posttreatment (light green), loss of response following treatment (black), or no change in response (dark green) for all subjects (in rows) and all antigens (in columns).

the three replicates. Heatmap Builder software (Version 1.1, Stanford University) was used to generate heatmaps displaying changes (gain, loss, or no change) of antibody immune responses following treatment.

2.3. Statistical Analysis. Our statistical analysis consisted of using a commercially available machine-learning software package (FasterAnalytics, DecisionQ Corporation, Washington, DC). Machine learning is a field of computer science that uses intelligent algorithms to allow a computer to mimic the process of human learning. Machine learning algorithms allow the computer to learn dynamically from the data that resides in the training dataset, detecting associations between features without human supervision. The machine learning heuristics generate hypothetical models with different conditional independence assumptions. DecisionQ software generates several networks simultaneously and then continues to generate new hypotheses for each network. The software promotes the network with the best score as determined by goodness of fit relative to compactness. This allows for *de novo* exploration of associations in complex data sets.

In preprocessing our data, we compared the pre- and post-treatment status of biomarkers and encoded the change in each biomarker as a feature. We then used these encoded features and clinical response (PSA decline) to train models. The output of our machine-learning algorithms is a Bayesian Belief Network (BBN). A BBN encodes the joint probability distribution of all the variables in the domain by building a hierarchical network of conditional dependence. The graphical nature of the network allows the user to query the structure of conditional dependence to identify those features which provide the most information content in the network. In order to select a subset of features for inclusion in a final model, we used a stepwise process and trained a series of machine-learned (ML)BBNs for feature selection. We used this stepwise process as a means of identifying nodes with relatively high information content given our statistically challenging biomarker data sets. Because these data sets have a very high degree of dimensionality (features) relative to evidence (number of subjects), finding those features with the highest information content can be very challenging. To address this challenge, we trained multiple BBN-ML models and identified those features which recurred across multiple models as evidence of high information content. We modeled each of our study cohorts (ADT, PSAV, PAPV) and then compared to the model structures between individual cohorts to identify shared nodes. We also identified high-content nodes (greater than 10 associations) and combined these with the shared nodes to create a selected subset or training a final model to evaluate a network of biomarkers to evaluate clinical response (PSA decline). We used our selected markers to then train three additional models: (i) a final subset model including clinical response (PSA decline) on the vaccine cohorts, (ii) a model of subjects in the vaccine studies who were immune responders, and (iii) a model of subjects who were not immune responders in the vaccine studies. Finally, we performed tenfold cross-validation on our clinical response subset model and used receiver operating characteristic (ROC) curve analysis to calculate an area-under-the-curve (AUC) metric for the feature PSA decline, to determine if the subset model could robustly classify clinical response given immune biomarkers.

The frequencies of IgG responses to individual antigens were compared between treatment study populations using a chi-square test.

3. Results

3.1. IgG Responses to Prostate-Associated Antigens are Elicited Following Prostate Cancer-Directed Immune Therapies. We have previously reported that antibody responses to prostate antigens can be detected in patients with prostate cancer or other inflammatory conditions of the prostate [15]. Moreover, a subset of these prostate-associated antigens was commonly recognized in patients, relative to men without prostate disease, suggesting that the detection of IgG responses to specific prostate-associated antigens might have diagnostic value. In the current analysis, we wished to determine whether the detection of IgG responses to a panel of prostate-associated antigens might have utility in the evaluation of vaccine or other immunomodulatory therapies aimed specifically at eliciting immune responses to the prostate. For this, we obtained sera from men with prostate cancer prior to and following three months of therapy with standard androgen deprivation therapy ($n = 34$), and from men with prostate cancer ($n = 52$) prior to and following three months of therapy with one of two different antigen-specific vaccines (Figure 1). Sera from these individuals were used to screen for IgG responses to a panel of 126 antigens by immunoblot, as previously described [15]. Responses to all antigens were evaluated in blinded fashion at both time points, and in Figure 2, changes in IgG responses (gain or loss of response) after 3 months were determined. As demonstrated, androgen deprivation elicited immune responses to multiple antigens, and in particular to antigens previously identified as antigens recognized in patients with chronic prostatitis [8, 15]. Responses to these prostatitis antigens were uncommon over a similar 3-month period in patients treated with either of the vaccines. Gain or loss of IgG responses to some antigens appeared to be shared by these different treatments, while responses to some appeared more specific for individual treatments. Of note, gain or loss of IgG responses to PSA, while detected in one individual treated with ADT, were not detected in patients receiving the PSA-TRICOM vaccine. Similarly IgG responses to PAP were not detected in any of the patients, including those receiving the PAP-targeted vaccine, as previously reported [6].

3.2. IgG Responses to Individual Antigens are Specific for the Type of Prostate-Directed Therapy. We next wanted to determine whether IgG responses observed were generally stable, or increased over time, and also identify more specifically whether responses to some antigens were more generally associated with different therapies. In the majority of patients treated with ADT and PAPV, sera samples were also available 12 months later. Evaluation of IgG responses gained or lost

Table 1: Biomarker co-occurrence among models. Feature comparison analysis describing which biomarkers have in population specific ML-BBNs. An IgG response change to Chromosome 1 gene contig CHANGE1, for example, has associations in all three population-specific ML-BBNs. Conversely, an IgG response change to Adducin 1 CHANGE only has an association in the ADT population ML-BBN.

	ADT	PAPV	PSAV	Total
Chromosome 1 gene contig CHANGE1	Yes	Yes	Yes	3
Prolactin-induced protein CHANGE	Yes	Yes	Yes	3
Acetyl-coenzyme A acyltransferase 1 CHANGE	No	Yes	Yes	2
BAC RP11-321G3 CHANGE	Yes	No	Yes	2
Cutaneous T cell CHANGE	Yes	No	Yes	2
neuronal PAS domain protein 2 CHANGE	No	Yes	Yes	2
o-fucosyltansferase CHANGE	Yes	No	Yes	2
Page 1 CHANGE	Yes	No	Yes	2
Recombination signal CHANGE	Yes	No	Yes	2
Adducin 1 CHANGE	Yes	No	No	1
caldesmon 1 (CALD1) CHANGE	No	Yes	No	1
carcimona-associated antigen 64 CHANGE	Yes	No	No	1
Chromosome 1 gene contig CHANGE	Yes	No	No	1
Chromosome 16 gene contig CHANGE	Yes	No	No	1
chromosome 17 CHANGE	Yes	No	No	1
Chromosome 20 gene CHANGE	No	Yes	No	1
Chromosome 4 gene contig CHANGE	No	Yes	No	1
FLJ10710 cDNA CHANGE	Yes	No	No	1
fms-related tyrosine kinase 3 ligand CHANGE	No	No	Yes	1
Helicase with SNF2 domain CHANGE	No	No	Yes	1
Lage 1 CHANGE	No	Yes	No	1
Mage A3 CHANGE	Yes	No	No	1
Ny-ESO1 CHANGE	Yes	No	No	1
PAP associated domain CHANGE	Yes	No	No	1
PAP ELISPOT 12 months	No	Yes	No	1
Plexin B2 CHANGE	No	Yes	No	1
polypeptide E (POLR2E) CHANGE	No	Yes	No	1
Prostate specific antigen (PSA) CHANGE	Yes	No	No	1
PSA ELISPOT 3m	No	No	Yes	1
Ribosomal protein S27a CHANGE	Yes	No	No	1
RP11-3J10 on chromosome 13 CHANGE	No	No	Yes	1
RP11-738B7 DNA on chromosome 7 CHANGE	Yes	No	No	1
RP11-746L20 DNA on chromosome 8 CHANGE	Yes	No	No	1
SPA17 CHANGE	No	Yes	No	1

after 12 months of ADT, or 12 months after PAP vaccine treatment compared with baseline demonstrated overall an increased number of antigens recognized (Figure 3). Interestingly, responses to individual antigens were observed to be highly specific for the treatment. For example, IgG responses were elicited to the ribosomal L5 protein in 8/24 patients receiving ADT, and 0/19 patients after receiving the PAPV ($P = .005$, chi-square test). Similarly, IgG responses elicited to the neuronal PAS domain protein 2 (NPAS2) antigen were observed more frequently in patients receiving the PAP vaccine (5/19) compared with patients receiving androgen deprivation (0/24, $P = .0075$, chi-square test). Even after one year, responses gained or lost to antigens previously identified as prostatitis antigens were not detected in patients treated with the vaccine. While we did not have access to a control population of sera from untreated men, given that these represented populations of subjects with nearly identical stage of disease, collected at the same institution, the differences in IgG response patterns to individual antigens appears most related to the difference in treatment. Moreover, these findings suggest that IgG responses are elicited to "off-target" antigens by means of prostate-directed therapies, and the patterns of IgG responses differ with respect to therapy.

3.3. Machine-Based Learning Algorithms can be Designed to Detect Early IgG Response Changes That Might have Predictive Value. The results above demonstrated that, indeed, IgG immune responses are elicited as a result of prostate-directed immune-active therapies. Moreover, antigen-specific IgG

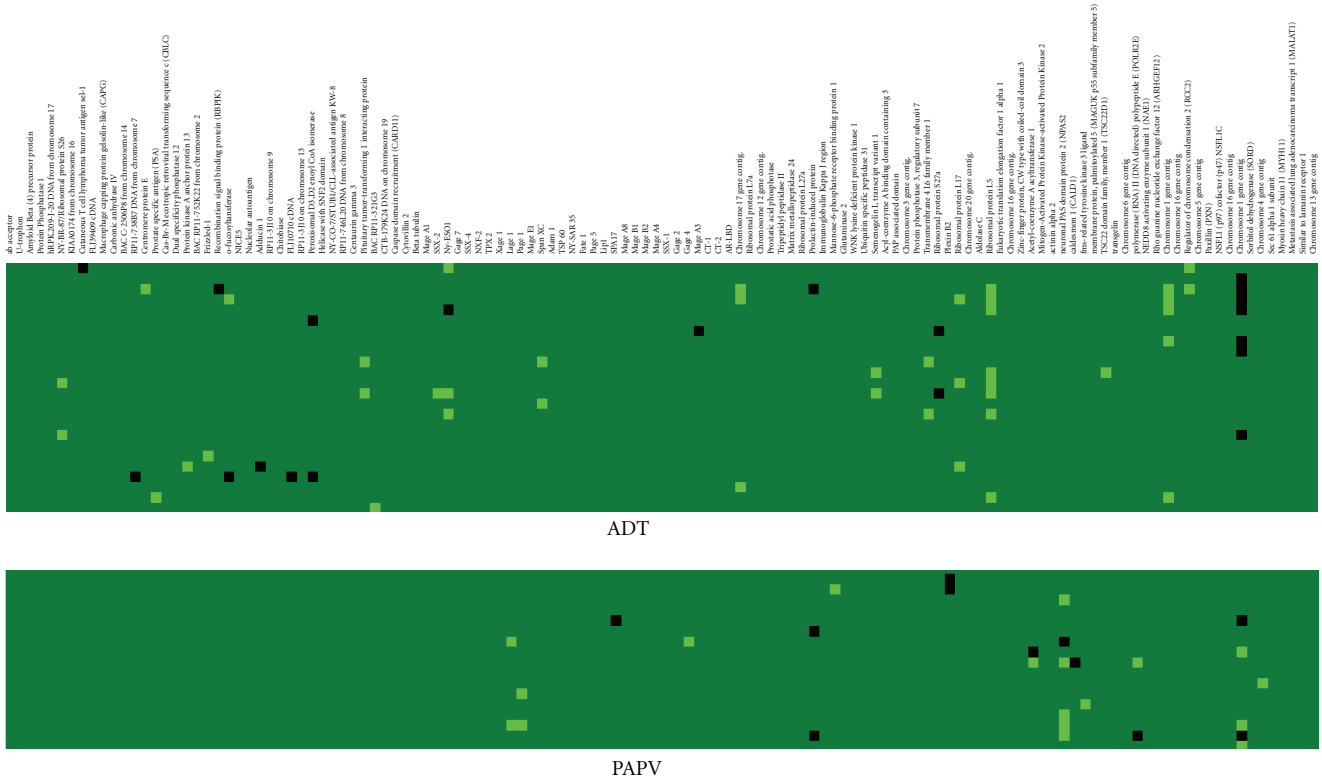

Figure 3: IgG responses to specific prostate-associated antigens are detectable several months after initiation of treatment with ADT or a PAP vaccine. Immunoblot analysis was performed with the same panel of antigens using sera from individual subjects for whom sera was available 12 months after beginning treatment with ADT (24 of the original 34) or the PAP vaccine (19 of the original 21). The heatmap similarly shows gain of response pretreatment to 12 months posttreatment (light green), loss of response following treatment (black), or no change in response (dark green) for all subjects (in rows) and all antigens (in columns).

immune responses were highly associated with specific treatments, suggesting that the generation of immune responses to these "off-target" antigens might be associated with other measures of immune response or clinical outcome. These responses, however, were most detectable at 12 months after therapy initiation, a time relatively late to be useful in most circumstances as a predictive biomarker. Responses detectable at three months would be more useful. However, the sample sizes for each individual trial were small, and multiple comparisons made by evaluating IgG responses to multiple antigens present difficulties in statistically assessing the importance of an individual marker. Consequently, we evaluated IgG responses to multiple antigens by training a ML-BBN model to determine whether we could identify groups of IgG responses that are associated with clinical response, using data obtained at three months. Because the vaccine trials were conducted in different patient populations where different definitions of clinical response were used, we defined it simply for this purpose as a serum PSA value at the 3-month time point lower than the baseline time point ($n = 1$ for the PAPV trial, and $n = 4$ for the PSAV trial subjects).

We trained classifiers on each cohort and compared classifier structure between cohorts. We identified nine (9) biomarkers that were shared between one or more model structures, as described in Table 1, as well as two high-content nodes in the all-cohort model. This resulted in a final subset of biomarkers to include in the final ML-BBN model: chromosome 20 gene contig CHANGE, RP11-738B7 DNA on chromosome 7 CHANGE, chromosome 1 gene contig CHANGE1, prolactin-induced protein CHANGE, acetyl-coenzyme A acyltransferase 1 CHANGE, BAC RP11-321G3 CHANGE, cutaneous T-cell lymphoma tumor antigen sel-1 CHANGE, neuronal PAS domain protein 2 CHANGE, o-fucosyltansferase CHANGE, PAGE 1 CHANGE, and recombination signal binding protein (RBPJK) CHANGE. The structure of the final subset model is displayed in Figure 4. This indicates that there are two first-degree associates of PSA decline, IgG responses to chromosome 1 gene contig 1 and BAC RP11-321G3, and three immune biomarkers features which can be used to estimate PSA decline: IgG responses to chromosome 1 gene contig 1, BAC RP11-321G3, and RP11-738B7 DNA on chromosome 7. Further, these biomarkers are associated with IgG responses to chromosome 20 gene contig, o-fucosyltansferase, PAGE 1, and cutaneous T-cell lymphoma tumor antigen sel-1. To evaluate the robustness of this model, we performed tenfold cross-validation and calculated an AUC for clinical response (PSA decline) of 0.357. This indicates that our first model is not a robust classifier, but is rather an exploratory model.

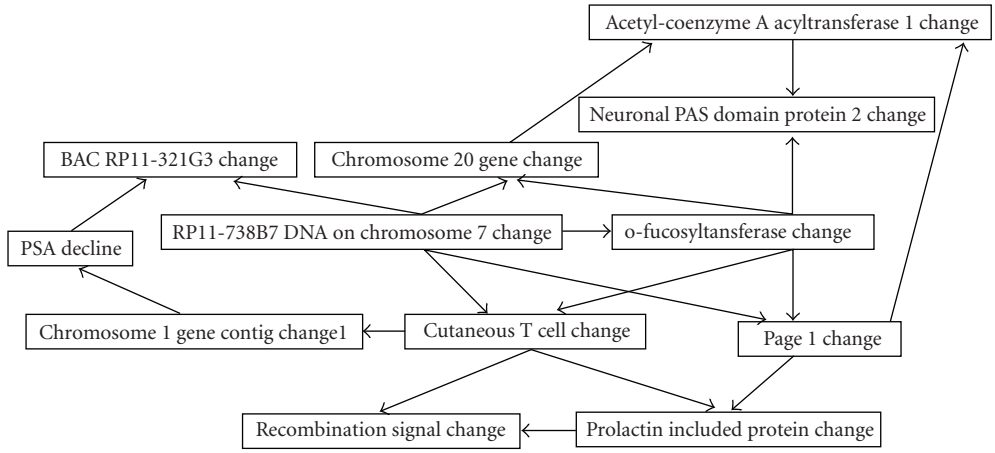

Figure 4: Structure of Bayesian Belief Network representing selected subset of biomarkers. The structure of the network represents the hierarchy of conditional dependence between features, hence we can identify that the two first degree associates of clinical response are change in IgG responses to BACRP11-321G3 and chromosome 1 gene contig 1. Further, because IgG response to BAC RP11-321G3 is a shared child between PSA decline and IgG response to RP11-738B7 DNA on chromosome 7, IgG response to RP11-738B7 DNA on chromosome 7 still influences the estimate of PSA decline even when IgG response to BAC RP11-321G3 is known.

4. Discussion

In the current report, we sought to determine whether serum antibody responses to a panel of prostate tissue- and prostate cancer-associated antigens might be developed as a diagnostic tool to evaluate immune responses elicited following immune-active therapies, and further to determine whether this might be developed in the future as a biomarker of clinical response. Using sera obtained from patients treated with three different therapies, we found that antigen-specific IgG responses could be detected, likely elicited as a result of therapy. The patterns of response differed with respect to the individual therapy, and recognition of specific antigens was most evident at a later (12 months following treatment) than at an earlier time point (3 months following treatment). Using a ML-BBN model to evaluate groups of IgG responses detected three months after treatment, we prioritized a cohort of antigens, immune responses to which were most associated with PSA decline. These findings suggest that, with data from larger populations of subjects, models could be developed to assist in the detection of potentially therapeutic immune responses resulting from immune-based therapies.

Our results demonstrate that immune-active therapies, including androgen deprivation, elicit IgG responses to individual prostate-associated antigens. This has already been demonstrated in previous studies [18, 20]; however, the IgG responses from androgen deprivation therapy were most obvious many months after treatment, where responses to individual antigens were common and predominantly induced rather than lost. It is conceivable that some immune responses wax and wane over time, and in fact the detection of frequent gains and losses of immune responses to individual antigens, common across treatment groups detected earlier at three months, suggests that this can happen with some antigens. Ideally, to control for this, we would have preferred to have sera samples from men without prostate cancer and/or not undergoing active treatment over the same periods of time. In the absence of this, however, we did have cohorts of subjects treated with different therapies. Given that different individual and sets of antigens were specifically recognized following these different therapies suggests that the responses observed were not purely by chance or due to the waxing and waning of responses to individual antigens. The antigens recognized following androgen deprivation, in particular, were ones previously demonstrated to be commonly recognized by IgG in patients with prostate cancer or inflammatory conditions of the prostate [8, 15]. We did not observe IgG responses to PAP in patients receiving the PAP-targeted vaccine, nor IgG responses to PSA in patients receiving the PSA-targeted vaccine. This was actually not unexpected, as we have previously reported that these vaccines, while able to elicit antigen-specific T-cell responses, do not elicit robust antigen-specific IgG in patients as detected by more sensitive ELISA methods [6, 22]. The observation of IgG responses elicited with these treatments to other antigens suggests that they may be presented by cross-presentation following immune-mediated tumor cell targeting.

Of interest was the observation that the antigens recognized following androgen deprivation were different from those recognized following vaccine treatment. Theoretically, the recognition of other nonvaccine target antigens represents antigen spread induced by immune targeting and presentation of other tissue-associated antigens. The recognition of different antigens suggests different mechanisms of antigen spread, or potentially recognition of other tissue-derived antigens, since most of the antigens in this panel are not prostate specific in expression. At present it is unclear whether the generation of such responses is favorable or not; at least one report has suggested that the generation of IgG responses to non-target antigens might be associated

Table 2: Estimate of PSA decline given biomarker evidence. Inference table describing estimates of the posterior distribution of PSA decline (target) representing estimates of likelihood of increase (0), decrease (1), or unknown (MISSING). For example, the most common case involves no change in any of the independent features (biomarkers), occurs 66.7% and results in a 64.1% posterior probability of PSA increase. The case representing an increase in IgG responses to BAC RP11-321G3, a decrease in IgG response to chromosome 1 gene contig and no change in IgG response to RP11-738B7 DNA on chromosome 7 occurs 1.1% of the time and results in a 6.9% posterior estimate of PSA increase.

Probability of case	Drivers			Target		
	BAC RP11-321G3 CHANGE	Chromosome 1 gene contig CHANGE1	RP11-738B7 DNA on chromosome 7 CHANGE	PSA Decline		
				0.0	1.0	MISSING
0.235%	0.0	−1.0	−1.0	28.6	14.3	57.1
0.168%	1.0	−1.0	−1.0	40.0	20.0	40.0
0.638%	0.0	0.0	−1.0	55.6	6.5	37.9
0.517%	1.0	0.0	−1.0	68.6	8.0	23.4
0.423%	0.0	1.0	−1.0	18.7	6.3	75.0
0.264%	1.0	1.0	−1.0	30.0	10.0	60.0
10.727%	0.0	−1.0	0.0	36.2	13.2	50.6
1.172%	1.0	−1.0	0.0	6.9	48.3	44.8
66.744%	0.0	0.0	0.0	64.1	5.4	30.5
4.311%	1.0	0.0	0.0	20.7	33.6	45.7
11.448%	0.0	1.0	0.0	24.8	6.0	69.2
1.101%	1.0	1.0	0.0	5.4	25.0	69.6
0.235%	0.0	−1.0	1.0	28.6	14.3	57.1
0.168%	1.0	−1.0	1.0	40.0	20.0	40.0
0.638%	0.0	0.0	1.0	55.6	6.5	37.9
0.517%	1.0	0.0	1.0	68.6	8.0	23.4
0.423%	0.0	1.0	1.0	18.7	6.3	75.0
0.264%	1.0	1.0	1.0	30.0	10.0	60.0

with a worse outcome [23]. Future studies will explore whether the antigens recognized are shared among different vaccine approaches, suggesting common mechanisms of antigen spread, or whether different vaccine therapies elicit specific responses to different "off-target" antigens. With larger group sizes, we also hope to address whether responses to these antigens are associated with measures of T-cell immune responses to the target antigen, further implicating antigen spread as the mechanism of their recognition. In addition, with larger group sizes we hope to answer whether these are clearly associated with improved clinical benefit or not, or whether this is dependent on treatment context and the specific antigen(s) recognized, as we expect.

Given the small sample size and the multitude of IgG response data points, we sought to identify if the use of ML-BBN modeling was feasible to identify biomarker cohorts in our study data. We were able to use a stepwise process and BBN model structures to identify those biomarkers which had high information content for use in a selected subset for ML-BBN modeling. We were subsequently able to use this subset to train an ML-BBN including clinical response, however on cross-validation, our AUC for clinical response was poor. This is likely due to the fact that of the 52 vaccine subjects we only had 5 "responders" as defined. This resulted in a very small set of training outcomes, making models very sensitive to record deletion, as in the case of cross-validation. PSA response has itself not been validated as a surrogate clinical endpoint, and ADT itself elicits initial PSA responses in the vast majority of patients. Consequently, future studies will explore other better markers of clinical response. In addition, as further data are collected from additional subjects treated by vaccines, we expect this will produce a more robust predictive model.

In any case, the use of ML-BBN modeling appears to provide a promising method for identifying biomarkers in complex data sets that can then be selected for further analysis, as the same subset of biomarkers appeared to produce high information content in models across different populations. Further, once we have sufficient subjects to produce a robust model, tables of posterior estimates for clinical response given combinations of IgG response biomarkers can be developed. An example inference table is provided in Table 2, where those biomarkers that are predictive of clinical response can provide a posterior estimate of response. This type of inference could support the translation of this research into a clinical application for determining whether an individual patient has "responded" from a particular vaccine therapy or potentially whether ongoing immunization should be performed. Future modeling might further permit the selection of patients who would be appropriate to

receive vaccine therapy based on pre-existing immunological response parameters.

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