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MINIREVIEW

Translating Tumor Antigens into Cancer Vaccines[▽]

Luigi Buonaguro,[1,2]* Annacarmen Petrizzo,[1] Maria Lina Tornesello,[1] and Franco M. Buonaguro[1]

Laboratory of Molecular Biology and Viral Oncogenesis & AIDS Reference Center, Istituto Nazionale Tumori "Fondazione G. Pascale," Via Mariano Semmola 1, 80131 Naples, Italy,[1] and Institute of Human Virology, University of Maryland School of Medicine, Baltimore, Maryland[2]

Vaccines represent a strategic successful tool used to prevent or contain diseases with high morbidity and/or mortality. However, while vaccines have proven to be effective in combating pathogenic microorganisms, based on the immune recognition of these foreign antigens, vaccines aimed at inducing effective antitumor activity are still unsatisfactory. Nevertheless, the effectiveness of the two licensed cancer-preventive vaccines targeting tumor-associated viral agents (anti-HBV [hepatitis B virus], to prevent HBV-associated hepatocellular carcinoma, and anti-HPV [human papillomavirus], to prevent HPV-associated cervical carcinoma), along with the recent FDA approval of sipuleucel-T (for the therapeutic treatment of prostate cancer), represents a significant advancement in the field of cancer vaccines and a boost for new studies in the field. Specific active immunotherapies based on anticancer vaccines represent, indeed, a field in continuous evolution and expansion. Significant improvements may result from the selection of the appropriate tumor-specific target antigen (to overcome the peripheral immune tolerance) and/or the development of immunization strategies effective at inducing a protective immune response. This review aims to describe the vast spectrum of tumor antigens and strategies to develop cancer vaccines.

CANCER IMMUNOTHERAPY

Cancer immunotherapy may be classified into passive as well as active strategies, with the latter being specific or nonspecific (117). Passive or "adoptive" immunotherapy is based on administration of antitumor antibodies or transfer of tumor-reactive lymphocytes. Active immunotherapy is aimed either at eliciting a specific *de novo* host immune response against selected tumor antigens (Ags) by employing cancer vaccines or at amplifying the existing antitumor immune response by administering nonspecific proinflammatory molecules or adjuvants. In this context, considering the disappointing results up to now, the quest for specific and selective tumor antigens for developing tumor-specific cancer vaccines, optimal delivery systems (i.e., dendritic cell [DC]-based vaccines), adjuvants, and strategies to overcome immune tolerance and regulatory T (Treg) cell responses is the main goal for several research groups and leading health care companies.

QUEST FOR THE APPROPRIATE TUMOR ANTIGEN

The role of the immune system in tumor containment and/or "rejection" has been studied for decades, showing the possibility of inducing an immune response able to reject an experimentally transplanted tumor. However, the "immunosurveillance of tumors" theory independently postulated by Burnet (19–21) and Thomas (173) has not held the original promise, and much skepticism has been raised by different authors. More recently, the original concept of immunosurveillance has been further elaborated by Schreiber et al. (53, 54) into the "cancer immunoediting" hypothesis, which postulates three main phases: elimination, equilibrium, and escape. In particular, in the elimination phase, cells of the innate and adaptive immune responses may eradicate the developing tumor and protect the host from tumor formation. If the elimination process is not successful, the tumor cells may enter the equilibrium phase and be immunologically shaped by immune "editors" to produce new populations of tumor variants. These variants may eventually evade the immune system and become clinically detectable in the escape phase (53, 54).

The cells playing a key role in this process have been identified in both the innate (e.g., natural killer cells, natural killer T cells, macrophages, and dendritic cells) and the adaptive (e.g., $CD4^+$ Th1 and $CD8^+$ T cells) immune systems, whose final goal is to kill the antigen-bearing tumor cells. More recently, a relevant role for an additional subset of $CD4^+$ T helper cells (named Th17) in the immune response to cancer has been proposed and described by several authors (reviewed in reference 203).

However, different approaches have failed to induce an effective antitumor immune response, suggesting the notion of "nonimmunogenicity" of tumors (69). However, more recently it has been shown that the low tumor immunogenicity is not due to the lack of target "tumor" antigens but to their inability to induce an effective immune response. Among several possible biological reasons, this would be consequent to the growth of tumors in the absence of an inflammation process necessary to establish the tissue microenvironment essential to

* Corresponding author. Mailing address: Laboratory of Molecular Biology and Viral Oncogenesis & AIDS Reference Center, Istituto Nazionale Tumori "Fondazione G. Pascale," Via Mariano Semmola 1, 80131 Naples, Italy. Phone: 39-081-5903273. Fax: 39-081-5451276. E-mail: irccsvir@unina.it.

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recruit and induce activation and maturation of the antigen-presenting cells (APCs), which represent the key point of initiating an effective adaptive humoral and cellular immune response.

In this perspective, the search for human tumor antigens as potential targets for cancer immunotherapy has led to the discovery of several molecules expressed mainly or selectively on cancer cells.

Antigens used in cancer vaccines, indeed, should preferably be molecules differently expressed on normal and tumor cells; however, most antigens are derived from mutated or modified self-proteins, which may induce immune tolerance (61). This aspect represents a challenge for the appropriate design of vaccines that have to overcome such tolerance in order to elicit specific antitumor immunity without "undesired" autoimmunity side effects (130).

MULTIPLE "UNDEFINED" ANTIGENS

It is well known that tumors show the accumulation of several genetic modifications in somatic cells (63, 186), which provide cancer cells with the selective growth advantage to initiate clonal expansion (66).

In this context, cancer genes have been originally studied based on their possible relation to cancer (59). More recently, high-throughput technologies have enabled the identification of mutated genes in cancers without any hypothesis-driven bias, whose number has resulted to be surprisingly high, with a functional heterogeneity broader than previously thought (140, 169). These studies have been performed in breast, colorectal, pancreatic, and lung cancers and glioblastoma and, overall, have identified almost 400 candidate cancer genes (CAN-genes) (48, 83, 134, 199). Interestingly, systems-level analyses show that, despite the low degree of overlap in terms of gene identity, cancer signatures converge on specific biological processes, as defined by significant molecular and functional associations between genes and/or proteins (68, 163, 170).

Considering the high number of potential tumor antigens for each individual type of cancer, the concept of immunizing with whole tumor cells to avoid the exclusion of potentially relevant antigens from the vaccine is still valid. A further advantage is that since whole tumor cells express an array of antigens, this vaccine approach circumvents the major histocompatibility complex (MHC) restriction and the need for specific patient-tailored epitope identification. The efficacy of autologous tumor cells as a cancer vaccine has been tested in several clinical trials targeting different tumor types, including colorectal cancer (67, 185) and melanoma (3, 12). Alternatively, to overcome the limitations of patient-tailored vaccines (e.g., standardization of large-scale production, variability in the quality and composition of the vaccines, and lack of reliable comparative analysis of clinical outcome), the use of allogeneic tumor cell lines as cancer vaccines has been tested for prostate cancer (114, 160, 162).

However, the effectiveness of such a vaccine strategy is dramatically hampered by the immune system's inherent tolerance to several tumor antigens, as they may be expressed by normal tissues or presented to T cells in a non-stimulatory context. As a consequence, the breaking of tolerance and the containment of immune suppression need a potent and specific immune stimulus combining antigens and immunological adjuvants (reviewed in references 32 and 192). Whole tumor cell vaccines can be made more immunogenic by modifying tumor cells to express costimulatory molecules and/or cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), which has proven to be more effective than others in inducing recruitment, maturation, and function of dendritic cells (DCs), the most potent type of antigen-presenting cell (APC) (50, 57, 81).

In particular, vaccination with genetically engineered, irradiated melanoma cells, modified to secrete GM-CSF, was shown to improve tumor antigen presentation through increased DC and macrophage recruitment, enabling the generation of effective melanoma-specific $CD4^+$ and $CD8^+$ T cells, CD1-restricted NKT cells, and antibodies (51, 57). Although the advantages of using GM-CSF as an adjuvant for cancer vaccines have been reported, recent observations have suggested the potential of GM-CSF to induce immune suppression, which may negatively impact the management of cancer patients (reviewed in reference 34). Whole tumor cell vaccines expressing high levels of costimulatory molecules are currently being pursued to treat aggressive cancers such as acute myeloid leukemia (AML) (26, 31).

Another approach based on multiple "undefined" antigens takes into account the heat shock proteins (HSPs), which are ubiquitous, intracellular molecular protein chaperons whose expression increases under conditions of elevated temperatures and metabolic stress (105) to enhance the antigen processing and presentation by MHC molecules (167). Consequently, HSPs are associated with a large repository of peptides consisting of self-peptides as well as the entire antigenic peptide repertoire of cancer cells. Therefore, the cross-presentation and cross-priming of tumor antigens mediated by HSPs may result in a valid strategy, especially when the amount of antigen is a limiting factor (reviewed in references 15 and 119). Heat shock protein-based vaccines have been shown to be effective in mice when the HSP was purified from tumor cells matching the implanted tumor (166, 171, 179). Vaccines based on proteins complexed with HSPs, purified from autologous tumors (HSP-protein complex), have been evaluated in clinical trials targeting different cancers. In particular, the immunogenicity and efficacy of HSP-protein complex 96 (HSPPC-96; vitespen) made of tumor peptides associated with the heat shock protein gp96 has been extensively assessed in preclinical and clinical trials for a wide range of cancers, including phase I and II trials in colorectal cancer (110), melanoma (7, 138), and renal cell carcinoma (82) and two phase III studies of melanoma and renal cell carcinoma (165, 172, 197, 198). Furthermore, a full-length human papillomavirus type 16 (HPV16) E7 antigen fused to HSP65 from *Mycobacterium bovis* BCG (HspE7) (33) has been evaluated in a phase II clinical trial, resulting in lesion regression in women with grade III cervical intraepithelial neoplasia (CIN III) (55, 183).

DEFINED ANTIGENS

Cancer vaccines based on defined specific tumor antigens should elicit a very specific effector and memory cell response

TABLE 1. List of most relevant TAAs recognized by T cells[a]

Shared Antigens		Type of tumor	Normal tissue distribution
Cancer-testis (CT) Ags	BAGE GAGE MAGE NY-ESO-1 SSX	melanoma, lymphoma, lung, bladder, colon and breast carcinomas	spermatocytes/spermatogonia of testis, placenta, ovary cells
Differentiation Ags	Gp100 Melan-A/Mart-1 Tyrosinase PSA CEA Mammaglobin-A	melanoma, prostate cancer, colon and breast carcinomas	melanocytes, epithelial tissues, prostate, colon
Overexpressed Ags	p53 HER-2/neu livin survivin	esophagus, liver, pancreas, colon, breast, ovary, bladder and prostate carcinomas	ubiquitous (low level)
Unique Antigens		**Type of tumor**	**Normal tissue distribution**
Unique Ags	β-catenin-m β-Actin/4/m Myosin/m HSP70-2/m HLA-A2-R17OJ	melanoma, non-small cell lung cancer, renal cancer	N/A
Unique/Shared Antigens		**Type of tumor**	**Normal tissue distribution**
Tumor-associated Carbohydrate Ags	GM2 GD2 GD3 MUC-1 sTn globo-H	melanoma, neuroblastoma, colorectal, lung, breast, ovarian and prostate cancer	epithelial tissues (e.g., renal, intestinal, colorectal)

[a] References for antigens listed here are reported in the text. N/A, not applicable.

with a limited chance of inducing autoimmunity. Such an approach may have opposite biological effects. One effect is the possible undesired selection and expansion of tumor variants which lack the target tumor antigen and are biologically resistant to the vaccine-induced immune response. Such tumor variants, however, may in turn induce a beneficial effect, broadening the immune response against newly expressed antigens not present in the original vaccine in a process defined "epitope spreading" (23, 144).

Since the identification of MAGE-1, the first gene reported to encode a human tumor antigen recognized by T cells (182), a large number of tumor antigens have been described (Table 1). Initial classification was based on expression profiles, with tumor-specific antigens (TSAs) being expressed only by cancer cells and tumor-associated antigens (TAAs) representing the mutated counterparts of proteins expressed by normal tissues. The currently accepted classification, however, includes only TAAs, which are divided into shared and unique TAAs and further classified into class I and class II HLA-restricted TAAs, according to the HLA allele restriction (reviewed in reference 125).

Among the shared TAAs, the following three main groups can be identified: (i) cancer-testis (CT) antigens, (ii) differentiation antigens, and (iii) widely occurring, overexpressed antigens.

Among shared tumor-specific antigens, cancer-testis (CT) antigens are expressed in histologically different human tumors and, among normal tissues, in spermatocytes/spermatogonia of the testis and, occasionally, in placenta. CT antigens result from the reactivation of genes which are normally silent in adult tissues (46) but are transcriptionally activated in different tumor histotypes (45). Many CT antigens have been identified and used in clinical trials, although little is known about their specific functions, especially with regard to malignant transformation. This group of TAAs includes MAGE-A1 (30, 177), NY-ESO-1 (78), and SSX-2 (1).

Differentiation antigens are shared between tumors and the normal tissue of origin and found mostly in melanomas and normal melanocytes (Gp100, Melan-A/Mart-1, and Tyrosinase) (4, 89–91, 131, 190), although they are also found in epithelial tissues and tumors such as prostate tumors (prostate-specific antigen [PSA]) (37, 38) and breast carcinomas (mammaglobin-A) (79). Moreover, expression of several oncofetal antigens appears to be increased in many adult cancer tissues, including carcinoembryonic antigen (CEA), which is highly expressed in colon cancer (178). TAAs from this group, despite

representing self-antigens, have been and still are commonly used in current cancer vaccination trials, often together with CT antigens.

Widely occurring, overexpressed TAAs have been detected in different types of tumors as well as in many normal tissues, and their overexpression in tumor cells can reach the threshold for T cell recognition, breaking the immunological tolerance and triggering an anticancer response. Among the most interesting TAAs of this group are the antiapoptotic proteins (livin and survivin) (154, 155), hTERT (116, 187, 188), and tumor suppressor proteins (e.g., p53) (2, 180).

Unique TAAs, on the other hand, are products of random somatic point mutations induced by physical or chemical carcinogens and therefore expressed uniquely by individual tumors and not by any normal tissue, representing the only true tumor-specific antigens (Ags) (reviewed in reference 133). Such Ags characterize each single neoplasm and were shown to be diverse between tumors induced in the same animal or even in different tissue fragments from the same tumor nodule (61, 141, 200). A relevant feature of unique Ags is their potential resistance to immunoselection if the mutated protein is crucial to the oncogenic process and thus indispensable for maintaining the neoplastic state. As a consequence, unique Ags should elicit an immune response clinically more effective than that of shared Ags. However, identification of unique tumor antigens for solid human tumors requires sequencing of the whole genome of each individual tumor in order to identify mutated genes and select peptides whose motifs are predicted to be presented by the patient's HLA alleles. Moreover, each tumor bears highly heterogeneous sets of defects in dozens of different genes (25, 83, 85, 142, 199) which need to be further verified for their substantial contribution to the tumor development and progression and, consequently, for their relevance as vaccine targets (58).

On the contrary, unlike for the solid tumors, the strategy of identifying unique TAAs is relatively easy and feasible for tumors of hematological origin, such as B cell lymphomas, for which the target antigen is well known, being represented by the immunoglobulin idiotype (Ig Id) included in the B cell receptor (BCR). Therefore, sequencing analysis for the identification of cancer-related mutations can be selectively focused on the Ig Id, which can be used for developing a patient-specific vaccine (8, 168). More recently, however, it has been demonstrated that the BCR repertoire expressed by clonal B cells sustaining hepatitis C virus (HCV)-associated non-Hodgkin's lymphoma (NHL) is not random, with a restricted representation of immunoglobulin idiotypes in different patients (28, 43, 137). As a consequence, it is possible to overcome the limitations of tailor-made individual vaccines by designing shared idiotype vaccines to elicit immunity, targeting the B cell clone sustaining the HCV-associated NHL in a broad spectrum of patients (18, 44).

An additional class of tumor antigens is represented by tumor-associated carbohydrate antigens (TACAs), which are glycans uniquely or excessively expressed on the cancer cell surface (143) and correlate with various stages of cancer development (42, 65). However, TACAs do not elicit T cell responses and are usually poorly immunogenic given their structural similarity to normal antigens (80). Nevertheless, conjugation to a carrier protein increases their immunogenicity, enhancing the presentation of carbohydrate antigens to antigen-presenting cells as well as induction of helper T cell activation (92, 106). In particular, keyhole limpet hemocyanin (KLH) has been shown to be the most effective carrier for TACAs (86), and KLH conjugates of GM2 for melanoma (27) as well as those of sTn for breast cancer (73) have both entered phase III clinical trials. However, the disappointing outcomes of these vaccine clinical trials, in terms of time-to-disease progression and overall survival, have driven the generation of several forms of fully synthetic carbohydrate vaccines, which have been shown to be immunogenic regardless of the use of a protein carrier or external adjuvant (6, 13, 22, 76, 176, 194). Unfortunately, despite all efforts, TACA-based cancer vaccines have failed to induce sufficient T cell-mediated immune responses in cancer patients, and none has been approved for clinical use yet (64).

APPLICATION OF DEFINED ANTIGENS AS CANCER VACCINES

Most cancer vaccine clinical trials have been performed with peptide-based vaccines, employing either cancer-testis antigens or differentiation TAAs, and despite the induction of a high frequency of specific T cells, the clinical outcomes have been disappointingly limited (29, 132, 136, 148, 149) (Table 2). There are many possible reasons for these unsatisfactory results, including immune tolerance induced by shared TAAs (35, 112) and limited cytotoxic T lymphocyte (CTL) expansion due to activation of regulatory T lymphocytes (122). Furthermore, single peptides elicit a $CD8^+$ T cell response with a narrow epitope specificity, which may result in limited immunological efficacy and in the induction of immune escape mechanisms (146).

Several strategies have been adopted to overcome such limitations, including the introduction of inflammatory cytokines in the vaccination protocol, such as alpha interferon (IFN-α) (94, 135) and interleukin-2 (IL-2) (104, 147, 150, 151), with conflicting results. An alternative strategy is to generate peptide variants of TAAs (47, 72, 84), including mimotopes, heteroclitic peptides, altered-peptide ligands, and superagonists, introducing MHC-anchor residue modifications (127, 181), systematic residue substitutions (161), combinatorial peptide libraries (111, 139), and genetically encoded peptide libraries (39, 191). However, considering the overall disappointing results of clinical trials testing such peptide variants, additional strategies have been developed to broaden the repertoire of responding T cells by introducing amino acid substitutions in the peptide-MHC binding surface (16, 74, 102).

Significant improvement in the immunogenicity of single-peptide vaccines has been achieved using long peptides deriving either from the chemical linkage of multiple immunogenic epitopes (71, 159) or from naturally occurring linked CTL and Th epitopes, as shown for human papillomavirus (HPV) E6-E7 proteins (93, 193), the CT antigen NY-ESO-1 (202), and HER-2/neu (49, 95). The enhanced immunological potency of long peptides, which do not bind directly to MHC class I molecules as 8-mer to 10-mer CTL epitopes do, is most likely due to their efficient presentation to CTL precursors through processing by DCs (9, 14, 113, 156), which should dramatically reduce transient CTL responses or tolerance (174, 175). Furthermore,

TABLE 2. Peptide-based cancer vaccine clinical trials for most representative tumors[a]

Tumor	N.	Antigen (# of trials)	Phase
Acute Myelocytic Leukemia	10	WT1 (5), PR1 (5)	I/II
Breast	32	E75 (2); p53 (2); HER-2/neu (9)	I/II
Colorectal	18	ras (5); CEA (4)	I/II
Liver	6	AFP (2); CEA (1)	I/II
Lung	20	URLC10 (6); ras (4); HER-2 (2); VEGFR1 and 2 (3); mutant p53 (2)	I/II
Melanoma	115	MAGE (13); gp100 (54); MART-1 (36); Tyrosinase (32); NY-ESO-1 (4)	I/II
Ovarian	16	p53 (4); NY-ESO-1 (3); HER-2 (3)	I/II
Uterine	8	HPV16 E7 (4); Survivin (1); mutant p53 (1)	I/II
Pancreas	14	ras (4); VEGFR1 and 2 (3); MUC-1 (1); Survivin (1)	I/II

[a] Further information on current cancer vaccine clinical trials is available at www.clinicaltrials.gov. N. = number of total clinical trials registered for the corresponding tumors.

long peptides may persist longer in inflamed lymph nodes in close proximity to the vaccination site, resulting in the clonal expansion of IFN-γ-producing effector T cells and improved antitumor CTL response (14). Finally, the presence of multiple immunogenic epitopes in long peptides would ensure the interaction with different HLA class I and class II alleles, eliciting a broad T cell response against many epitopes, including the immunodominant ones, and reducing the emergence of tumor escape variants.

DENDRITIC CELLS AS AN ANTIGEN DELIVERY SYSTEM

An effective vaccine needs to efficiently hit the innate immune system and, downstream, the adaptive humoral and cellular immunity to elicit an adequate level of effector cells and establish the immunological memory. To this aim, vaccine strategies effective in activating both innate and adaptive immunity are actively pursued by several groups, and among the different possible strategies, dendritic cell (DC)-based vaccines represent one of the most promising strategies (reviewed in references 96 and 126).

Dendritic cells can be generated *in vitro* from $CD34^+$ progenitor cells derived from the patient's bone marrow or peripheral blood, and maturation can be obtained *ex vivo* with a cocktail of several cytokines.

The original protocol for DC generation has been designed, including GM-CSF and IL-4 (121, 157), but it has been shown that functionally distinct DC subsets can be generated by using different cytokines. Indeed, activated monocytes induced with IFN-α/β, thymic stroma lymphopoietin (TSLP), tumor necrosis factor (TNF), or IL-15 will differentiate into IFN DCs, TSLP DCs, TNF DCs, or IL-15 DCs, respectively, able to induce different types of immune responses (5). For example, melanoma-peptide-pulsed IL-15 DCs are much more efficient than IL-4 DCs in inducing antigen-specific CTL differentiation *in vitro* (52), whereas IFN-α DCs show improved activation of T helper cells (128). Similarly, different DC maturation pathways may significantly impact their capacity to elicit T cell immunity (99). Indeed, GM-CSF/IL-4 DCs activated with a cocktail of IFN-α, poly(I:C), IL-1β, TNF, and IFN-γ are much more effective in inducing specific CTLs than "gold standard" DCs matured with a cocktail of macrophage cytokines, including IL-1β/TNF/IL-6/prostaglandin E2 (PGE2) (109). Moreover, PGE2 can skew the differentiation of T helper cells to Th2 cells, blocking the production of IL-12 p70 (87, 88).

Matured DCs are then loaded with tumor antigens as peptides (24), tumor lysates (121), or apoptotic debris (129), which are processed and presented on the DC surface in the context of MHC class I and II molecules. The matured and antigen-loaded DCs are then transferred back to the patients for the generation of an antitumor immune response (reviewed in references 5 and 157) (Fig. 1).

Concerning the strategy of using tumor lysates or apoptotic debris as source of tumor antigens, the original and standard procedure is to use autologous DCs loaded with autologous tumor cells, both derived from the treated patient. However, the preparation of sufficient amounts of autologous tumor cells might be a significant limiting factor, and to overcome this hurdle, the use of allogeneic tumor cell lines as sources of tumor antigens has been proposed. Tumor cell lines, indeed, share many TAAs with the patients' autologous tumor cells and can be efficiently expanded *in vitro*. Cellular fusions generated by autologous DCs and allogeneic tumor cell lines have been shown to induce antigen-specific polyclonal CTLs, with cytotoxic activity against autologous tumor cells (10, 97, 98). A further possible alternative is to use allogeneic DCs from healthy donors as a fusion partner, given that unprimed T cells from an individual react against the foreign MHC antigens of another individual. It has been demonstrated, indeed, that fusions of both autologous and allogeneic DCs are effective in inducing antitumor immunity in humans (62), although this approach can be applied only in selected situations (reviewed in reference 96).

The effectiveness of DC-based vaccines has been demonstrated in specific tumor stages, and very recently, the first autologous cellular immunotherapy has received FDA approval for the treatment of asymptomatic or minimally symptomatic metastatic hormone-refractory prostate cancer (HRPC). Sipuleucel-T consists of autologous PBMCs loaded with recombinant human prostatic acid phosphatase (PAP) linked to granulocyte-macrophage colony-stimulat-

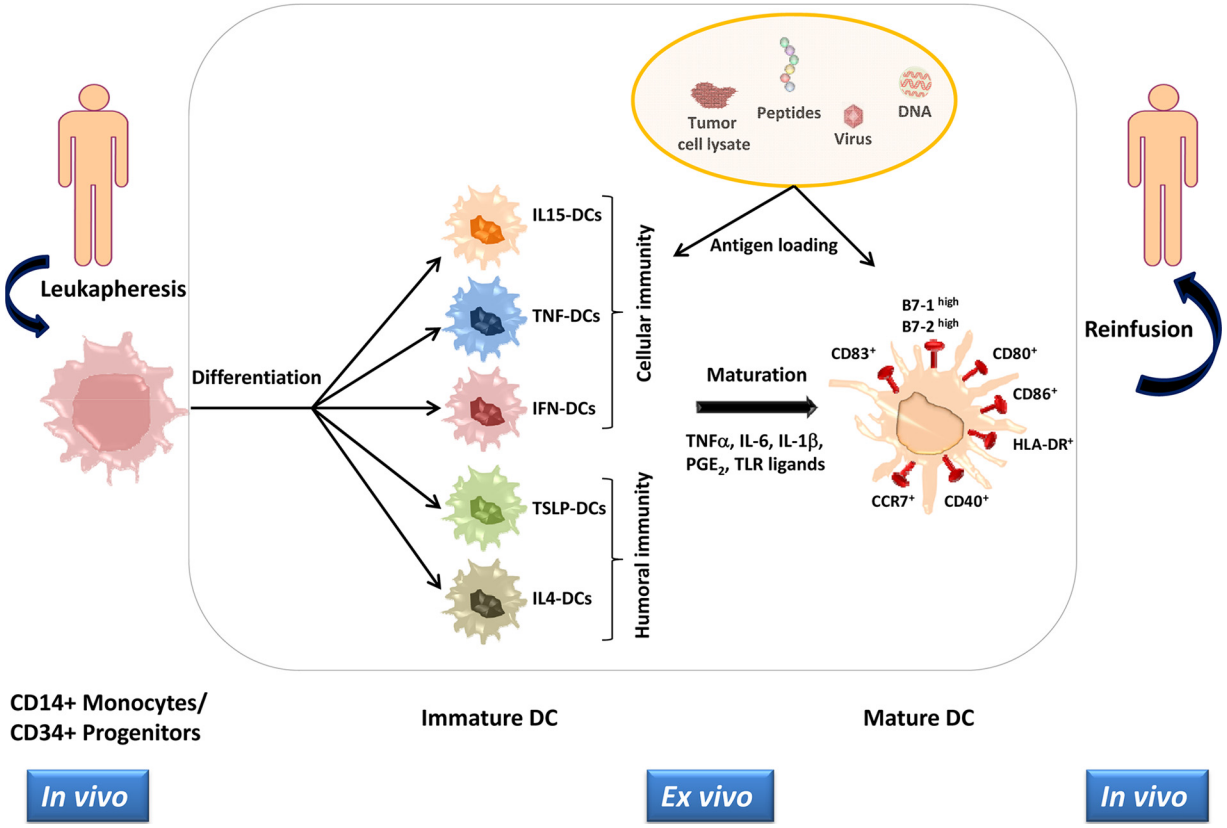

FIG. 1. Schematic representation of a DC-based vaccine preparation. $CD14^+$ monocytes or $CD34^+$ hematopoietic progenitors are derived from patients. Different DC subsets are generated *in vitro*, with distinct specialization in driving adaptive immunity to the Th1 or Th2 response. Mature DCs are loaded with one of the indicated sources of tumor antigens and reinfused in the patient. The most relevant cell markers characterizing the different activation stages of DCs are indicated.

ing factor (PAP–GM-CSF), which has proven to be effective in phase III clinical trials (70).

However, results from multiple clinical trials with DC-based cancer vaccines have been contradictory, and only fractions of the enrolled patients show potent antitumor immune responses (reviewed in references 96 and 103). Several reasons may account for this modest clinical outcome, including the reproducible efficiency of DC generation and the possible induction of adaptive $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T (Treg) cells in the presence of transforming growth factor β (TGF-β) or IL-10 derived from the tumor microenvironment (189).

HARNESSING IMMUNE TOLERANCE AND Treg CELLS

Naturally occurring regulatory T (Treg) cells account for 5% to 10% of peripheral $CD4^+$ T cells (60, 152), whose key role is to inhibit self-reactive effector T cells, inducing peripheral T cell tolerance (152). Moreover, Treg cells have been found to be increased in peripheral blood and tumors in a variety of human cancers (75, 107, 196), resulting in poorer prognosis and reduced survival (153, 158, 195, 201). The presence of an increased percentage of circulating Treg cells may, indeed, represent a major obstacle to the success of cancer vaccines, and partial depletion of Treg cells has been shown to enhance DC vaccine-induced immune responses in cancer patients (41, 123). In this perspective, cancer vaccines may be more effective when combined with therapeutic interventions aimed at eliminating and/or controlling naturally occurring $CD4^+$ $CD25^+$ regulatory T cells. Studies done in the 1980s showed that pretreatment with cytostatic drugs (i.e., cyclophosphamide) was significantly enhancing the efficacy of adoptive cancer immunotherapy in preclinical (124) as well as clinical (11) settings. Several clinical trials are currently ongoing to assess the efficacy of cyclophosphamide to control Treg cells and improve the immune response to cancer vaccines in humans (56). Alternative strategies to eliminate and/or control naturally occurring Treg cells are represented by the use of a recombinant IL-2 diphtheria toxin conjugate (Ontak), which has been shown to enhance tumor-specific T cell responses to vaccines (41, 118) as well as to improve immune responses in patients with metastatic melanoma (108).

Furthermore, depletion of $CD4^+$ $CD25^+$ Treg cells may also be achieved using an anti-CD25 monoclonal antibody (MAb), as shown for melanoma or breast cancer vaccine (77, 115, 145).

The role of Toll-like receptor (TLR) agonists as inhibitors of Treg cell function (36, 184) is still controversial. It has been reported, indeed, that different TLR agonists can be effective in limiting tumor progression when used as adjuvants to coadministered a cancer vaccine (120, 164) or as stand-alone immunotherapeutics, eliciting an immune response to tumor self-antigens (17, 100, 101). Nevertheless, such effects are not

univocal, given that TLR agonists may induce differentiation, proliferation, or activation of Treg cells. Human $CD4^+$ $CD25^+$ Treg cells stimulated with the TLR5 agonist flagellin, indeed, show enhanced expression of Foxp3 and increased suppressive function (40).

CONCLUSIONS AND FUTURE DIRECTIONS

The cancer vaccine field is constantly growing and generating a considerable amount of information in terms of antigen target identification and delivery as well as immune modulation, which will represent the knowledge platform to accomplish the ultimate aim of developing an effective cancer vaccine. However, large-scale clinical trials of current strategies and protocols have not yet proved to be as efficacious as needed for complete tumor regression.

Several reasons may account for these disappointing results, including (i) tumor evasion from immune recognition, (ii) inefficient induction of high-affinity adaptive immunity, and (iii) tumor-induced immunosuppression. Each of these aspects needs to be addressed and possibly solved in order to increase the chances of success.

In this discovery process, the systems biology approach can have a great impact not only on the comprehension of multiple pathways involved in tumor development and progression but also on the dissection of molecular mechanisms involved in the efficient induction of effective innate and adaptive immunity. Such an approach, ultimately, will have a significant impact on cancer vaccine development for the identification of both novel potential target antigens and molecular prediction markers of immunogenicity.

This would represent the real switch from the "empirical" to the "knowledge-based" age of cancer vaccinology, enabling the development of strategies with enhanced therapeutic efficacy to significantly improve the quality of life of cancer patients.

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