Haematologica 2000; 85:1172-1206
trends in hematology

Hematology & Medicine

Antitumor vaccination: where we stand

MONICA BOCCHIA,* VINCENZO BRONTE,° MARIO P. COLOMBO,# ARMANDO DE VINCENTIIS,@ MASSIMO DI NICOLA,^ GUIDO FORNI,§ LUIGI LANATA,** ROBERTO M. LEMOLI,°° MASSIMO MASSAIA,## DAMIANO RONDELLI,°° PAOLA ZANON,** SANTE TURA°°

**Department of Hematology, University of Siena; °Department of Oncology, University of Padova; #Division of Oncology, Istituto Nazionale dei Tumori, Milan; @Dompè-Biotec, Milan; ^Bone Marrow Transplant Unit, Istituto Nazionale dei Tumori, Milan; §Department of Clinical and Biological Sciences, University of Turin; **Amgen Italia, Milan; °°Institute of Hematology and Medical Oncology "Seràgnoli", University of Bologna; ##Division of Hematology, University of Turin, Italy*

ABSTRACT

Background and Objectives. **Vaccination is an effective medical procedure of preventive medicine based on the induction of a long-lasting immunologic memory characterized by mechanisms endowed with high destructive potential and specificity. In the last few years, identification of tumor-associated antigens (TAA) has prompted the development of different strategies for antitumor vaccination, aimed at inducing specific recognition of TAA in order to elicit a persistent immune memory that may eliminate residual tumor cells and protect recipients from relapses. In this review characterization of TAA, different potential means of vaccination in experimental models and preliminary data from clinical trials in humans have been examined by the Working Group on Hematopoietic Cells.**

Evidence and Information Sources. **The method employed for preparing this review was that of informal consensus development. Members of the Working Group met four times and discussed the single points, previously assigned by the chairman, in order to achieve an agreement on different opinions and approve the final manuscript. Some of the authors of the present review have been working in the field of antitumor immunotherapy and have contributed original papers to peer-reviewed journals. In addition, the material examined in the present review includes articles and abstracts published in journals covered by the Science Citation Index and Medline.**

State of the art. **The cellular basis of antitumor immune memory consists in the generation and extended persistence of expanded populations of T- and B-lymphocytes that specifically recognize and react against TAA. The efficacy of the memory can be modulated by compounds, called "adjuvants", such as certain bacterial products and mineral oils, cytokines, chemokines, by monoclonal antibodies triggering co-stimulatory receptors. Strategies that have been shown in preclinical models to be efficient in protecting from tumor engraftment, or in preventing a tumor rechallenge, include vaccination by means of soluble proteins or peptides, recombinant viruses or bacteria as TAA genes vectors, DNA injection, tumor cells genetically modified to express co-stimulatory molecules and/or cytokines. The use of professional antigen-presenting cells, namely dendritic cells, either pulsed with TAA or transduced with tumor-specific genes, provides a useful alternative for inducing antitumor cytotoxic activity. Some of these approaches have been tested in phase I/II clinical trials in hematologic malignancies, such as lymphoproliferative diseases or chronic myeloid leukemia, and in solid tumors, such as melanoma, colon cancer, prostate cancer and renal cell carcinoma. Different types of vaccines, use of adjuvants, timing of vaccination as well as selection of patients eligible for this procedure are discussed in this review.**

Perspectives. **Experimental models demonstrate the possibility of curing cancer through the active induction of a specific immune response to TAA. However, while pre-clinical research has identified several possible targets and strategies for tumor vaccination the clinical scenario is far more complex for a number of possible reasons. Since experimental data suggest that vaccination is more likely to be effective on small tumor burden, such as a minimal residual disease after conventional treatments, or tumors at an early stage of disease, better selection of patients will allow more reliable clinical results to be obtained. Moreover, a poor correlation is frequently observed between the ability of TAA to induce a T-cell response *in vitro* and clinical responses. Controversial findings may also be due to the techniques used for monitoring the immune status. Therefore, the development of reliable assays for efficient monitoring of the state of immunization of cancer patients against TAA is an important goal that will markedly improve the progress of antitumor vaccines. Finally, given the promising results, identification of new or mutated genes involved in neoplastic events might provide the opportunity to vaccinate susceptible subjects against their foreseeable cancer in the next future.**

Key words: antitumor vaccination.

Correspondence: Prof. Sante Tura, Istituto di Ematologia e Oncologia Medica "Seràgnoli", Università di Bologna, Policlinico S.Orsola-Malpighi, via Massarenti 9, 40138 Bologna, Italy. Phone: international +39-051-6363680 – Fax: international-39-051-6364037 – E-mail: tura@orsola-malpighi.med.unibo.it

Antitumor vaccines: the meaning

As illustrated in a previous paper of this series, many strategies are being used to try to cure cancer, each one based on different theoretical and experimental grounds.[1] Active immunotherapy strategies elicit specific or non-specific antitumor reactions by stimulating the patient's immune system. Alternatively, lymphocytes collected from patients are stimulated *in vitro* and re-injected into the patient (adoptive immunotherapy). Lastly, passive immunotherapy consists in the administration of antitumor antibodies to the patient. However, dealing with such a dramatic issue as is cancer, emotional empiricism spurs the adoption of distinct strategies or their mixing in an apprehensive pursuit of efficacy. Indeed, emotional empiricism has been and still is a deadly sin of tumor immunology. In the long run, only rational considerations lead to clinical progress.

The issue

Vaccination is an effective medical procedure characterized by being a) predominantly a maneuver of preventive medicine; b) based on the induction of a long-lasting immunologic memory that is c) characterized by mechanisms endowed with high destructive potential and specificity.[2] It rests on an artificial encounter of a sham pathogen with the immune system. The sham pathogen elicits a strong host reaction and leaves a persistent memory of this first artificial fight. If the real pathogen enters the immunized organism it becomes the target of a much stronger and precise reaction than that put up by a non-immunized organism. If the pathogen had a small possibility of escaping the reaction of a naive immune system, very seldom would it evade a memory reaction.[3] The cellular basis of immune memory consists in the extended persistence of expanded populations of T- and B- lymphocytes that specifically recognize and react against the pathogen. Memory lymphocytes are also experienced *veterans*, able to detect a pathogen promptly and fight effectively against it.

There is a large universe of sham pathogens that are used for vaccination, named *antigens* or *immunogens*. A killed or inactivated pathogen, or a non-pathogenic organism sharing critical molecules with the real thing can be a good immunogen. Memory can also be induced by a protein from the pathogen. In addition, a virus engineered with the DNA coding for a protein of the pathogen or even the mere naked DNA induces an effective memory.[4]

The efficacy of the memory is modulated by numerous compounds, called *adjuvants* and *danger signals*.[5,6] These provide additional activation signals, recruit reactive leukocytes at the immunization site and delay antigen catabolism. Bacterial products and mineral oils are typical conventional adjuvants. Cytokines and chemokines also act as adjuvants. As will be discussed in detail, their use allows the induction of selective mechanisms of immune memory.[7]

Antitumor vaccination has a defined goal: to provoke specific recognition of tumor-associated antigens (TAA) in order to elicit a persistent immune memory. Many experimental data have shown that following immunization, the growth of tumor cells expressing the same TAA as the vaccine can be impaired. In patients, the immune memory elicited by vaccines is sometimes fast and strong enough to hamper the growth of their tumor.[8]

A brief history

Interest in antitumor vaccination arose around 1900 when a series of microbial vaccines proved to be effective. The idea was straightforward: to apply the same intervention to tumor. «*If .....it is possible to protect small laboratory animals in an easy and safe way against infectious and highly aggressive neoplastic specimens, then it will be possible to do the same for human patients*». These words of 1897 by Paul Ehrlich[9] ignited a series of studies with transplantable mouse tumors. However the underlying issue turned out to be more complex than had first been presumed. More than one century was required to elucidate its molecular and genetic features.

The first outcome was not a progress in antitumor vaccination, but instead the definition of a few rules of allograft rejection. Transplanted tumors were rejected by immunized host not because they expressed a particular TAA but because they were from histoincompatible mice and displayed normal allogeneic histocompatibility antigens.[10] Later studies with syngeneic mouse strains showed the feasibility of immunizing a mouse against a subsequent tumor challenge.[11] However, the suspicion that residual unnoticed histocompatibility differences were involved in these vaccination-rejection studies was not ruled out until experiments by George Klein in 1960.[12] Carcinoma was induced by methylcholanthrene in syngeneic mice. The carcinoma was then surgically removed, and its cells were cultured *in vitro* and used to repeatedly immunize the mouse in which the tumor had arisen. Finally the immunized mouse and a few control syngeneic mice were challenged with the carcinoma cells of the original tumor maintained *in vitro*. While these cells gave rise to a carcinoma in control mice, they were rejected by the immunized mouse in which the carcinoma had arisen originally. This evidence of the possibility of immunizing against a lethal dose of own

tumor was of seminal importance and had notable consequences. A very large series of subsequent studies established a few basic foundations of tumor vaccination.[13]

Looking back at tumor immunology over the last 20-30 years, the importance of models in influencing immunologic beliefs is strikingly evident. Inappropriate use of an experimental model may produce wrong beliefs, from which it is then very hard to escape. Virus- and chemically-induced tumors form highly immunogenic models. Since they are easy to handle, these were used to establish the rules of tumor vaccination. However, it was disputable whether the information from these models was relevant to the situation of patients with cancer. Using a series of murine *spontaneous* tumors, Hewitt concluded that it was not possible to immunize against these tumors.[14] This observation had crucial importance in shaping subsequent studies. The possibility that the experimental work done with high immunogenic transplantable tumors had little relevance to human tumors was a dark shadow that hindered the progress of tumor immunology. Later, more careful use of these spontaneous tumors and more refined immunization techniques showed that Hewitt's conclusions were wrong.[15]

Boon led the genetic and molecular identification of a large series of TAA. Initially his studies were performed using conventional transplantable mouse tumors.[16] Then, tumor antigens were detected on the same spontanous tumors that had previously been classified as non-immunogenic by Hewitt.[15] Now many antigens associated with human tumors have been identified.[17,18]

The targets

Boon and others[18,19] provided an unambiguous definition of TAA, an important finding that definitively laid to rest the doubts on the foundations of tumor immunology in man.[20] In many cases TAA are peptides presented by class I and class II glycoproteins of the major histocompatibility complex (MHC). Things that may give rise to these tumor-associated peptides are enhanced or diminished expression of some normal proteins and the new expression of altered or normally repressed molecules. Less frequently these antigens are tumor-specific as they derive from mutated proteins. Lastly, various TAA are shared by tumors with distinct histology and origin (Table 1). Telomerase catalytic subunit looks like another widely expressed TAA recognized by T-lymphocytes. It is markedly activated in most human tumors while it is silent in normal tissues.[21]

Why?

The central tenet of antitumor vaccination is that the immune system is able to destroy tumor cells and to retain a long-lasting memory provided that TAA are first efficiently recognized. While the studies aimed at the definition of TAA progressed quickly, investigations of lymphocyte receptors and their idiotype network, co-stimulatory molecules, and cytokines were leading to a more exact description of the requirements for the induction of an immune response.[22] Finally, technical refinement of genetic engineering is making the development of new cancer vaccines easier .[23] The convergence of these issues is once again placing antitumor vaccination at the cutting edge of biological research. A survey by Science[24] indicates that antitumor vaccination is expected soon to become an established therapeutic option.

When?

Whereas individuals are immunized with microbial vaccines prior to encountering the pathogen, cancer patients have to be immunized when a tumor has been already detected. It is not yet possible to predict which combination of gene mutations will give rise to cancer. Therefore, the common clinical setting is elicitation of an immune response in a tumor-bearing patient, rather than prior to tumor development. The very concept of vaccine is somewhat distorted since it has moved from being preventive to being therapeutic.[23]

The kind of patients who should be considered eligible for tumor vaccination is not a minor issue. In many trials patients with advanced diseases are enrolled both for compassionate reasons and because of the constraints imposed by ethical considerations. But, do experimental data suggest that vaccination could be effective in advanced stages of neoplastic progression? The experimental data provide an unambiguous picture of the potentials and limits of vaccination. This picture is not, however, generally taken into account. Perhaps unconscious reasons lead to experimental data being assessed with optimistic superficiality.[25] Many experimental

Table 1. Cross-expression of some tumor-associated antigens among histologically different human tumors from distinct organs.

bladder	BAGE	GAGE					
breast	BAGE		MAGE	CEA	p53	ras	MUC-1
colon				CEA	p53	ras	
lung	BAGE			CEA	p53		
melanoma	BAGE	GAGE	MAGE			ras	
pancreas				CEA	p53	ras	MUC-1
sarcomas		GAGE					

studies have shown that an antitumor response can be elicited by new vaccines. This results in strong resistance to a subsequent tumor challenge and inhibition of minimal residual disease remaining after convention therapy. The pitfall hidden in the evaluation of these vaccine-re-challenge experiments is that successful immunization of healthy mice against a subsequent re-challenge with tumor cells does not demonstrate a true therapeutic effect.[26] Examination of more realistic studies of the ability of vaccines to cure existing tumors shows that only a minority of tumor-bearing mice could be cured. Furthermore, the limited therapeutic efficacy of vaccines was lost when they were not given in the first few days after the implantation of tumor cells.[26]

A similar picture is emerging from phase I studies on vaccination of cancer patients. The vaccination is safe, but the results suggest that only a minority of patients (about 10%) display an objective response. The immunologic performance status of these patients is obviously suboptimal for this type of therapy. Even so, one would have expected a greater number of responders to support the promise of new sophisticated vaccines.[23]

Therapeutic vaccination has not had much success in the management of infectious diseases. Its use against the progression of an established tumor is very challenging, since it must secure an effective immune response capable of getting the better of a well-established, proliferating tumor. Of the several objectives that have been made approachable by antitumor vaccines, the cure of advanced tumors is both the most difficult and at the same time the most common in clinical trials. The strong emotions kindled by cancer suffering provide the main justification for these attempts.[27] Perhaps, major improvements in antitumor vaccination will make this goal approachable. However, the data reviewed show that this is far from the present reality.

Nevertheless it should be considered that most tumor lethality depends on a few neoplastic cells remaining after surgical excision of a tumor mass or after having escaped direct killing by chemo- and radiotherapy. Many experimental findings[28-30] suggest that a stage of minimal residual disease is one in which it is possible to foresee a significant cure by immunization. After successful conventional management the tumor burden may be low, and the tumor may reappear after a long dormancy. This is a realistic setting in which vaccination could lead to the induction of anti-tumor immunity capable of extending the survival of patients. As there are grounds for believing that antitumor vaccines could be used as an effective anticancer tool, the purpose of this review is to describe the types of vaccines that are being experimented, emerging clinical results and the new perspectives opened by this scientific endeavor.

Common tools

Cytokines and cellular signals

The immunologic attempt of the immune system to prevent the development of a neoplastic disease may be ineffective due to either a lack of immunogenicity of tumor cells, or to a weak reaction unable to contrast the neoplastic proliferation. In both cases, it is likely that most of the physiologic mechanisms of priming of the immunologic effector cells may be impaired or absent. In fact, initiation of immune responses requires that professional antigen-presenting cells (APC) deliver a first signal to T-lymphocytes through the binding of the T-cell receptor by the peptide enclosed in the HLA molecule, that is responsible for the specificity of the immune response, and a second or co-stimulatory signal that is not antigen-specific but it is required for T-cell activation[31,32] mainly through CD80 (B7-1) and CD86 (B7-2) binding to CD28 receptor, or the CD40:CD40L pathway. Moreover, the capacity of dendritic cells (DC) to activate natural killer (NK) cells by ligation of the CD40 molecule with its counter-receptor has recently been demonstrated.[33,34] Immunocompetent cells may also determine the type of immune response by the expression of chemokines and by the release of pro-inflammatory, or anti-inflammatory cytokines which drive T-cells to different activities or even to suppression.[35]

Therefore, given the complex network of regulatory signals by professional APC and naive and memory lymphocytes occurring in antigen-specific immune responses, it is not surprising that tumor cells may fail to induce efficient humoral and cellular immune reactions even when a well known TAA is present. In this review, several strategies to overcome the immune escape mechanisms of tumor cells will be considered, such as the direct use of TAA to elicit specific reactions, the use of dendritic cells to present TAA in order to enhance the immune response, and the use of tumor cells genetically modified to function as professional APC or to release soluble factors. Animal models have been widely used for many years to demonstrate the effect of different cytokines, added to or secreted by tumor cell-based vaccines, in increasing the *in vitro* and *in vivo* cytotoxicity against tumor challenge. The role of the main cytokines involved in activation of humoral and cellular immune responses is represented in Figure 1. On the basis of cytokine functions it has been previously shown in experimental models

that: the number of APC in the site of tumor infiltration can be increased by cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4, which should allow also the differentiation of DC precursors; B- and T-cell responses are potently increased by IL-2, IL-12, or GM-CSF;[36] and in particular T-cell cytotoxicity is enhanced by GM-CSF, IL-2, IL-12, interferon (IFN-γ), tumor necrosis factor (TNF-α), while NK activity is enhanced by IL-12 or FLT 3-L.[36,37] However, different models and different TAA resulted in controversial findings. Furthermore, since these cytokines are likely to be more effective when released within the tumor area, the transduction of cytokine genes into tumor cells and their use as cellular vaccines after irradiation has been tested in animal models and in humans.[38-40]

Initial clinical experiences in patients with advanced melanoma or renal cell carcinoma suggest that tumor cell-based vaccines, either engineered to produce GM-CSF or IL-12 or with exogenous GM-CSF, may facilitate marked infiltration of DC and $CD4^+$ and $CD8^+$ T-lymphocytes into tumor lesions, potentially improving the antitumor effect. These data provided evidence of the feasibility of the approach but were unlikely to be able to address the point of efficacy, due to the large tumor burden of these patients. Future immunotherapy attempts should, in fact, focus on the possibility of eradicating minimal residual disease. More recent data demonstrated the role of GM-CSF as a useful adjuvant in peptide-based vaccines in ovarian and breast cancer and in follicular lymphoma, as will be described later in this review.

Figure 1 also shows that cell-to-cell contact via CD40:CD40L plays a pivotal role in activating specific T-cell, B-cell and NK-cell responses. On the other hand, T-cell tolerance can be obtained by blockade of CD40L receptor in non-human primates undergoing solid organ allogeneic transplantation, and in mice receiving either allogeneic bone marrow or solid organ transplantation.[41-43]

Recent experiments demonstrated that stimulation, via an activating anti-CD40 antibody, resulted in the activation of host APC and could convert lymphocytes of mice treated with a tolerogenic peptide vaccine into cytotoxic T-cells. Moreover, this stimulation induced the regression of established tumors that had not been affected by previous vaccination alone,[44,45] thus showing that triggering the CD40 molecule may both overcome T-cell tolerance in a tumor-bearing animal and greatly potentiate a peptide-based vaccine. Moreover, gene transfer by an adenovirus vector of CD40L in human B-cell chronic lymphocytic leukemia (B-CLL) allowed the activation of bystander non-infected B-CLL cells that upregulated co-stimulatory molecules such as CD80 and CD86 and stimulated autologous cytotoxic T-cells.[46]

Another important issue concerns the way T-

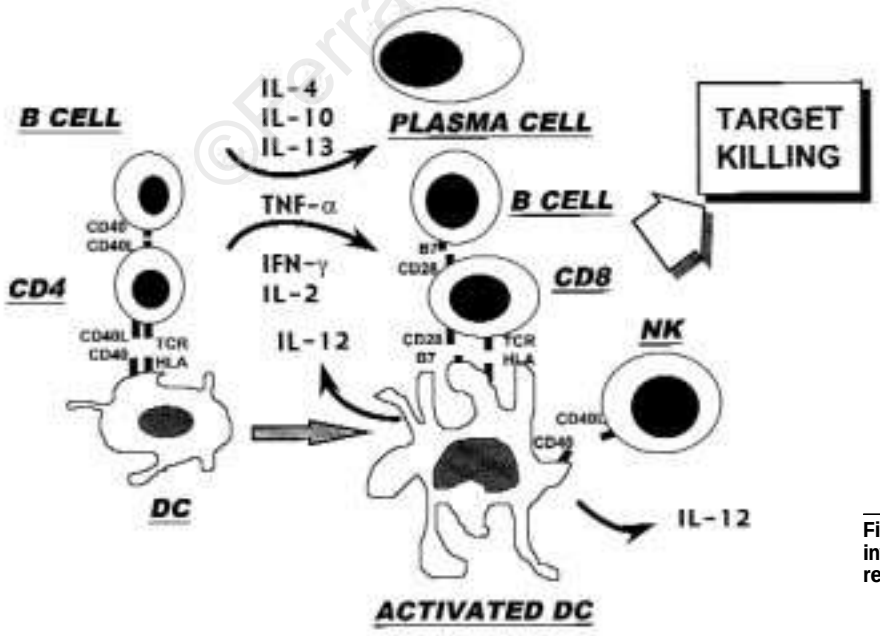

Figure 1. Principal cytokines involved in the antitumor immune response.

cells are turned off by CTLA-4 receptor following activation via CD28. In fact, both CD28 and CTLA-4 bind with high affinity to CD80 and CD86 and CTLA-4 physiologically blocks T-cell activation. In the case of antitumor T-cell response it has been demonstrated that blockade of negative regulatory signals by an anti-CTLA-4 monoclonal antibody may retard tumor growth in experimental systems.[47,48] More recent studies in mice suggested that this molecule was extremely efficient in causing tumor regression when used in combination with subtherapeutic doses of melphalan, or with a GM-CSF-expressing tumor.[49,50]

Altogether, these studies strongly support the role of cytokines or immunomodulatory molecules in anticancer vaccine strategies. However, they do not clarify whether a strict T-helper (Th1) response is required to achieve tumor killing, or whether a humoral response induced by anti-inflammatory cytokines should also be pursued. Finally, future directions of anticancer vaccine research are likely to deal with monoclonal antibodies enhancing or blocking specific receptors.

Dendritic cells as initiators of immune response

Dendritic cells (DC) represent a heterogeneous population of leukocytes defined by morphologic, phenotypic and functional criteria which distinguish them from monocytes and macrophages.[32] From among the professional APC, DC are the most potent stimulators of T-cell responses and play a crucial role in the initiation of primary immune responses.[32]

The DC system comprises at least three distinct subsets, including two within the myeloid or non-lymphoid lineage, and a third represented by lymphoid DC.[32,51] There is also a continuum of differentiation within each of these subsets, from precursors circulating through blood and lymphatics, to immature DC resident in peripheral tissues, to mature or maturing forms in the thymus and secondary lymphoid organs. Recent studies have focused on the different roles of lymphoid and myeloid DC: more resident lymphoid DC induce tolerance to self, whereas migratory myeloid DC, including Langherans cells, are activated by foreign antigens in the periphery and move to lymphoid organs to initiate an immune response.[32]

DC have always been described as having two distinct functional stages: 1) immature, with high antigen uptake and processing ability, and poor T-cell stimulatory function; 2) mature, with high stimulatory function and poor antigen uptake and processing ability. Bacterial products such as lipopolysaccharides, and inflammatory cytokines such as IL-1, TNF-α, type I interferons (IFN α or β) and prostaglandin E_2 (PGE_2) stimulate DC maturation, whereas IL-10 inhibits it.[52] Interestingly, human and murine DC upregulate the synthesis of HLA class I and II molecules, and B7-1, B7-2 and CD40 molecules, after ingestion of bacteria or bacterial products, such as lipopolysaccharides, can prime naive T-cells.

An emerging concept is that APC activate T-helper (Th) cells not only with antigen and co-stimulatory signals, but also with a polarizing signal (signal 3). This signal can be mediated by many APC-derived factors, but IL-12 and PGE_2 seem to be of major importance. As for Th cells, APC can be functionally polarized. *In vitro* experiments with monocyte-derived DC showed that the presence of IFN-γ during activation of immature DC induces mature DC with the ability to produce large quantities of IL-12 and, consequently, a Th1-driving capacity (APC1 or DC1). In contrast, PGE_2 primes for a low IL-12 production ability and Th2-driving capacity (APC2 or DC2).[32,53] DC-stimulated $CD4^+$ cells upregulate CD40L/ CD154 that reciprocally activates DC via CD40. This renders DC more potent stimulators of $CD8^+$ cytotoxic T-cell (CTL).[54] This novel concept is in contrast to simultaneous stimulation of $CD4^+$ and $CD8^+$ T-cells by DC, whereby the $CD4^+$ T-cells secrete helper lymphokines in support of $CD8^+$ CTL development.[55] Together with CD40 L and CD40, two different groups have discovered another paired member of the TNF-TNF receptor family. This factor, termed TNF-related activation induced cytokine (TRANCE) or receptor activator of NF-kB ligand (RANK-L), is expressed by T-cells.[56] Its corresponding receptor, receptor activator of NF-kB (RANK7) or TRANCE R, is expressed by mature DCs but not on freshly isolated B-cells, macrophages, or T-cells.[57] Ligation to this receptor causes either activation of T-lymphocytes or enhancement of DC survival. In addition, IL-12 is a critical mediator of DC-supported differentiation of naive, but not memory, B-cells,[58] indicating that direct interactions occur between DC and B-cells, apart from those that occur via cognate $CD4^+$ T-cell help.

Lastly, it should be mentioned that the primary and secondary B cell follicles contain another population of DC, the follicular DC (FDC). The origin of these cells is not clear, and most investigators believe that they are not leukocytes. FDC trap and retain intact native antigen as immune complexes for long periods of time, present it to B-cells and are likely to be involved in the affinity maturation of antibodies, the generation of immune memory and the maintenance of humoral immune responses.[59]

In conclusion, there is a general agreement in

considering DC as very important players in the game of immune responses against foreign antigens either of infectious agents or of neoplastic cells. Many developing immunotherapeutic strategies against *danger* antigens are aimed at exploiting the powerful antigen-presenting properties of DC by an *in vivo* or *ex vivo* engineering of the DC system. In fact, subcutaneous or intramuscular injection of antigens relies on the local recruitment and activation of DC to capture and present antigens to the immune system. Although the techniques for targeting tumor antigens to DC *in situ* might eventually obviate the need for *ex vivo* manipulation of DC, novel methods for *ex vivo* generation and activation of large amounts of human DC have been developed.

Antitumor vaccination: types and formulations

Killing for priming and killing to destroy

The way cell vaccines die when injected *in vivo* influences DC loading. The initial activity of cytokines transduced into the cell vaccine is to select the leukocyte type recruited and stimulated at the site of injection. Tumor cells are killed by effectors of the innate response; NK and polymorphonuclear cells also produce secondary cytokines that set up a local inflammation recruiting DC, whereas T-cell response is activated later. Of note, gene engineering allows the manipulation of the first phase of the process through the choice of cytokine and/or co-factor and by deciding the level of cytokine to be produced. Once the infiltrate leukocytes are activated, their response to the triggering cytokine is physiologic and independent. They produce other cytokines thus amplifying the magnitude and the complexity of response. The initial stage is finalized to T- and B-cell activation, killing of cell vaccine is to provide the antigen(s) and the inflammatory response should provide the right environment for such activation.[60]

The final stage is aimed at the destruction of existing tumor. Specific immune response is often insufficient to fight solid tumor nodules. Among the possible causes, immunosuppression, low effector-target ratio, MHC downregulation on tumor cells are the most common. Animal studies have shown that these problems can be overcome by general inflammation associated with neutrophil influx. This combination may destroy the tumor-associated blood vessels[61] in a way that may resemble tissue damage in vasculitis. In this setting a specific immune response is not directly responsible for tumor elimination but should be strong enough to at least begin and direct the inflammatory response to the tumor site. In this perspective, tumor vessels are the main target of a non-specific immune response, their functional impairment increasing the effect of either T- or B-cell-mediated specific immune responses.

An add to DC-common link?

Although DC have been indicated as central in alerting and activating the immune system, it is now clear that certain peptides are not and can not be presented by mature DC. This observation, made by Van den Eynde and colleagues,[62] concerns autoantigens and T-lymphocytes that recognize them without being deleted in the thymus and normally without provoking autoimmunity. In fact, APC differ from other cell types by the proteosome that digests protein into immunogenic peptides to fit the MHC groove. APC immunopreoteosomes have three catalytic subunits substituted by those induced by IFN-γ, thus generating slightly different peptides from those generated by non-APC cells.

Several self-antigens identified as tumor-associated because of CTL recognition may not be processed by immunoproteosome. The implication is that such CTL were not generated through DC presentation or at least not through DC processing unless this happened during transition from immature to mature DC.[63] Perhaps free peptides can be captured on the surface of DC for presentation, or perhaps other as yet unknown mechanisms are involved.

Genetically modified tumor cell vaccines

Old and recent discoveries confirm the possibility of a cancer vaccine made of tumor cells.

An empirical approach, such as the use of allogeneic whole-cell vaccine composed of 3 allogeneic melanoma cell lines established *in vitro*, allowed a 3-fold increase of the five-year survival of patients with stage IV melanoma as reported by Morton *et al*.[64] The most active component of Morton's vaccine has been identified by Livingston and colleagues[65] to be a ganglioside (GM2). Patients who develop antibody response to ganglioside showed a significant survival advantage. Whether a CTL response was also activated has not been investigated but does probably exist. However, this finding prompted a phase III study in patients with stage III disease.

In the autologous setting, irradiated melanoma cells were modified with dinitrophenyl and used to treat patients with metastatic disease. Clinical evidence of inflammatory response to superficial metastases was reported. The same treatment administered to phase III patients who remain tumor-free after resection of lymph node metastases has resulted in 50 and 60% 4-year relapse-free and overall survival, respectively.[66] Immunostaining, TCR repertoire analysis and functional

data of node-metastases post-vaccination have shown that treatment with autologous dinitrophenyl-modified melanoma cells can expand certain T-cell clones at the tumor site.[67]

The above results bring up two issues: autologous versus allogeneic tumor cells and chemical or genetic (see below) modifications of tumor cells to be used as cancer vaccines.

Before discussing these issues we should, however, address a more basic question, that is how to use cancer cells as vaccine. Well before identification of tumor antigens, their existence was inferred in melanoma on the basis of expansion and characterization of cytotoxic T-lymphocytes recognizing autologous tumor,[68] whereas antibodies against a variety of tumor types were isolated from patient sera. Antigens recognized by CTL can be tumor-specific and even restricted to the autologous tumor or cross-react among different neoplasms of the same or different tissue origin depending on the mechanism generating such an antigen: point mutation, incorrect splicing, over-expression, translocation and other (see Table 2). The number of tumor antigens is always increasing thus suggesting that our knowledge of the antigenic repertoire of tumor cells is partial. In addition, which antigen among those already characterized should be used in a vaccine?

These problems can be solved altogether by using tumor cells that would represent the entire antigenic repertoire with a single drawback, that is immunoselection of certain antigens. Tumor cells from different patients may undergo different processes of immunoselection and therefore a pool of these tumors would be ideal for preparing an allogeneic vaccine. The finding that antigens belonging to allogeneic cells are processed by host antigen-presenting cells,[69] so that they can be recognized by host T-lymphocytes (a phenomenon called *cross-priming*), removes any conceptual obstacles to the use of allogeneic cells. Allovaccines have several advantages over autologous vaccines: cell lines that are extensively characterized *in vitro* can be used to treat all the patients included in a clinical study. Genetic modifications of tumor cells have been widely studied as a way to increase immunogenicity. These modifications can be easily made to a cell line, thus avoiding the need to isolate cells from every tumor. The rate of success in culturing tumor cells from a primary tumor varies according to the type of tumor and experience of the operator. Transduced cell lines can be selected for production of a certain amount of transgene thus assuring that the same vaccine will be given to all patients. Which modification is most efficient in inducing tumor immunity has not been unequivocally determined since variability among different tumors has been described.

Table 2a. Tumor-associated antigens recognized in class I HLA restriction.

Antigen defined	*Type of antigen and distribution*	*Type of tumor*	*HLA-restriction allele*
gp 100	Melanocyte diff.	Melanoma	A2, A3, A24, Cw8
Melanocortin receptor 1	Melanocyte diff.	Melanoma	
MART-1/MelanA	Melanocyte diff.	Melanoma	A2, B45
Tyrosinase	Melanocyte diff.	Melanoma	A1, A2, A24, B44
TRP-1 (gp 75)	Melanocyte diff.	Melanoma	A31
TRP-2	Melanocyte diff.	Melanoma	A2, A31, Cw8
p15	Widely expressed	Melanoma	A24
SART-1	Widely expressed	Lung carcinoma	A2601
PRAME	Widely expressed	Melanoma, renal	A24
NAG-V*	Melanoma sheared	Melanoma	A2.1
β catenin	Unique tumor specific	Melanoma	A24
CDK4-Kinase	Unique tumor specific	Melanoma	A2
MUM-1	Unique tumor specific	Melanoma	B44
TRP2/INT2	Unique tumor specific	Melanoma	A6801, Cw8
CASPASE-8	Unique tumor specific	Head/neck cancer	B35
HLA-A*201 mutated	Unique tumor specific	Renal cancer	A2.1
KIAA0205	Unique tumor specific	Bladder cancer	B44*03
BAGE	Cancer/ testis	Melanoma	Cw 1601
GAGE-1/2	Cancer/ testis	Melanoma	Cw 6
MAGE-1	Cancer/ testis	Melanoma	A1, Cw16
MAGE-3	Cancer/ testis	Melanoma	A1, A2, B44
RAGE	Cancer/ testis	Renal cancer	B7
NY-ESO-1	Cancer/ testis	Melanoma, ovarian, esophageal cancer	A2
K-RAS-D13 mutated	Shared tumor-specific	Colon cancer	A2.1
p53 mutated	Shared tumor-specific	Colon and lung	A2.1
Bcr/abl	Shared tumor-specific	CML	A2.1, A3, A11, B8
MUC-1	Shared tumor-specific	Breast, colon, pancreatic cancer	A11
HPV16E7	Viral related	Cervical cancer	A2.1

Table 2b. Human tumor antigens recognized by HLA class II-restricted CD4+ T cells.

Antigen	Tissue distribution	Class II restriction
Tyrosinase	Melanoma/melanocytes	DRb1*0401
Tyrosinase	Melanoma/melanocytes	DRb1*1501
Triosephosphate isomerase mutated form	Melanoma, unique	DRb1*0101
MAGE-3	Melanoma and other tumors, testis	DRb1*1301
MAGE-1, -2 or -6	Melanoma and other tumors, testis	DRb1*1301
MAGE-3	Melanoma and other tumors, testis	DRb1*1101
RAS-D12 mutated	Colon and pancreatic cancer	DR1
HER-2/neu	Breast and ovarian cancer	DR11
PML/RARα	Acute promyelocytic leukemia	DR2
Bcr/abl	Chronic myeloid leukemia	DR1, DR4, DR11
CDC27	Melanoma	DR4

**N-acetylglucosaminyl transferaseV. CML: chronic myeloid leukemia.*

Chemical modification, also called haptenization, aims at adding helper determinant to tumor cells,[70] although the exact mechanism and the downstream pathways activated in this way are not clearly understood. Genetic modification is now preferred since the effect of several cytokines and co-stimulatory molecules are known at molecular level. Their genes can be

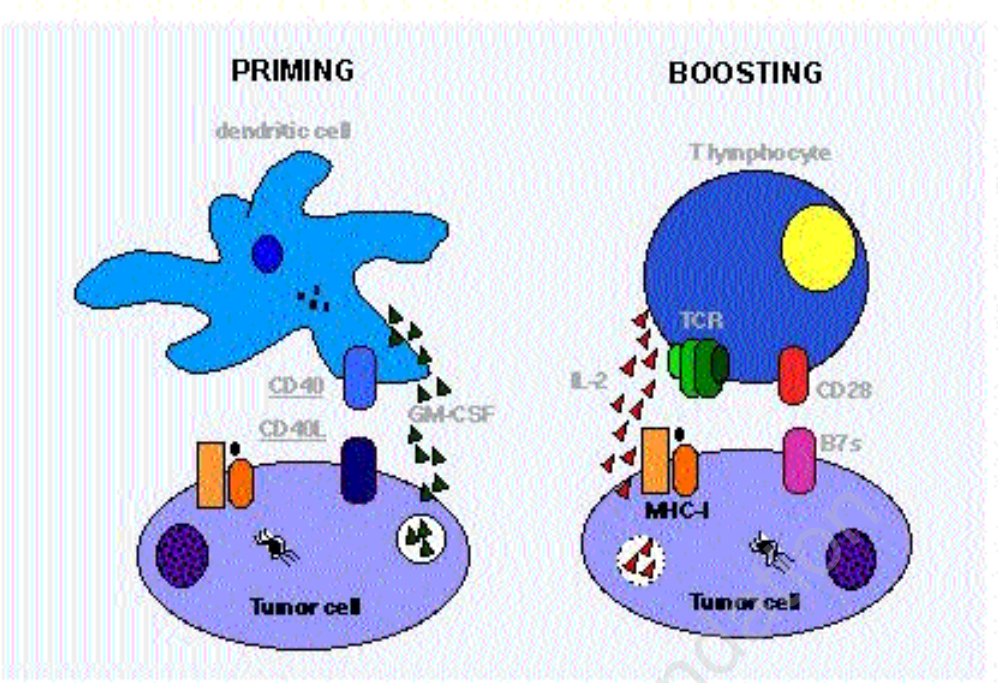

Figure 2. Priming of DC and boosting of T-cell responses by transduced tumor cells as vaccine.

transduced into tumor cells that acquire new immunoregulatory functions. In this way a desired immune response can be fine-tuned through gene dosage, recruitment of certain cell types, deflection of Th1 or Th2 type of response and several others mechanisms.[60]

To summarize the most recent and promising approaches we may consider two strategies aimed at favoring vaccine interaction with DC or directly with T-lymphocytes. The two approaches can be viewed for priming or boosting (Figure 2). A combination of cytokine and co-stimulatory factors is likely to be synergistic as generally occurs when soluble and cell-contact signals are given together. Tumor cells transduced with both GM-CSF and CD40L have been shown to be heavily infiltrated by DC. GM-CSF induces proliferation and maturation of hematopoietic cells, and has been shown to stimulate DC accessory properties and enhance the immune response initiated by these cells.[71]

The CD40/CD40L interaction plays a critical role in cell-mediated immunity, and in proliferation and activation of APC, as shown for B-cells, monocytes and, more recently, DC.[72] Ligation of CD40 on monocytes and DC results in the secretion of several cytokines, including IL-1, IL-6, IL-12, and TNF-α. The murine tumor transduced with both GM-CSF and CD40L genes showed that tumor infiltrating DC can take up cellular antigen from the tumor and can present it to T-lymphocytes *in vitro*.[73] In this setting, DC bridges cell vaccine-T lymphocytes interaction and can be envisaged as the way to prime patients against weaker antigens otherwise ignored by the immune system. A complementary approach would consider the possibility of boosting the primed or the existing immune response. In this case DC that re-encounter activated T-cells can be lysed and may not be appropriate for boosting. Boosting can be done using tumor cells transduced with IL-2 and B7-1 such as to provide both cell expansion and co-stimulation from the tumor cell vaccine directly to T-lymphocytes (Figure 2). The way cellular antigen can be captured by DC for priming of T-lymphocytes depends on how tumor cells die after injection. Irradiation of the cell vaccine induces apoptotic cell death and apoptotic bodies can be captured by DC.[74] Others suggest that peptides of cellular protein complexed to heat shock protein (HSP), the natural *chaperon* of peptide from proteosome to membrane-associated TAP, leave the cells upon necrosis to be taken up by DC.[75,76] DC loading must be followed by DC maturation and migration to the lymph node to ensure correct antigen presentation.[32]

The way cytokines and co-signals modulate

vaccine-host interactions may determine the extent and the efficacy of treatment. Systemic activation of the immune response (T-cell cytotoxicity, antibodies) is easy to measure but can not be used as a read-out system to predict whether the tumor will be rejected. Tumor nodules might be reached by circulating lymphocytes which, however, may be neutralized because of either immunosuppression or peripheral tolerance. The former is likely dependent on tumor size and is less expected when a small tumor, minimal residual disease or prevention of recurrence is the target to be treated. The latter could be surmounted by appropriate co-signals, for example CD40L.[45]

Soluble proteins as immunogens

Soluble proteins derived from autologous cancer cells are not as immunogenic as proteins derived from infectious agents. The degree of foreign-ness, which depends on the reciprocal distribution between epitopes subject and not subject to self-tolerance, is low in TAA. Moreover, TAA do not have the particulate nature of infectious agents that is a key factor in increasing their immunogenicity. Finally, infectious agents are rich in molecules that are able to raise immune responsiveness without being themselves immunogenic. A significant effort has been made over the last few years to translate the immunogenic properties of infectious agents into cancer vaccines for clinical use. The most suitable TAA should be directly involved in the malignant behavior of tumor cells; contain multiple immunodominant B cell and T cell epitopes, including both helper and cytotoxic T-cell epitopes; contain a very high degree of foreignness; be unprotected by self-tolerance mechanisms. Of course, HLA haplotype remains a major constraint in determining whether TAA-derived immunodominant peptides can elicit tumor-specific immune responses in a given individual (see also below).

Building up immunogenicity of soluble proteins

Even the most suitable TAA is not immunogenic unless it is processed and presented by professional APC to the host immune system. To this aim, TAA should interact with APC directly. The route of administration and adjuvants are key factors in determining the final outcome of immunization. Intravenous injection of soluble antigens induces tolerance, whereas subcutaneous or intramuscular injection of the same antigens results in immunity because of the interaction with epidermal Langerhans cells or dermal dendritic cells. Accordingly, delivery of antigens via mucosal surfaces may result in immunity because antigens may interact with the numerous DC located just beneath the epithelium of mucosal lymphoid organs. The association between soluble antigens and APC is made much more intense by adjuvants. Adjuvants act via different principles and pathways, but the common goals are to prolong the interaction with APC by promoting a slow antigen release, and functionally activate APC themselves by delivering danger signals. Cytokines have also emerged as potent immunoadjuvants since they can influence the immune responses at different levels (see above).

Particulation of soluble proteins

Precipitation with aluminium hydroxide or aluminium phosphate has been used to particulate antigens in diphtheria, tetanus, hepatitis B, and other vaccines. These types of vaccines induce antibody formation, but very little delayed cutaneous hypersensitivity (DTH) or cell-mediated cytotoxicity. In a pilot study, five stage I-III patients with multiple myeloma were immunized with autologous idiotype (Id) precipitated in aluminium phosphate suspension. Three patients developed idiotype-specific T- and B- cell responses, but these responses were transient and their amplitude was low.[77]

The use of immunostaining complexes (ISCOMs) is another strategy that has been used to particulate antigens. ISCOMs are cagelike structures made of cholesterol, saponin, phospholipid, and viral envelope proteins to which other proteins can be associated. Saponin is a plant derivative that is critical to the efficacy of ISCOMs. QS21 is the most effective fraction of saponin and is currently under clinical investigation in several trials.[78] ISCOMs may reach the endocytic pathway and induce DTH and CTL responses other than antibody production. Liposomes, virosomes, and proteasomes are alternative strategies to ISCOMS. Experimental data have recently shown in the 38C13 mouse B-cell tumor that liposomal formulation of autologous Id converts this weak self-antigen into a potent tumor rejection antigen.[79]

Promoting slow release of soluble antigens

A slow release of antigen is one of the major goals of adjuvants. Antigen polymerization and emulsifying agents have been put together to achieve this goal. Polymerization can be obtained by association with non-ionic block polymers or by association with carbohydrate polymers. Non-ionic block polymers have been used as components of water-in-oil or oil-in-water emulsions. SAF-1 (Syntex Adjuvant Formulation-1) is an oil-in-water adjuvant formulation containing non-ionic block polymers, squalene, and Tween 80. SAF-1 has been used by Kwak *et al.* in their pioneering study on idiotype vaccination in follicular lymphoma

patients.[80] Chemical immunomodulators such as derivatives of muramyl dipeptide, the smallest subunit of the mycobacterial cell wall that retains immunoadjuvant activity, can be added to oil-in-water adjuvants.[81] This approach was used in the study by Hsu *et al*. in which idiotype/KLH conjugates were delivered to follicular lymphoma patients after mixing with SAF-165 that contained muramyl dipeptide as immunomodulator.[82] Lipid components of bacteria have also been used as adjuvants in experimental models. These are portions of the LPS endotoxins of Gram-negative bacteria. These molecules are, however, too toxic and chemically modified derivatives have been developed for clinical use. So far, monophosphoryl lipid A is the least toxic derivative capable of promoting cell-mediated immunity.[83]

Polysaccharide polymers have also been used as a sustained-release vehicle for TAA. Poly-N-acetyl glucosamine is a highly purified, biocompatible polysaccharide matrix that has recently become available for this purpose.[84]

Peptide vaccines

T-lymphocytes recognize small peptides that represent the degradation products of a complex intracellular process and are presented on the cell surface complexed to 1 of 2 types of histocompatibility leukocyte antigen molecules (HLA classes I or II). CTLs ($CD8^+$) mainly recognize peptides of 8 to 10 amino acids derived from intracellular or endogenous proteins and complexed to HLA class I molecules.[85-89] $CD4^+$ T-lymphocytes recognize exogenous proteins which are ingested by APC, degraded to peptides of 12-24 amino acids and complexed to HLA class II molecules.[90,91]

Class I and Class II peptides that are presented on the cell surface, although randomly derived from the original protein, must contain specific amino acids in 1 or 2 critical positions in order to be able to bind the appropriate HLA molecules. Thus, peptide binding is HLA-restricted. The amino acid motifs responsible for the specific peptide-binding to HLA class I and class II molecules have been determined for the common HLA types by analyzing acid-eluted naturally processed peptides and by using cell lines defective in intracellular peptide loading and processing.[92-94]

Several tumor-specific and some leukemia-specific peptides have so far been identified, and studies aimed at evaluating the potential clinical benefit of peptide vaccines in cancer patients have begun. Among the reasons that make a peptide vaccine strategy interesting are several unique advantages that peptide immunization offers over other vaccine approaches: 1) peptide vaccines permit specific targeting of the immune response against 1 or 2 unique antigens (thus limiting the potential autoimmune cross-reactivity or immunosuppressive activity often observed with more complex immunogens); 2) emerging technology has made it simple, rapid and inexpensive to sequence and prepare larger quantities of tumor antigen peptides for both laboratory and clinical use; 3) use of synthetic peptides greatly reduces the possible risk of bacterial or viral contamination that might derive from autologous or allogeneic tissue for immunization. On the down side, the main disadvantages of peptide immunization are: 1) lack of universal applicability as each peptide is restricted to a single HLA molecule; 2) poor immunogenicity of most native peptides; 3) risk of inducing antigenic tolerance. Successful attempts to enhance HLA binding affinity have been based on synthetically generating peptides with amino acid deletions or substitutions while maintaining antigen specificity.[95,96] In initial studies, synthetic substituted peptides appeared to enhance immunogenicity and also to overcome the host immune tolerance that exists to native peptides.[97,98] Conversely it has been reported that changes in the fine specificity of modified peptide-reactive T-cells following vaccination may occur with subsequent loss of tumor cell recognition.[99]

A peptide vaccine approach involves several steps which are aimed firstly at identifying the appropriate peptide, secondly at checking for its immunogenicity and relevance as a tumor-associated antigen (TAA) *in vitro*, and thirdly at formulating a safe product to be used clinically.

Identification of the appropriate peptide

1) From protein to peptide. This approach involves the screening of potentially HLA-binding peptides within the sequence of a known tumor-specific protein by using HLA anchor motifs and epitope selection. Peptides derived by mut RAS,[100] melanoma-associated MAGE protein,[101] prostate specific antigen[102] and chronic myelogenous leukemia (CML) specific P210 were identified by this approach.[103,104] In CML, for example, a possible total of 76 peptides, 8 to 11 amino acids in length, spanning the b2a2 and b3a2 junctional regions of bcr-abl were screened for HLA class I-binding motifs and tested for the effective binding property to purified HLA molecules. Four of them, all derived from the b3a2 breakpoint, were found to be able to bind with either intermediate or high affinity to purified HLA A3, A11 and B8.[103] A similar approach allowed identification of class II b3a2 breakpoint peptides capable of binding HLA DR11,[105] DR4[106] and DR1[107] This method for identifying tumor-specific peptides is relatively simple, fast and suitable for any known intracellular protein that may be a potential TAA. Never-

theless, it bears the disadvantage that it cannot, by itself, predict whether the identified peptide is found on HLA molecules of the leukemia or cancer cells that contain the parent protein.

2) From peptide to protein. Another strategy used to identify suitable peptides for cancer vaccines involves the structural analysis of naturally processed peptides (NPPs) bound to HLA class I and class II molecules of cancer cells. NPPs were first isolated and sequenced by acid-elution from immunoaffinity purified HLA molecules and subsequently compared with existing protein sequences.[108] Alternative approaches are to obtain NPPs by mechanically destroying and acid-treating whole tumor cells and/or by exposing living tumor cells to rapid acid treatment.[109] These procedures should characterize tumor-, differentiation stage- and tissue-specific self-antigen MHC-bound peptides as well as the naturally processed proteins from which they are derived and use them as tools for immunotherapy. The main disadvantage of this approach is that many tumor cells express low levels of HLA molecules and the yield of NPPs can be scarce. Nevertheless some immunogenic peptides derived from wild-type p53 protein, melanoma associated MART-1 and gp100 proteins were identified by these methods,[110,111] and naturally processed peptides from acute myeloblastic leukemia cells and CML blasts are now under evaluation.[112,113] Advantages and disadvantages of synthetic versus natural tumor peptides have been recently reviewed.[114]

3) *From tumor infiltrating lymphocytes to peptide.* Probably the most clinically relevant tumor peptides are those identified from the epitope analysis of tumor infiltrating lymphocytes (TIL).[115-118] In preliminary experiments HLA class I restricted CTL lines were derived by repetitive *in vitro* stimulation of TIL with autologous tumor cells. Subsequently, by transfection of a tumor cDNA library and *in vitro* sensitization assays, the peptide sequences recognized by the tumor-specific CTLs were identified as were the parent proteins (i.e. peptides derived from melanoma associated gp100, tyrosinase and the MAGE family). Most TIL-derived tumor peptides found in the past few years are MHC class I-restricted; however, a novel melanoma antigen resulting from a chromosomal rearrangement and recognized by a HLA-DR1-restricted CD4+-TIL has recently been identified.[119]

Checking for peptide immunogenicity

Except for TIL-derived peptides, all other tumor-specific, HLA binding, synthetic or naturally expressed peptides still need to be tested for immunogenicity. The ability of inducing CTL or specific CD4+ proliferation has been evaluated for all tumor-specific peptides that were subsequently used in clinical trials. P210-derived peptides, for example, were able to elicit peptide-specific T-cell immunity both in normal donors,[105,120] and CML patients.[121] Their relevance as TAAs has been further confirmed by observing peptide-specific HLA restricted CTLs and CD4+ cells able to mediated killing of b3a2-CML cells and proliferation in the presence of b3a2 containing cell lysates, respectively.[106,107] The latter findings were the indirect proof of *natural processing* of P210 and of HLA presentation of breakpoint-derived peptides. Although strong peptide-specific CTL and CD4+ responses have been shown *in vitro* for most tumor peptides so far identified, few data on T-cell induced immunity after peptide vaccination in patients have been generated.[100,122,123] Thus, strategies to improve peptide immunogenicity, by using different adjuvants and delivery systems, are currently under evaluation.

Peptide vaccine formulation

The goal of experimental clinical protocols using peptide antigens for active vaccination is to induce a strong CTL response against the immunizing antigen and thereby against tumor cells expressing the antigen. The mode of peptide-based cancer vaccination critically affects the clinical outcome. The synthesis of a peptide on a large scale, its purification and testing for common Quality Control/Quality Assurance compliance are simple and fast procedures but the choice of an effective delivery system for the peptide is crucial. Peptide vaccination strategies currently being evaluated include: 1) direct peptide vaccination with immunologic adjuvants and/or cytokines;[124] 2) lipopeptide conjugates;[125] 3) peptide loading onto splenocytes or DC;[126] 4) lysosomal complexes.[127] Recently, a specific formulation of the polysaccharide poly-N-acetyl glucosamine has been found to be an effective vehicle for sustained peptide delivery in a murine vaccine model able to generate a primary CTL response with a minimal peptide dose.[84] Finally, triggering CD40 *in vivo* with an activating antibody considerably improved the efficacy of peptide-based anti-tumor vaccines in mice, converting a peptide with minimal immunogenicity into a strong CTL inducer.[44]

Recombinant viruses

The molecular identification of the antigens on human tumors recognized by T- and B-lymphocytes offers the opportunity to design novel cancer vaccines based on recombinant forms of TAA. Genes coding for TAA can be inserted into the genome of attenuated micro-organisms such as bacteria and viruses. Viruses are among the most interesting vectors since they are able

to induce antibody, Th, and CTL responses in the absence of co-stimulation.[128] Their long-lasting cohabitation with human beings has likely favored the evolution of specific patterns recognized by the innate immune system which create an immunostimulatory environment for optimal immune responses. The vector choice, however, is limited by the possible disadvantages of recombinant virus utilization, such as recombination with wild-type viruses, oncogenic potential, or virus-induced immunosuppression.

Vaccinia virus (VV) belongs to the *Poxviridae* family, and its worldwide use in the smallpox eradication campaign demonstrated that it was safe and very effective. To date, no other large-scale vaccination program has had such an impact on human diseases, because smallpox has been virtually eliminated from the world population. Large amounts of foreign DNA can be stably inserted into the VV genome by homologous recombination.[129] VV employs a built-in transcriptional and post-translational apparatus to produce large amounts of the protein encoded by the inserted gene. VV sojourns within the host cell cytoplasm and does not integrate nor is it oncogenic.[129] The induction of potent cellular and humoral immune responses with recombinant (r)VV was observed in several tumor systems.[130-132] Preclinical studies in models of pulmonary metastatization caused by tumors bearing a prototype TAA have revealed some features of rVV that are important in determining successful therapy. In particular, TAA gene must be expressed under the control of a strong, early promoter which allows its expression in professional APC such as DC.[133] Moreover, while prevention from tumor challenge requires only the synthesis of the TAA by the recombinant virus, genes encoding immunostimulatory molecules inserted in the recombinant poxvirus,[134] exogenous cytokines,[131,135] or their combination[136] are required to induce eradication of established tumors.

Encouraging results have also been obtained in mouse models more relevant to the therapy of human cancer. Immunization of mice transgenic for the human HLA-A*0201 allele with an rVV encoding a form of the melanoma antigen gp100, which had been modified to increase epitope binding to the restricting class I molecule, elicited $CD8^+$ T-lymphocytes specific for the epitope that is naturally presented on the surface of an HLA-A*0201-expressing mouse melanoma.[137] Repeated inoculations of an rVV encoding the mouse tyrosinase-related protein-1 (TRP-1/gp75) caused autoimmune attack of normal melanocytes manifested by hair depigmentation (vitiligo) and $CD4^+$-mediated melanoma destruction in mice.[138] In a clinical trial, administration of VV encoding human carcinoembryonic antigen (CEA) proved effective in inducing both humoral and cellular immune responses in patients with colorectal cancer.[139]

Poxviruses are not the only choice for tumor immunologists. Adenoviruses in which critical genes that enable viral replication have been deleted and replaced by genes encoding heterologous antigens, have been generally used in gene therapy studies, but have also provided antitumor activity when employed as immunogens.[140] Liver toxicity was described following systemic administration of high titers of first generation E1-deleted Adenovirus vectors optimized for gene therapy.[141] These side effects, which certainly raise some concerns about Adenovirus administration in patients, might not restrain their use in cancer immunotherapy since systemic delivery of high viral titers would certainly not be the favored immunization route.

Initial clinical trials have unveiled some of the intrinsic limitations of recombinant viruses. Many patients, in fact, have high neutralizing antibodies against VV as a consequence of its use as a vaccine for smallpox prevention, and against Adenoviruses, which cause upper respiratory tract infections throughout life. High doses of recombinant adenoviruses expressing the human melanoma antigens MART-1 and gp100 could be safely administered to cancer patients, but the high levels of neutralizing antibodies present in their sera likely impair the ability of these viruses to immunize against the melanoma antigens.[142]

These results, largely expected, have not put an end to the clinical use of recombinant viruses, as various strategies have been exploited to overcome pre-existing immunity. Several groups have engineered non-replicating viruses which normally do not infect human beings. The *Avipoxviridae* family comprises viruses, such as fowlpox and canarypox, which can productively infect avian but not mammalian cells, and are not cross-reactive with VV.[143] A recombinant fowlpox virus expressing a model TAA was able to cure established tumors in mice;[144] an important aspect of this study was the observation that prior exposure to VV did not abrogate the immune responses induced by the recombinant fowlpox virus. A different non-replicating virus, canarypox virus (ALVAC), has also been employed to elicit immune responses against a variety of antigens.[145,146]

A highly attenuated strain of VV, known as modified VV Ankara (MVA), has been inoculated as smallpox vaccine into more than 120,000 recipients without causing any significant side effect.[147] Replication of MVA is blocked at the step of virion assembly and for this reason the MVA vectors produce recombinant proteins

expressed under the control of both early and late viral promoters, thus mimicking the expression in wild-type virus. An MVA vector, and a fowlpox virus vector expressing a model TAA showed better therapeutic effects on pulmonary metastasis than a VV encoding the same TAA.[148] MVA is a very promising vector for the development of recombinant vaccines for cancer, and can be efficiently used in combination with DNA vaccines.[149]

As an alternative approach, the mucosal route of administration was recently shown to overcome pre-existing immunity to VV. Intrarectal immunization of vaccinia-immune mice with rVV expressing HIV gp160 induced specific serum antibody and strong HIV-specific CTL responses in both mucosal and systemic lymphoid tissue, whereas systemic immunization was ineffective under these circumstances.[150]

Direct immunization of mice with recombinant adenoviruses resulted in the induction of high titers of neutralizing antibodies, which precluded a boost of CTL responses after repeated inoculations. The presence of neutralizing antibodies did not, however, affect the immunogenicity of infected DC, as repeated administration of virus-infected DC boosted the CTL response even in mice previously infected with the recombinant vector.[151] It was also shown that protective immunity against mouse melanoma "self" antigens, gp100 and TRP-2, could be obtained by DC transduced with Adenovirus vector encoding the antigen. Importantly, immunization with Adenovirus-transduced DC was not impaired in mice that had been pre-immunized with Adenovirus.[152] With the help of these novel strategies, recombinant vectors could be used in the general population, including those individuals previously exposed to the viruses. Gene transfer to different human DC subpopulations by vaccinia and adenovirus vectors is a conceivable strategy for TAA loading.[153]

DNA vaccines

Following the first and somewhat shocking demonstration that the intramuscular injection of *naked* DNA (i.e., DNA devoid of a viral coat) encoding the influenza A nucleoprotein could induce nucleoprotein-specific CTL, and protect mice from challenge with heterologous influenza strains,[154] DNA immunization has become a rapidly developing technology. This vaccination method provides a stable and long-lasting source of antigen, and elicits both antibody- and cell-mediated immune responses. Compared to recombinant viruses, DNA vaccines offer a number of potential advantages because they are cheap, easy to produce, and do not require special storage or handling. DNA vaccines express virtually only the heterologous gene, therefore, they should induce an immune response selective for the antigen and not the vector, thus supplying a source of antigen suitable for repeated boosting. DNA vaccination has proven to be a generally applicable approach to various preclinical animal models of infectious and non-infectious diseases,[155] and several DNA vaccines have now entered phase I/II human clinical trials. Although the clinical application of DNA vaccines is a very young practice, some trials have already demonstrated that is possible to elicit a specific CTL response against malaria and HIV proteins in human volunteers.[156,157]

This novel vaccination approach involves different steps: 1) cloning of a heterologous gene under the control of a viral promoter (ordinarily derived from the CMV immediate early region); 2) purification of the endotoxin-free DNA plasmid from bacteria *factories*; 3) administration of the expression vectors by direct intramuscular or intradermal injection with a hypodermic needle or using a helium-driven, *gene gun* to shoot the skin with DNA-coated gold beads. Heterologous DNA can also be introduced into recombinant Salmonella,[158,159] or Listeria strains[160] that can be thus administered by a mucosal route (Figure 3). In addition to these classic routes of DNA delivery, plasmid-based gene expression vectors have also been admixed with polymers and administered with a needle-free injection device, achieving high and sustained levels of antigen-specific antibodies.[161] The route of DNA delivery can profoundly influence the type of immune response by preferentially activating different Th populations: gene gun bombardment elicits a Th2 response, while intramuscular inoculation induces Th1 activation, even though the antigen form (i.e., membrane-bound vs secreted) can also exert some effect.[162,163]

The immunostimulatory activity of DNA vaccines has been associated with the prokaryotic-derived portion of the plasmid, which contains a central CpG motif in the sequence PuPuCpG-PyPy.[164,165] In their unmethylated form, these hexamers stimulate monocytes and macrophages to produce different cytokines with a Th1 promoting activity including IL-12, TNF-α, and IFN-γ.[166,167] A plasmid that incorporated several CpG islands in the prokaryotic ampicillin-resistance gene induced a stronger immune response when compared to a second plasmid carrying the kanamycin-resistance gene which possesses none.[168] To date, it is not known whether the CpG motifs will have the same immunostimulatory properties when applied to vaccination of human beings. However, it was recently reported that CpG motifs can activate

in vitro subsets of freshly isolated human DC to promote Th1 immune responses.[169]

The mechanism of DNA-induced immunization has not yet been fully elucidated. An exclusive role for the direct transfection of normal tissue cells, such as myocytes or keratinocytes, has been debated because surgical ablation of the injected muscle within 1 minute of DNA inoculation did not affect the magnitude and longevity of DNA-induced antibodies.[170] Moreover, studies with bone-marrow chimeras clearly indicated that bone-marrow-derived APC, either transfected by the DNA plasmid or able to capture the antigen expressed by other transfected cells, were necessary to prime T- and B-lymphocyte responses.[171] Indeed, more recent evidence suggests that Th and CTL are activated by DC directly transfected *in vivo* following DNA immunization.[172,173]

The first applications of DNA immunization to preclinical models of tumor growth revealed some interesting aspects. In general, the potency of naked DNA does not equal that of recombinant viruses, probably because DNA does not undergo a replicative amplification in the transfected cells, which in turn limits the amount of heterologous antigen produced. Inflammatory responses caused by DNA inoculation are more contained than those occurring during infection with viruses; for this reason, repeated inoculations of plasmid DNA, or the use of adjuvants such as cardiotoxin are generally required for the induction of an optimal response. Another emerging issue is that the efficacy of the vaccination approach depends more on the type of antigen than on the route of administration (Table 3). Vaccines based on shared viral antigens, or model TAA artificially introduced in the experimental tumors can be used to induce a strong, and often therapeutic immune response. Using a gene gun for DNA immunization, Irvine *et al.*[174] observed effective treatment of established pulmonary metastases, but recombinant cytokines were necessary to enhance the therapeutic effects. Unlike viral and model TAA, *self* TAA fail to induce *sterilizing* immunity since therapy of established tumors has been rarely reported, and prevention from challenge is often partial.[175-178] Central and peripheral tolerance to *self* antigen has thus emerged as the main limitation to the successful application of DNA vaccines to the therapy of cancer. This conclusion seems to apply to several mouse melanocyte differentiation antigens, a class of molecules that is expressed in both melanomas and melanocytes and includes tyrosinase, TRP-1/gp75, TRP-2, and gp100/pmel 17. However, tolerance can be broken by the use of a xenogeneic source of TAA. While immunization with mouse TRP-1/gp75 or TRP-2 antigens failed to induce a detectable immune response, vaccination with a plasmid DNA encoding the human homologous antigens elicited autoantibodies and CTL in C56BL/6 mice:[178,179] immu-

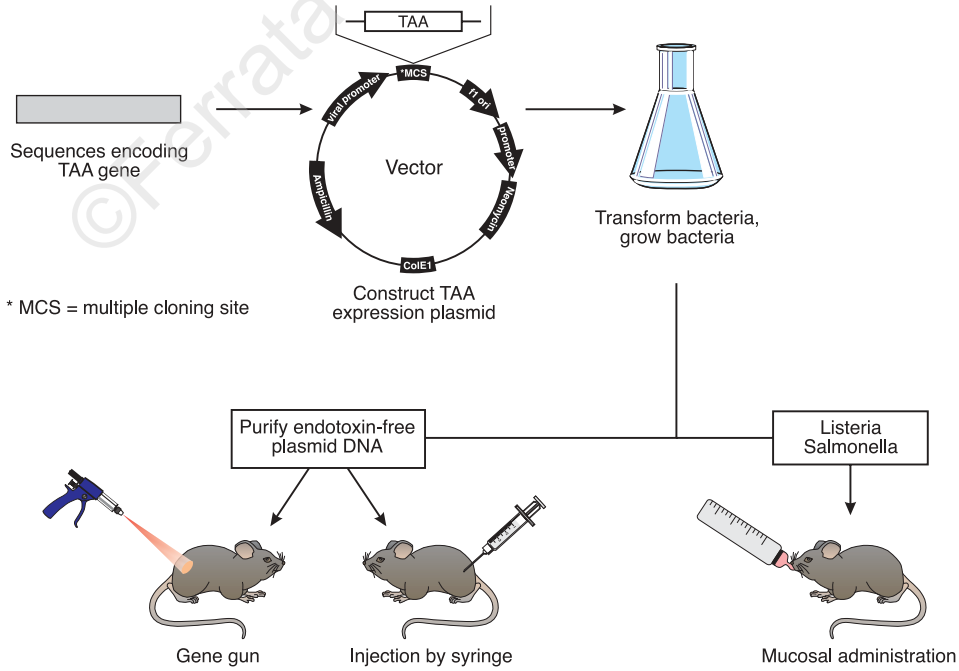

Figure 3. DNA immunization protocols.

nized mice rejected metastatic melanoma and developed patchy depigmentation of their coats (vitiligo). This "obligatory" association of vitiligo and antitumor response was recently questioned by a study showing that immunization with mouse TRP-2-encoding plasmid could protect CB6 F1 mice in the absence of overt vitiligo, suggesting a role for the genetic background in controlling both the extent and the consequences of the immune activation against *self* TAA.[180]

A novel vaccine that combines the properties of viruses and DNA is based on antigen production in the context of an alphavirus replicon. These new vectors rely on the ability of the alphavirus RNA replicase to drive the replication of its own gene, as well as of subgenomic RNA encoding the heterologous antigen. This loop of self-replication results in a several-fold amplification of protein production in infected cells.[181] Compared with traditional DNA vaccine strategies, in which vectors are persistent and the expression constitutive, the expression mediated by the alphaviral vector is transient and lytic, resulting in a decrease of biosafety risks as well as the risk of inducing immunologic tolerance due to long-lasting antigen expression. A single intramuscular injection of a self-replicating RNA immunogen at doses lower than those required for standard DNA-based vaccines elicited antibodies, $CD8^+$ T-cell responses, and prolonged the survival of mice with established tumors.[182] Interestingly, the enhanced immunogenicity of these vectors correlated with the apoptotic death of transfected cells, which facilitated their uptake and presentation by DC.

Methodology for ex vivo generation of DC

Investigators working in human and murine systems have discovered culture conditions that use hematopoietic cytokines to support the growth, differentiation, and maturation of large amounts of DC. Therefore, DC can also be purified from peripheral blood after removal of other defined T, B, NK and monocyte populations by using antibodies and magnetic beads or a cell-sorter.[183] However, the very low frequencies of DC in accessible body samples, especially blood, limits the use of these DC for vaccination protocol. The *ex vivo* differentiation of DC progenitors can be traced easily by monitoring changes in some key surface molecules such as CD1a (acquired by DC) and CD14 (expressed by monocytes and lost by DC). Furthermore expression of co-stimulatory molecules such as CD40, CD80, CD86, as well as HLA antigens, can be used to evaluate the stage of differentiation and the degree of maturation of DC during *in vitro* culture. In addition, two new markers, CD83 and p55, have been shown to be selectively expressed by a small subset of mature DC differentiated in *in vitro* culture.[184,185] According to the knowledge of DC ontogeny, two major strategies are used. The first is based on the ability of $CD34^+$ progenitors iso-

Table 3. DNA vaccines in active immunotherapy of experimental mouse tumors.

TAA	*Experimental tumor*	*Route of inoculation*	*Adjuvant*	*Vaccine effects*	*Reference*
Beta-galactosidase (model TAA)	adenocarcinoma	gene gun bombardment followed by cytokine i.p.	cytokines (IL-2, IL-12)	treatment of 2-day-old pulmonary metastases	(47)
gag from M-MuLV	leukemia	i.m., 3 inoculations every 10 days	none	complete protection from challenge	(56)
HPV-E7	sarcoma	3 gene gun bombardments, every 2 weeks	none	complete protection from challenge	(48)
Neu	spontaneous mammalian tumor	i.m., 4 weekly inoculations	IL-2-encoding plasmid	partial protection from challenge	(50)
P1A	mastocytoma	i.m., 3 inoculations every 10 days	none	partial protection from challenge	(49)
Idiotype/GM-CSF fusion protein	B-cell lymphoma	i.m., 3 inoculation every 3 weeks	none	partial protection from challenge	(57)
mouse TRP-2	melanoma	3 gene gun bombardments, every 2 weeks	IL-12-encoding plasmid	partial protection from challenge	(48)
human TRP-1	melanoma	5 gene gun bombardments, weekly	none	reduction of pulmonary metastases upon challenge; vitiligo	(52)
human TRP-2	melanoma	1-4 gene gun bombardments	GM-CSF (in therapy setting)	reduction of pulmonary metastases (prevention and therapy); vitiligo	(51)
mouse TRP-2	melanoma	i.m.	cardio-toxin	protection from challenge; no vitiligo	(53)

lated from bone marrow,[186] peripheral blood,[187] or neonatal cord blood[188] to differentiate *ex vivo* within 12-14 days into mature CD1a$^+$/CD83± HLA-DR$^+$ DC in the presence GM-CSF and TNF-α. Both stem cell factor (SCF) and FLT3 ligand are able to augment the DC yield if these key factors are present in the culture.[189]

The maturation of DC from progenitors is influenced not only by cytokines, but also by extracellular matrix (ECM) proteins, such as fibronectin which has been reported to enhance DC maturation by mediating a specific adhesion through the a5b1 integrin receptor.[190] The choice of culture conditions, especially the cytokine combination, will influence DC purity, maturation and function, and this is a consideration of prime importance before starting a DC-based immunotherapy strategy. A more practical approach is the production of DC from CD14$^+$ monocytes, in the presence of GM-CSF and IL-4.

A future strategy for easy achievement of large amounts of DC is the *in vivo* injection of the same cytokines utilized for *ex vivo* DC generation. In fact, the administration of FLT3 ligand either in animals[191] or in humans[192] results in a reversible accumulation of functionally active DC in both lymphoid and non-lymphoid tissues. Therefore, in murine models it has been demonstrated that FLT3 ligand caused the regression of various tumors supporting the suggestion that DC may be directly involved in the antitumor effect of FLT3 ligand.[193,194]

Strategies for delivery of TAA into DC

Several approaches for delivery of TAA into DC have been utilized. To date, in the clinical protocol of vaccination by DC both synthetic peptides corresponding to known tumor antigens and tumor-eluted peptides have been used for DC-mediated antigen presentation.[195] While synthetic peptides represent only the limited antigenic repertoire of the presently known tumor antigens, tumor-eluted peptides, though originating from unknown proteins, may reflect a wider antigenic spectrum. Another potential disadvantage of using defined synthetic peptides to activate tumor-reactive T-cells is that the generated peptide-specific T-cells may not recognize autologous tumor cells expressing the antigen of interest. Loading DC with cocktails of different synthetic peptides, corresponding to different tumor antigens expressed by the same tumor, has been demonstrated to be a clinically effective procedure. Nevertheless it is possible that the synthetic peptide-approach will limit patient selection, on the basis of the HLA phenotype, and will prevent the possibility of activating both CD4 and CD8 T-cells directed to different epitopes of the same antigen. To by-pass these disadvantages, several alternative methodologies using a mix of TAA have been developed. DC are able to internalize complete tumor lysates or apoptotic cells and to present derived antigen in an HLA I-restricted manner.[196] In addition, DC secrete antigen-presenting vesicles, called exosomes, which express functional HLA class I and class II, and T-cell co-stimulatory molecules. Tumor peptide-pulsed DC-derived exosomes prime specific cytotoxic T-lymphocytes *in vivo* and eradicate or suppress growth of established murine tumors in a T-cell-dependent manner.[197] However, a possible limitation of these approaches is the need for large numbers of primary samples or tumor cell lines and the complete lack of control of the nature of the antigens that are being presented by the DC. The use of RNA instead of protein could constitute a good alternative since it could be amplified *in vitro*. In addition, substractive hybridization could allow the enrichment of tumor-specific RNA, thus limiting immune response against self antigen.[198] A further possibility is the engineering of DC with expression vectors carrying TAA genes. Among the viral vectors, retroviral, adenoviral, and vaccinia vectors have been widely utilized to transduce either monocyte or CD34$^+$ cell-derived DC. Many authors have chosen retroviral vectors because retroviral transduced-DC should be able to constitutively express and process TAA to produce long-term antigen presentation *in vivo*. Specific CTLs against transduced-TAA are elicited by retrovirus-engineered DC.[199] However, their low efficiency of transduction limits the clinical use of retroviruses.

In contrast, adenoviral vectors infect replicating and non-replicating cells, are easy to handle, and supernatant with clinical grade high titer is readily achievable. Both monocyte and CD34$^+$ cell-derived DC can be transduced with high efficiency by adenovirus combined with polycations.[200,201] Moreover, DC transduced by adenovirus maintain their APC functions.[202] However, the use of adenovirus vectors is hampered by their immunogenicity which causes the rapid development of a CTL response that eliminates virus-infected cells and generation of neutralizing antibodies in recipients. Moreover, vaccinia virus which is a member of the poxvirus family, is not oncogenic, does not integrate into the host genome, is easy to manipulate genetically and is capable of accepting large fragments of heterologous DNA.[203] The transduction of CD34$^+$ cell-derived DC is feasible but their use is limited by the narrow therapeutic index between optimal transduction and target cell viability.[153] The presence of acquired genetic abnormalities in myeloid hematologic malignancies might represent the basis for innovative immune-based anti-

leukemic strategies. In fact, intracellular proteins can be processed and presented on the cell surface by HLA molecules indicating the possibility that leukemia-specific genetic abnormalities may be targets for cytotoxic T-cells. The generation of functional monocyte-derived DC carrying the specific genetic lesion has been reported for both acute myeloid and lymphoid leukemia (AML),[204,205] as well as chronic myelogenous leukemia (CML).[206] Current protocols for *ex vivo* DC generation from CD34+ cells does not allow large numbers of *leukemic* DC to be produced, probably because of a defective proliferative and/or maturative capacity of transformed CD34+ cells.

Recently, a protocol which allows the optimal generation of BCR/ABL-positive DC from CML-derived CD34+ cells has been reported.[207]

Antitumor vaccination: emerging clinical results

Clinical trials in solid tumors

Despite the fact that the large number of ongoing clinical trials which can be derived from the Physician Data Query (PDQ) of NCI (Figure 4) suggests a diffuse interest in immunotherapy, there is still a strong need to define the clinical impact of immunotherapy in the treatment of solid tumors. Table 4 summarizes the already published vaccination trials carried out using a) autologous or allogeneic neoplastic cells, b) synthetic peptides corresponding to defined TAA, alone or pulsed on autologous monocytes or DC.

Melanoma

Melanoma is the most striking example of a non-virus-induced immunogenic tumor in man that is able to elicit T-cell-mediated antitumor immunity. The majority of tumor antigens defined by T-cells have been identified utilizing patients' T-lymphocytes as effector cells and tumor cells obtained from autologous (metastatic) tumor deposits as the target.[68] Several investigators have isolated cross-reactive tumor-specific CTLs from peripheral blood, lymphocytes, or tumor-infiltrating lymphocytes of melanoma patients, and these CTLs are able to recognize common tumor antigen expressed in melanomas that share the restricting HLA class I allele.[19] Thus, large numbers of ongoing or published clinical trials have been carried out on patients with metastatic melanoma. Experimental strategies are encouraged since with current standard therapies the prognosis of patients with metastatic melanoma is poor with a median survival of about 6 months.[208] Several approaches to induce antitumor immune response have been reported.

Irradiated tumor cells. The initial anti-melanoma vaccines were similar in their preparation to the vaccines against infectious diseases. Crude preparations of homogenized tumor cells were mixed with immune-stimulating adjuvants such as viral or bacterial particles. Some of these early vaccines were tested in clinical trials and induced objective clinical responses in about 25% of the cancer patients.[209] The most impressive trial was reported by Morton *et al.*[64] who administered, to 136 stage IIIA and IV (American Joint Commitee on Cancer, AJCC) melanoma

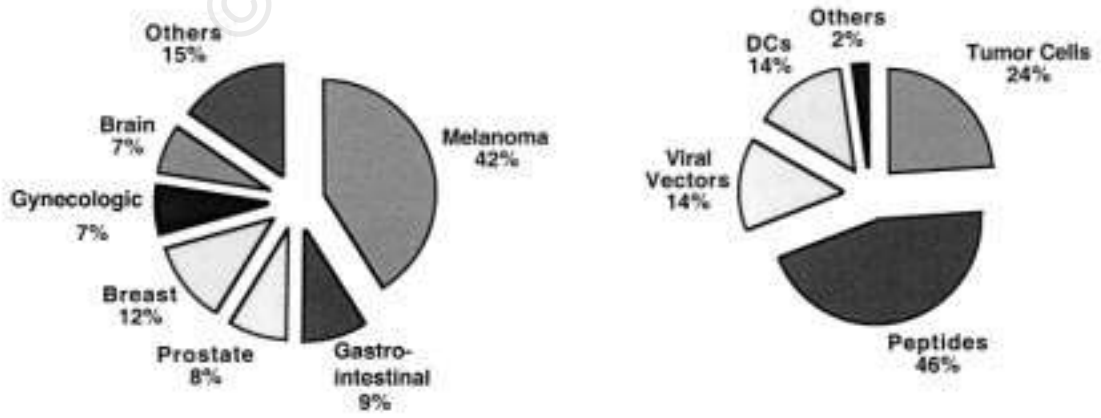

Figure 4. NCI-registered immunotherapeutic trials according to diseases and strategies.

Table 4. Phase I/II trials of vaccination in patients with solid tumor.

Disease	Stage	Pts	Vaccine	Route of Admin.	TA	Ads	Side Effects	Clinical Results	Immunol Results	Authors
Melanoma	IIIA/IV	136	Melanoma Cell Vaccine (MCV)	ID	Human Melanoma Cell Lines	Yes/No	Mild (Erithema, Fever)	9/40 evaluable pts (3 CR, 6 PR)	↑Cell mediated and humoral responses to MCV ; ↑ Activation of TIL	Morton, 1992
	IV	12	IL-2 Transduced-Melanoma Cell Line	SC	Melanoma Cell Line	No	Mild (Erithema, Fever)	3 MR; 1 SD	↑ Melanoma-Specific CTLp	Arienti, 1996
	IV	10	IL-7 Transduced-Autologous Melanoma Cells	SC	Autologous Melanoma Cells	No	Mild (Fever)	2 MR 5 SD	↑ Melanoma-Specific CTLp	Moller, 1998
	IV	21	GM-CSF Transduced-Autologous Melanoma Cells	ID and SC	Autologous Melanoma Cells	No	Mild (Erithema, Induration, Itching)	1 PR; 1 MR; 3 Minor Res	↑ Melanoma-Specific CTLp; 80% Tumor Destruction into Metastases	Soiffer,1998
	IV	31	Peptide+IL-2	SC	modified g209-2M	IL-2 and IFA	Mild (Erithema)	6 PR, 3 MR, 3 SD	↑ Melanoma-Specific CTLp	Rosenberg, 1998
	IV	39	Peptide	SC and ID	MAGE-3 (HLA-A1)	No	Mild	3 CR; 4 PR	No Increase of anti MAGE-3 CTLs even in Responders	Marchand,1999
	IV	16	Ag Pulsed Autologous Monocyte- derived DCs	Lymph nodes	Peptide Cocktail or Autologous Tumor Lysate	KLH	Mild (Fever)	2 CR; 3 PR;1 MR	DTH to KLH in 16; DTH to peptide-pulsed DCs in 11	Nestle, 1998
	IV	17	Ag Pulsed Autologous Monocyte- derived DCs	ID	Autologous Tumor Lysate	No	No	1 PR	DTH to Vaccine in 9/17; CD8 Cells in expanded-VIL	Chakraborty, 1998
Colon Cancer	II and III	254	Irradiated Autologous Tumor Cells	ID	Autologous Tumor Cells	BCG	Mild	Stage II: 61% Risk Reduction for Recurrences; Stage III: no Significant Benefits	DTH+: ≥90% Pts	Vermorken,1999
Prostate Cancer	Locally Advanced	82	Ag Pulsed Autologous Monocyte- derived DCs	Lymph nodes	Peptide Cocktail or Autologous Tumor Lysate	KLH	Mild (Fever)	2 CR; 3 PR; 1 MR	DTH to KLH in 16; DTH to peptide-pulsed DCs in 11	Salgallar, 1998
Renal Cell Cancer	IV	12	Ag Pulsed Monocyte derived-DCs	IV	Autologous Tumor Lysate	KLH	Mild (Fever)	1 PR	DTH to KLH after 1° and 2° Vaccination	Holt, 1999

Pts: Patients; TA: Tumor Antigens; Ads: Adjuvants; SC: Subcutaneous; MR: Mixed Response; SD: Stable Disease; CTLp: Cytotoxic T Lymphocyte precursor; ID: Intradermic; PR: Partial Response; TIL: Tumor-Infiltrating LymphocytesIFA: Incomplte Freund's Adjuvant; KLH: Keyhole Lympocianin; DTH: Delayed Type Hypersensitivity; IV: Intravenous

patients, a polyvalent melanoma cell vaccine (MVC) comprising 3 allogeneic melanoma cell lines. Of 40 patients with evaluable disease, 9 (23%) had regressions (3 complete). Induction of cell-mediated and humoral immune responses to common melanoma-associated antigens present on autologous melanoma cells was observed in patients receiving the vaccine. Survival correlated significantly with DTH and antibody response to MCV and there was a 3-fold increase in 5-year survival of patients with stage IV melanoma. Livingston *et al*.[65] randomized 122 stage III melanoma patients free of disease after surgery to receive treatment with the ganglioside GM2/BCG vaccine or with BCG alone. All patients were pretreated with low-dose cyclophosphamide. In most patients vaccinated with GM2/BCG, an antibody production against ganglioside was demonstrated and this was associated with a prolonged disease-free interval and survival, although the improvement did not reach statistical significance.

Gene-modified tumor cell vaccine. Autologous or allogeneic tumor or fibroblast cells have been modified to express cytokines and/or co-stimulatory molecules and/or a suicide compound. This genetic engineering is mainly performed *ex vivo* using retroviral vectors. In the published human trials, tumor cells have been transduced to express several cytokines. Arienti *et al.*[210] described 12 stage IV melanoma patients who underwent vaccination with HLA-A2-compatible allogeneic human melanoma cells ($5x10^7$ or $15x10^7$ cells) engineered to release IL-2. Little toxicity with three mixed clinical responses was recorded. Among the nine patients immunologically evaluated, peripheral blood lymphocytes from three patients displayed enhanced non-HLA-restricted cytotoxicity, and two of those individuals had an increased reactivity against tyrosinase peptide or gp-100 peptide after immunization. In the only patient for whom the autologous melanoma line was available, and following *in vitro* stimulation of the PBLs after vaccination, the frequency of CTL precursor (CTLp) was significantly enhanced. Other groups utilized a similar approach transducing different cytokines, i.e. IL-12,[40] IL-7,[211] and GM-CSF.[39] Despite documented specific antitumor reactivity with an increased frequency of anti-melanoma cytolytic precursor cells, negligible clinical results were demonstrated. To summarize, the advantage of a genetically modified autologous

cell vaccine is that it contains the whole collection of tumor proteins and therefore has the greatest chance of inducing an immune response against relevant tumor antigens. However, growing autologous tumor cells *in vitro* to establish tumor cell lines is time-consuming and often unsuccessful. These studies are relevant since they show that injection of gene-modified cells into a patient a) is safe; b) is followed by efficient and variable transduction rate of host tissues; c) is associated with transgene expression in the patient; d) is associated with biological activity of the transgene product in most instances.

Synthetic and natural peptides. Phase I clinical trials have been carried out using synthetic peptides corresponding to defined TAA. The clinical trial using melanoma differentiation antigen (MAGE-3.A1) by Marchand *et al*.,[123] enrolling 39 chemoresistant stage IV melanoma patients, was encouraging because monthly injection of 100-300 µg peptide alone was associated with tumor regression in 7 out of 26 patients who received the complete treatment. All but one of these regressions involved cutaneous metastases. No evidence for CTL response was found in the blood of the 4 patients who were analyzed, including 2 who displayed complete tumor regression. In contrast, Jager *et al*.[212] immunized similar patients with gp100 peptide along with GM-CSF and, in some patients, were able to document an increase in the specific CTL activity against the immunizing peptide. Rosenberg *et al.*[213] reported that 31 metastatic melanoma patients were immunized with the modified g209-2M peptide in incomplete Freund's adjuvant (IFA) along with IL-2 obtaining tumor regression in 42% of patients. Peripheral blood mononuclear cells harvested from these patients after, but not before, immunization exhibited a high degree of reactivity against the native g209-217 peptide, as well as against HLA-A2+ melanoma cells.

These studies indicate that vaccination with synthetic peptides is well tolerated, with occasional occurrence of mild fever and inflammation at the site of injection. Nonetheless, it should be pointed out that there is usually a poor correlation between induction of specific T-cells and the clinical response. The reasons for this discrepancy might be the selection of the patients enrolled in the trials since the majority of patients were in stage IV with large amounts of disease.

Dendritic cells. Autologous DC generated from peripheral blood monocytes have been utilized as antigen-presenting cells after their loading with specific melanoma antigens. Chakraborty *et al*.[214] found that intradermal administration of DC pulsed with a MAGE-1 HLA class I-restricted peptide could elicit peptide and autologous melanoma reactive-CTLs in patients with advanced melanoma. However, despite the presence of these CTLp in the vaccination site, peripheral blood, and distant tumor sites, no significant therapeutic responses were seen.

Nestle *et al*.[195] recently described the immunization of 16 melanoma patients using DC loaded with melanoma peptides or tumor lysates. DC were pulsed with a cocktail of gp100, MART-1, tyrosinase, MAGE-1, or MAGE-3 peptides chosen to suit the individual patient class I HLA molecules. Four patients whose HLA haplotype was inappropriate for peptide pulsing received DC pulsed with autologous tumor lysate. Keyhole limpet hemocyanin (KLH) was included during antigen pulsing. DC were administered by direct injection into uninvolved lymph nodes via ultrasound guidance to facilitate entry into the lymphatics and to minimize DC loss. Patients received 6-10 injections of $1x10^6$ cells every 1-4 weeks. Toxicity was limited to mild local reactions at the injection sites. Immunologic monitoring revealed DTH skin reactions to peptides in 11 cases, and peptide-specific CTLs could be recovered from the skin biopsies of some patients. Regression of tumor was seen in 5 out 16 patients, including 2 complete responses lasting over 15 months. Responding tumor sites included skin, lung, soft tissue, bone, and pancreas. Importantly, two of the responding patients received only tumor lysate-pulsed DC, suggesting an approach applicable to cancers lacking defined tumor antigens.

In a similar but smaller trial at the University of Pittsburgh,[215] 6 HLA-$A2^+$ patients with metastatic melanoma received four weekly intravenous injections of $1\text{-}3x10^6$ monocyte-derived DC pulsed with HLA-A2-restricted peptides derived from MART-1, gp100, and tyrosinase. Complete regression of a subcutaneous mass lasting more than one year has been observed in one patient.

Colon cancer

Colon cancer is potentially curable by surgery; the cure rate is, however, moderate to poor depending on the extent of disease. Adjuvant chemotherapy with 5-fluorouracil plus levamisole or folinic acid is the standard treatment for stage III colon cancer based on the results of numerous co-operative and intergroup clinical studies. In contrast, adjuvant chemotherapy for stage II disease has no benefit.[216] Despite several immunotherapeutic approaches having been tested for colon cancer patients, only one study has reported clinical results. In a prospective randomized study,[217] 254 patients with stage II or III post-surgery colon cancer were randomly

assigned to receive active specific immunotherapy, namely autologous tumor cell-bacille Calmette-Guèrin (BCG) or no adjuvant treatment. The immunotherapy program comprised three weekly intradermal injections starting 4 weeks after surgery, with a booster vaccination at 6 months with 10^7 irradiated autologous tumor cells. The first vaccination contained 10^7 BCG organisms. The 5-year median follow-up showed a 44% reduction of risk of recurrence in all patients receiving the vaccinations. The major impact of immunotherapy was evident in patients with stage II disease, who had a significantly longer disease-free period and 61% risk reduction. In addition, no patient discontinued treatment early because of side effects.

Recently, Foon *et al.*[218] generated anti-idiotype antibody, designated CeaVac, that is an internal image of CEA. Thirty-two patients with resected Dukes' B, C, and D, and incompletely resected Dukes' D disease were treated with 2 mg of CeaVac every other week for four injections and then monthly until tumor recurrence or progression. Fourteen patients were treated concurrently with a 5-FU chemotherapy regimen. All 32 patients entered into this trial generated a potent anti-CEA humoral and cellular immune response. Interesting, the 5-FU regimen did not affect the immune response. A phase III trial for patients with resected colon cancer is ongoing.

Prostate cancer

Several prostate-tissue-associated antigens, including prostatic alkaline phosphatase (PAP), prostate-specific membrane antigen (PSMA), and prostate-specific antigen (PSA), are now being explored as targets for prostate cancer immunotherapy.[219-221] Valone *et al.*[221] have carried out a dose-escalation trial of peripheral blood DC pulsed with recombinant PAP protein in 12 patients with advanced prostate cancer. Intravenous administration of 0.3, 0.6, and $1.2x10^9$ pulsed cells/m^2 monthly for three months resulted in T-cell proliferative responses against PAP in all patients, the magnitude of which was related to cell dosage. Toxicity was limited to myalgias in three patients. Clinical outcomes have not been reported.

Salgallar *et al*.[222] have recently updated the results of another trial[223] in which monocyte-derived DC pulsed with HLA-A2-binding peptides derived from PSMA were administered to 82 patients with advanced, hormone-refractory prostate cancer. Six infusions of up to $2x10^7$ DC were administered every six weeks, with half of the patients also receiving systemic GM-CSF. The treatment was well tolerated. However, only two patients mounted T-cell responses against the PSMA peptides as measured by enzyme-linked immunospot (ELISPOT) and DTH skin testing. Although four patients had reductions in serum tumor markers following vaccination, the concurrent administration of radiotherapy and hormonal therapy makes interpretation of the vaccine's effects difficult.

Renal cell carcinoma

Renal cell carcinoma accounts for 2% of all malignancies and many patients have metastatic disease at diagnosis and the prognosis is unfavorable. At present, neither chemotherapy nor radiation therapy has any significant influence on the course of disease or the survival time. Immunotherapy using recombinant IL-2 alone[224] or combined with interferon-α[225] is currently the standard therapy for metatastic renal cell carcinoma. Cellular immunotherapy includes the adoptive transfer of *in vitro* expanded tumor infiltrating lymphocytes[226] as well as active immunotherapy with an autologous tumor cell vaccine engineered to secrete GM-CSF.[38] Although each of these attempts generated promising results neither attempt met the expectations. Recently, Holtl *et al*.[227] have administered, to 4 metastatic renal cell carcinoma patients, autologous monocyte-derived DC pulsed with autologous tumor cell lysate. Each patient received 3 monthly intravenous infusions with the immunogeneic KLH. Initial results have shown that a potent immunologic response to KLH and, most importantly, against cell lysate could be measured *in vitro* after the vaccinations. In addition, the treatment was well tolerated with moderate fever as the only side effect. In contrast, only one partial response after 2 vaccinations was observed.

Recently, Kugler *et al*.[228] vaccinated 17 patients with metastatic renal cell carcinoma using hybrids of autologous tumor and allogeneic DC generated by an electrofusion technique. After vaccination, and with a mean follow-up time of 13 months, four patients completely rejected all metastatic tumor lesions, one presented a *mixed response*, and two had a tumor mass reduction of greater 50%. These promising data indicate that hybrid cell vaccination is a safe and effective therapy for renal cell carcinoma and may provide a broadly applicable strategy for other malignancies with unknown antigens.

Hematologic malignancies
Tumor vaccines in B-cell lymphoproliferative disorders

Multiple myeloma (MM) and low-grade non-Hodgkin's lymphomas (NHL) are clonal expansions of lymphoid cells that have rearranged immunoglobulin (Ig) genes. Early during devel-

opment, pre-B-cells become committed to the expression of a heavy and light chain Ig variable region. The heavy chain derives from the recombination of variable (V) with diversity (D) and joining (J) region genes with a constant region (C). The V-D-J joins occur with a variable number of nucleotide insertions or deletions resulting in a unique sequence which creates the third hypervariable region (CDR III) and contributes to the antigen-binding site. These antigenic regions (idiotype; Id) are characteristic for any given Ig-producing tumor (e.g. MM and NHL) and can be recognized by an immune response consisting of anti-Id antibodies and/or by Id reactive T-cells.[229-235] The tumor-derived Id is a self protein which, in most circumstances, is poorly immunogenic. However, haptens and adjuvants, including cytokines, have been used in several animal models to increase Id immunogenicity and establish protective anti-Id-immunity.[236] Lately, Id vaccines have come into medical use in patients with lymphoma and MM.

Idiotype vaccination in human lymphoma

A pioneering study was carried out in 9 lymphoma patients in CR or partial remission. They were immunized with subcutaneous injections of autologous Id, conjugated to KLH and emulsified in an oil-in-water emulsion containing non-ionic block polymers.[80] Specific anti-Id humoral and/or cellular responses were observed in 7/9 patients. Two patients with measurable disease showed a clinical improvement. These results have been confirmed in a larger series of patients.[82] Following standard chemotherapy, 41 patients with B-cell lymphoma received subcutaneous injections of autologous Id-KLH conjugates mixed with an oil-in-water emulsion containing non-ionic block polymers and threonyl-muramyl dipeptide. Approximately 50% of the patients generated specific anti-Id responses and isolated tumor regressions were observed. In particular, 11/16 patients had a significant increase in the frequency of tumor-specific cytotoxic T-lymphocytes precursor (CTLp).[237] The median duration of freedom from cancer-progression was significantly prolonged and this resulted in a survival advantage, especially in patients who generated cell-mediated anti-Id immunity.

These pioneering studies did not prospectively investigate the effect of Id vaccines on tumor burden, since most patients were already in clinical remission, and standard tumor regression criteria could not be used. A recent study has directly evaluated the ability of Id vaccines to eradicate residual t(14;18)$^+$ lymphoma cells in 20 patients in first remission after ProMACE-based chemotherapy.[238] These patients received multiple injections of Id /KLH conjugates in the presence of GM-CSF. Eight of eleven patients with detectable translocations in the peripheral blood converted to a PCR negative status after vaccination. Tumor-specific cytotoxic CD8+ and CD4$^+$ T-cells were uniformly seen in most patients. Antibodies to autologous Id were also detected, but they were apparently not required for molecular remission since the latter was achieved in some patients without a detectable antibody response. Clinical monitoring indicates a 90% disease-free survival after a median follow-up of 3 years. This is encouraging compared with the 44% disease-free survival (after a median follow-up of 3 years) in another series of patients treated at the same Institution with anti-B4-blocked ricin in first remission after ProMACE-based chemotherapy. The encouraging results obtained in lymphoma patients have provided the rationale for exploring the use of Id vaccination in MM.

Id vaccination in human MM

MM has several biological features that can advantageously be exploited in the setting of active specific immunotherapy. Among others, pre-existing tumor-specific T-cell immunity,[229-232] the possibility of using clonal markers to track the fate of residual tumor cells,[239] and the preserved susceptibility of chemoresistant myeloma cells to the effector mechanisms of cytolytic T-cells[240] may, together, represent a favorable basis for the efficacy of Id vaccines.

In a pilot study, five MM patients were repeatedly immunized with autologous Id precipitated in aluminium phosphate suspension.[77] Four patients were previously untreated and one patient was in stable-partial remission following chemotherapy. Three patients developed specific anti-Id T- and B-cell responses, but these responses were transient and their magnitude was low. A more effective immunization schedule was developed with the goal of achieving long-lasting T-cell anti-Id immunity. This effort was focused on early stage MM, based on the assumption that Id-specific T-cells are present at higher frequency mainly in patients with early stage MM or MGUS.[231] Most of these Id-reactive T-cells are Th1-type cells in early stage MM, whereas they are predominantly Th2-type in patients with advanced disease. Thus, a more effective antitumor T-cell immune response may be expected if vaccines are delivered when the frequency of T-cells with the potential to develop cytotoxic activity is higher. In this series, patients received subcutaneous injections of autologous Id precipitated in aluminium phosphate suspension, together with free GM-CSF.[241] Long-lasting Id-specific T-cell responses were induced in all five immunized patients. More-

over, one patient showed a decrease of circulating Id upon immunization.

A vaccination trial in which Id/KLH conjugates and GM-CSF were administered subcutaneously as a maintenance treatment after high-dose chemotherapy and PBPC infusion has recently been published.[242] Most patients generated Id-specific DTH reactions. DTH specificity was confirmed in one patient by investigating the reactivity to synthetic peptides derived from the VDJ sequence of the tumor-specific Ig heavy chain. In 3 patients with minimal residual disease, the DTH skin tests remained positive up to two years after the last immunization, but residual tumor cells were not eliminated by these long-lasting immune responses. Nevertheless, these patients remained in clinical remission without any further maintenance treatment. Thus, it is possible to generate anti-Id immune responses that are not potent enough to eliminate residual tumor cells, but are sufficient to hold the disease in check for extended periods.

These results have been confirmed in a preliminary report of 18 patients with MM receiving Id/KLH conjugates and GM-CSF in first remission after high-dose chemotherapy and tandem transplantation followed by PBPC infusions. In particular, 50% of the patients generated positive DTH reactions and 2 patients, in partial remission at the time of vaccination, achieved a complete remission following vaccination. A retrospective pair-mate analysis has shown a trend for a better clinical outcome in MM patients receiving Id vaccines compared to those receiving IFN-α alone. This tendency is particularly evident in patients who generated positive Id-specific T-cell responses. Although preliminary and retrospective, this is the first study providing clinical evidence that the generation of T-cell immune response against tumor cells may positively influence the clinical outcome of MM patients in the remission phase (*N. Munshi and L. Kwak , personal communications*).

DC-based anti-Id vaccination

As previously discussed, a proportion of lymphoma and MM patients enrolled in clinical trials mounted an Id-restricted antibody and T-cell response and some of them showed tumor regression. However, there are important differences between lymphoma cells and MM plasma cells. In the case of lymphomas, the cells are characterized by high surface expression and little antibody secretion whereas myeloma cells have very low levels of cell surface Ig with high levels of antibody secretion. Thus, it is unlikely that the sole generation of an antibody-based anti-Id immune response will be beneficial for MM patients. In fact, anti-Id antibodies may be blocked from reaching the tumor cells by the high levels of circulating Id. Moreover, despite the existence of a pre-plasma cell stem cell compartment in MM with a higher expression of surface Ig, it is possible that tumor cells would not express enough target protein for the antibodies to be effective. Conversely, an Id specific T-cell response would not need to bind to cell surface Ig to be active. T-cells do not recognize intact protein, but are specific for processed peptide fragments of the Id expressed on class I or II molecules. The advantage of a cytotoxic T-cell response is that it would not be blocked by free circulating paraprotein and would not depend on the expression of the native protein on the surface of tumor cells. Moreover, B-cells, including putative myeloma stem cells, are known to process and present peptides of the Ig on their membrane associated with class I and II molecules. Therefore, optimal strategies for Id vaccination may require the induction of a T-cell-mediated immune response which is best achieved by the use of APC. In this view, the rapid generation of a T-cell immunity in healthy volunteers after a single injection of mature DC has recently been described.[243] Nine normal subjects were given subcutaneous injections of monocyte-derived mature DC unpulsed or pulsed with KLH, tetanus toxoid (TT) or HLA-A*0201-positive restricted influenza matrix peptide. Four other individuals received these antigens without DC. Of note, administration of unpulsed DC or antigens alone failed to induce any T-cell response. Conversely, a $CD4^+$ T-cell response was observed in 9/9 and 5/6 subjects injected with KLH or TT-pulsed DC, respectively. Moreover, a significant stimulation of effector and memory $CD8^+$ CTLs was also reported. This feasibility trial provides the first controlled evidence of the capacity of DC to stimulate T-cell immunity.

DC-based anti-Id vaccination has been reported In B-cell malignancies in a few papers. Hsu *et al.* have[244] described 4 low-grade non-Hodgkin's lymphoma (NHL) patients resistant to conventional chemotherapy or relapsed who were injected intravenously with Id-pulsed DC freshly isolated from PB by subsequent enrichment steps. A tumor-specific T-cell response was observed in all cases associated, in one case, with tumor regression. Sixteen patients have been treated so far and an anti-Id restricted cellular response has been observed in 8 subjects (*R. Levy, personal communication*). The same strategy of targeting the Id has been applied by the same group to induce a T-cell immune response in MM patients.[245] Twelve patients were injected, 3 to 7 months after autologous stem cell transplantation, with Id-pulsed DC followed by

5 subcutaneous boosts of Id/KLH administered with adjuvant. Whereas 11/12 patients developed a strong KLH-specific cellular proliferative response, thus suggesting immunocompetence after high-dose chemotherapy, only 2 individuals generated an anti-Id restricted T-cell proliferation and only 1/3 patients showed a transient Id-specific CTLs response. This approach raises concerns about the efficacy of uncultured blood DC of stimulating efficiently T-cells, the capacity of Id-loaded DC to reach secondary lymphoid tissues to prime T-cells escaping the entrapment of the lungs and the role of Id-KLH boosts after DC administration.

Wen *et al.*[246] reported immunization of one MM patient injected with Id-pulsed DC derived from adherent mononuclear cells in the presence of appropriate cytokines. In this paper, Id-specific T-cell proliferation and secretion of IFN-γ were reported, as were the production of anti-Id antibodies. Similar results have recently been reported by Cull *et al.* who treated two patients with advanced refractory myeloma with a series of four vaccinations using autologous Id-protein pulsed DC combined with adjuvant GM-CSF.[247] DC were derived from adherent mononuclear cells. Both patients generated a specific T-cell proliferative response that was associated with the production of IFN-γ, indicating a Th-1-like response. However, no Id-specific cytotoxic T-cell response could be demonstrated. Lim and Bailey-Wood have also treated 6 MM with DC generated from adherent mononuclear cells.[248] DC were pulsed with the autologous Id and KLH as a control vaccine. All patients developed both B- and T- cell responses to KLH, suggesting the integrity of the host immune system. Id-specific responses were also observed. In one patient, a modest but consistent drop in the serum Id level was observed. Lastly, a vaccine formulation based on CD34 stem cell-derived DC pulsed with Id-derived peptides has recently been used in 11 MM patients with advanced disease.[249] Five patients generated Id-specific immune responses and one patient showed a decreased plasma cell infiltration in the bone marrow.

New strategies in Id vaccination

The generation of an effective antitumor response greatly depends on the final activation of tumor-specific cytolytic CD8 cells. This is the final event resulting from a series of cognate and non-cognate interactions occurring among tumor cells, professional APC, CD4, and CD8 cells. Each of these cell populations plays a unique role, and may represent a possible target of immune intervention to improve the efficacy of vaccination. Malignant B-cells can be modified to become efficient APCs themselves and present peptides from their own tumor-specific antigens to autologous T cells. To this end, a number of strategies are currently under preclinical and clinical evaluation. One possibility is to fuse tumor cells with dendritic cells. The fusion product will combine the functional properties of DC with the full antigenic repertoire of tumor cells.[250] As an alternative, malignant B-cells can be turned into effective APC by stimulating cell surface CD40 with its specific counter-receptor CD40 ligand.[251,252] Genetic engineering is another approach that may turn malignant B-cells into effective APC. Transfection with immunologically relevant DNA sequences coding for cytokines or co-stimulatory molecules greatly enhances the ability of malignant B-cells to activate antitumor immune responses. This approach has recently been used in MM taking advantage of the selective expression of functional adenoviral receptors on the cell surface of myeloma cells.[253]

Interestingly, there has been a description[254] of the immunization of a matched related donor of allogeneic bone marrow with the myeloma derived Id (conjugated with KLH) isolated pre-transplant from patients. After transplantation, a $CD4^+$ T-cell line was established from the peripheral blood of the recipient and found to be of donor origin and proliferate specifically in response to the myeloma Id. Thus, this experience demonstrates the principle of transfer of donor immunity with the advantage of immunizing a tumor naive donor who may be more likely to be able to generate an immune response against the tumor Ig without interference or suppression by the malignant cells. However, the lack of an anti-Id CTL response and ethical concerns on the immunization of healthy donors with tumor-derived products, suggest that in the future an alternative strategy based upon the generation, *ex vivo*, of Id-reactive CTL clones by means of APC, will be needed.

The prerequisite for any vaccine-based strategy is the possibility of differentiating tumor cells from normal cells. Id is absolutely tumor-specific, but is not directly related to the malignant phenotype of myeloma cells, and is self-Ag. As such, it is protected by self-tolerance mechanisms. There is a growing list of alternative tumor-specific antigens that can be exploited as targets for active specific immunotherapy. Among others, the core protein of Muc-1,[255,256] antigens encoded by MAGE-type genes,[257] overexpressed or fusion proteins resulting from chromosomal abnormalities may all represent alternative immunogens.[258,259] Compared to Id, some of these antigens may be more intrinsically related to the malignant phenotype of tumor cells.

Polymorphism of the HLA molecules is a major obstacle in the outcome of vaccines aimed at triggering cytolytic T-cell responses. By combining

amino acid sequencing of tumor-specific antigens and HLA typing, it is now possible to predict whether the HLA alleles of a given individual can bind tumor-derived peptides. Several groups are planning to use Id vaccination only in those patients for whom preliminary sequencing demonstrates a compatible restriction between HLA and peptides.

Finally, a new generation of immunogens has been developed using DNA-based technologies. The whole tumor-specific immunoglobulin or the variable region sequences of both heavy- and light-chains have been used as immunogens or to produce recombinant proteins in bacteria. However, it has soon become clear that naked DNA is not immunogenic *per se* and additional sequences are required to elicit protective immune responses.[260] Sequences coding for cytokines, chemokines or xenogeneic proteins have been included in these constructs and used as adjuvants.[261-264] Experimental data indicate that these fusion genes have indeed the potential to raise protective immunity in both NHL and MM.

Vaccine trials in chronic myelogenous leukemia

Chronic myelogenous leukemia (CML) is a biphasic neoplastic disorder with a prolonged indolent phase lasting an average of 4 years followed by an acute phase of blastic transformation which inevitably leads rapidly to death. There is no curative therapy for CML other than allogeneic bone marrow transplantation, an option that is available only to a small fraction of patients who have both a matched donor and are young enough to tolerate the procedure.

Recently, IFN-α has been shown to induce hematologic remissions in most CML patients, with a relevant portion of them also experiencing several degrees of cytogenetic response and ultimately a statistically significant prolongation of their chronic phase. However, still too few CML patients are long survivors if not *cured* regardless of the treatment option they received. Because of the unique features of this disease, the hallmark translocation that characterizes all neoplastic cells, a therapeutic targeting approach only the Ph+ clone could be a powerful tool in the treatment of CML.

The first direct evidence of the immune system's crucial role in recognizing and eliminating Ph+ CML cells came from the demonstration that infusion of large doses of peripheral blood leukocytes from the marrow donor induced durable remission in patients with CML who had relapsed following a T cell depleted marrow allograft.[265,266] This latter finding proved that in CML the graft-versus-tumor effect is mediated by the cellular arm of the immune system. While the nature of the response is likely to be largely allogeneic a possible role for specific anti-CML responses is suggested by the lack of this observation in patients with other myeloid leukemias undergoing the same treatment.[267]

The hypothesis that CML cells could be recognized by the immune system through the presentation of P210, the tumor-specific product of the bcr/abl hybrid gene, was first tested. The evidence that P210 b3a2-breakpoint peptides were able to bind HLA class I and HLA class II molecules and to elicit specific T cell responses in normal donors provided the rationale for a peptide vaccine in CML patients.[103-106] Pinilla-Ibarz *et al.*[124] have recently completed a phase I dose escalation trial (5 doses over 10 weeks) of a multivalent peptide vaccine (5 peptides) plus QS21 in patients with CML and b3a2 breakpoint. Patient characteristics included hematologic remission, IFN-α therapy and no HLA restriction. In a preliminary report the peptide vaccine appeared safe with patients experiencing only minimal discomfort at the site of injection.

With regards to the immune response, peptide-specific delayed hypersensitivity (DTH) *in vivo* and peptide-specific proliferation *in vitro* were shown but no peptide-specific CTL response was induced. Bocchia *et al.* are currently conducting a multicenter phase I/II trial of a pentavalent peptide vaccine plus QS21 and GM-CSF in b3a2-CML patients expressing any of HLA A3, A11 B8 or DR11. Patient characteristics also include major or complete cytogenetic response with or without IFN-α maintaining therapy. The protocol comprises 6 s.c. vaccinations with peptides + QS21 at 2 weekly intervals, with GM-CSF injected at the vaccine site for 4 consecutive days starting the day before each vaccination. Goals of the study are the evaluation of the induction of peptide-specific T-cell response, of the role of GM-CSF as immunologic adjuvant in CML patients and the impact of the peptide vaccine on minimal residual disease.

As in other cancers, the use of DC as powerful inducers of an active specific immune response in CML is now under *in vitro* evaluation.[107,268] Interestingly, most CML-derived DC carry the t(9;22) translocation and therefore could *naturally* present P210 derived peptides. In fact, CML derived Ph^+ DC were able to strongly stimulate autologous T-cells that displayed vigorous cytotoxicity activity against autologous CML cells but low reactivity to HLA-matched normal bone marrow cells or autologous remission state bone marrow mononuclear cells.[206] P210-derived peptides could have a role in inducing this leukemia-specific response and a vaccine strategy which combines Ph^+ DC and breakpoint peptides will be investigated.

Conclusions

This review shows that there are many prospects of curing cancer through the active induction of a specific immune response to TAA. The terms of the matter are now defined with molecular and genetic details for melanomas. Ongoing research is aimed at defining TAA on other forms of tumors. Indeed, experimental data and very recent clinical evidence suggest that antitumor vaccines will soon be a new form of tumor treatment that will be able to be adopted for the management of defined stages of neoplastic disease, in sequential association with conventional treatments.[269]

Prediction of when the efficacy of antitumor vaccination will be assessed and will become a routine procedure is beyond a simple scientific evaluation. While pre-clinical research has identified several possible targets and strategies for tumor vaccination, the clinical scenario is far more complex and as yet no specific clue has emerged to clearly envisage a clinical development strategy which could make biotechnology investments in this area attractive enough to pharmaceutical companies. Patent issue complexity further contributes to slowing down the development of expensive clinical trial programs. A cautious, yet attentive attitude seems, at the moment, to be the general behavior of the pharmaceutical industry.

At present peptide vaccination may appear of more immediate application. Several phase I clinical trials have already been carried out using synthetic peptides from defined TAA. These peptides have been administered alone[123] or combined with adjuvants, or *presented* by monocytes or DC.[269] Nearly all studies indicate that this form of vaccination is well tolerated, mild fever and inflammation at the site of injection being the only occasional side effects observed. Nonetheless it should be pointed out that there is usually a poor correlation between peptide ability to induce a T-cell response and clinical response. Among the several reasons that may account for this discrepancy, the choice of the peptides may have a critical importance. Most peptide-based vaccines have considered HLA class I restricted peptides only, whereas there is increasing evidence that tumor-specific $CD4^+$ T-cells may be important in inducing an effective antitumor immunity. The addition of peptides that bind class II HLA glycoproteins to peptide vaccines could lead to an amplification of the immune response as well as to better clinical effect.

A survey of the outcomes of vaccination trials shows that the poor correlation between induction of immunologic responses and the clinical results is a consistent finding, independently of the immunizing strategy adopted. Many factors may contribute to this poor correlation, e.g.:

a) the selection of the patients enrolled in the trial: tumor burden, stage of disease, and others, as discussed above;

b) the techniques used for the immune monitoring *in vitro*: most of the current studies evaluate T-cell induction through *in vitro* peptide stimulation of PBMC, while the use of tetrameric soluble class I-peptide complexes[270] or reverse solid phase ELISPOT analysis[271] may provide complementary information;

c) the assays for immune monitoring *in vivo*: often a positive DTH test does not correlate with evident tumor regression.

Perhaps, fine needle biopsies at the site of regressing and non-regressing tumors could provide a more direct insight into the events associated with the clinical outcome. The *immune pattern* within the tumor should be compared to the one found in the skin. This should allow evaluation of the exact role of vaccine-induced T-cells. Which of the existing *in vitro* and *in vivo* assays correlates most accurately with clinical responses remains to be established.

Several recent studies showing that the immune system recognizes TAA during tumor growth did not clarify whether such recognition was indeed associated with subsequent tumor cell destruction. The development of reliable assays for efficient monitoring of the state of immunization of cancer patients against TAA is as an important goal that will markedly affect the progress of antitumor vaccines. A major problem in testing the efficacy of antitumor vaccination in adjuvant settings depends on both the long period of time and large number of patients required. The possibility of effectively monitoring the immune response induced acquires critical importance since it may provide a much earlier surrogate end-point, predictive of the clinical outcome.

The downregulation of the expression of TAA represents another crucial issue for vaccination therapies, since it may lead to the immunoselection of tumor cell clones that hide the target TAA. An ideal TAA is a protein that is essential for sustaining the malignant phenotype, and that is not stripped or downmodulated by the immune reaction. Mutations that give rise to TAA of this kind have been described.[272] However, they will be an appropriate target only for the tumors expressing these particular mutations and will not be suitable for more general cancer vaccines. Improvements in the identification of tumor-associated mutations that may be potentially recognized by the immune system may also open up the possibility of tailoring individual cancer vaccines. The recent report of the construction of fusion cells composed of autol-

ogous tumor cells and DC or TAA pulsed-DC represents a step forward towards the quick manufacture of tumor-specific and individualized vaccines. In contrast, the characterization of the telomerase catalytic subunit (hTERT) expressed in more than 85% of human cancers appears to open the way to a novel strategy for a general anticancer vaccination targeting a widely distributed TAA.[273]

The *in vivo* or *ex vivo* introduction of TAA genes into DC through recombinant viral vectors is still hampered by the lack of an ideal viral vector and by the induction of an immune response against the viral proteins. Nonetheless, many viral vectors successfully used in animal models and currently tested in clinical trials appear to be safe vehicles for gene transfer, without any major toxicity. To control transgene expression levels better, investigators are exploiting tissue- or cell-specific regulatory elements such as cytomegalovirus promoters and enhancers that are preferentially expressed in tumor cells.

Certainly the new prospects opened by antitumor vaccines are fascinating. When compared with conventional cancer management, vaccination is a *soft*, non-invasive treatment free from particular distress and iatrogenic side effects. Antitumor vaccines can be expected to have a considerable social impact, but a few large clinical trials enrolling the appropriate patients are now necessary to assess their efficacy.

This review will end considering their use not in the treatment of cancer patients but to prevent cancer in healthy persons, a so far neglected prospect. Current studies are leading to the detection of gene mutations that predispose to cancer.[274] Identification of the gene at risk and its mutated or amplified products would provide the opportunity to vaccinate susceptible subjects against their foreseeable cancer. Molecular characterization of altered gene products predictably destined to become a tumor antigen will be the first step towards the engineering of effective vaccines to be used for this purpose.[25]

An unrestrained imagination may picture an even broader application of antitumor vaccines, i.e. their use to prevent tumors in the general population. Molecular and genetic data suggest that the number of TAA is not endless. Several of the TAA detected so far are shared by histologically distinct tumors arising in different organs (Table 1). The possibility of vaccinating against most common human cancers by using not many more than twenty TAA may perhaps be conceivable. Experimental data suggest that the immunity elicited by specific vaccination is much more effective in the inhibition of incipient tumors than in the cure of those that have already progressed.[275] The risk of inducing an autoimmune disease would be a major worry since not rarely antigens acting as TAA are expressed by normal tissues.[276] This risk would be much harder to accept when treating healthy individuals than in the vaccination of cancer patients, where the scales of risk-benefit are biased by a short life-expectancy. On the other hand failure to intervene when a disease so diffuse and dramatic as cancer can be prevented could also be viewed as harmful.[277] Lastly, it should be considered that the same or even a higher risk of inducing autoimmune reactions is associated with many antimicrobial vaccines. Fortunately, they started to be used when this risk was not yet perceived.

In conclusion, even if cancer vaccines are an old dream,[278] only recently has their design become a rational enterprise. There are now many ways of constructing vaccines able to elicit a strong protective immunity. This progress is offering ground for optimism.

Contributions and Acknowledgments

The authors were a group of experts and representatives of two pharmaceutical companies, Amgen Italia Spa and Dompé Biotech Spa, both from Milan, Italy. This co-operation between a medical journal and pharmaceutical companies is based on the common aim of achieving optimal use of new therapeutic procedures in medical practice.

Funding

The preparation of this manuscript was supported by educational grants from Dompé Biotech Spa and Amgen Italia Spa.

Disclosures

Conflict on interest: Dompé Biotec Spa sells G-CSF and rHuEpo in Italy, and Amgen Italia Spa has a stake in Dompé Biotec Spa.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received August 7, 2000; accepted October 24, 2000.

References

1. Bordignon C, Carlo-Stella C, Colombo MP, et al. Cell therapy: achievements and perspectives. Haematologica 1999; 84:1110-49.
2. Ada GL. The immunological principles of vaccination. Lancet 1990; 335:523-6.
3. Sprent J. Lifespan of naive, memory and effector lymphocytes. Curr Opin Immunol 1993; 5:433-8.
4. Rabinovich NR, McInnes P, Klein DL, Hall BF. Vaccine technologies: view to the future. Science 1994; 265:1401-4.
5. Medzhitov R, Janeway CA Jr. Innate immunity: the

virtues of a nonclonal system of recognition. Cell 1997; 91:295-8.
6. Matzinger P. Tolerance, danger, and the extended family. Annu Rev Immunol 1994; 12:991-1045.
7. Musiani P, Modesti A, Giovarelli M, et al. Cytokines, tumor-cell death and immunogenicity: a question of choice. Immunol Today 1997; 18:32-6.
8. Pardoll DM. Cancer vaccines: a road map for the next decade. Curr Opin Immunol 1996; 8:619-20.
9. Ehrlich P. In: Collected papers of Paul Ehrlich. London: Pergamon Press; 1960.
10. Snell GD. The histocompatibility systems. Transplant Proc 1971; 3:1133-8.
11. Gross L. Intradermal immunization of C3H mice against a sarcoma that originated in an animal of the same line. Cancer Res 1963; 3:326-33.
12. Klein G, Sjogren HO, Klein E. Demonstration of resistance against methylcholanthrene-induced sarcomas in the primary autochthonous host. Cancer Res 1960; 20:1561-72.
13. Jaffe EM, Pardoll DM. Murine tumor antigens: is it worth the search? Curr Opin Immunol 1996; 8:622-7.
14. Hewitt HB, Blake ER, Walder AS. A critique of the evidence for active host defence against cancer, based on personal studies of 27 murine tumors of spontaneous origin. Br J Cancer 1976; 33:241-59.
15. Boon T. Antigenic tumor cell variants obtained with mutagens. Adv Cancer Res 1983; 39:121-51.
16. De Plaen E, Lurquin C, Van Pel A, et al. Immunogenic (tum-) variants of mouse tumor P185: cloning the gene of tum- antigen P91A and identification of the tum- mutation. Proc Natl Acad Sci USA 1988; 85:2274-8.
17. Traversari C, van der Bruggen P, Luescher IF, et al. A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E. J Exp Med 1992; 176:1453-7.
18. Coulie PG. Human tumour antigens recognized by T cells: new perspectives for anti-cancer vaccines? Mol Med Today 1997; 3:261-8.
19. Robbins PF, Kawakami Y. Human tumor antigens recognized by T cells. Curr Opin Immunol 1996; 8:628-35.
20. Nossal GJV. The case history of Mr. T.I. - terminal patient or still curable. Immunol Today 1980; 1:5-6.
21. Vonderheide RH, Hahn WC, Schultze JL, Nadler LM. The telomerase catalytic subunit is a widely expressed tumor-associated antigen recognized by cytotoxic T lymphocytes. Immunity 1999; 10:673-9.
22. Lanzavecchia A. Identifying strategies for immune intervention. Science 1993; 260:937-44.
23. Colombo MP, Forni G. Immunotherapy I: Cytokine gene transfer strategies. Cancer Metast Rev 1996; 15:317-28.
24. Immunity future, Science, http://www.aaas.org/science/immunology.
25. Lollini PL, Forni G. Specific and nonspecific immunity in the prevention of spontaneous tumours. Immunol Today 1999; 20:347-50.
26. Nanni P, Forni G, Lollini PL. Cytokine gene therapy: hopes and pitfalls. Ann Oncol 1999; 10:261-6.
27. Colombo MP, Forni G. Cytokine gene transfer in tumor inhibition and tumor therapy: where are we now ? Immunol Today 1994; 15:48-51.
28. Allione A, Consalvo M, Nanni P, et al. Immunizing and curative potential of replicating and nonreplicating murine mammary adenocarcinoma cells engineered with interleukin (IL)-2, IL-4, IL6, IL-7, IL-10, tumor necrosis factor α, granulocyte-macrophage colony-stimulating factor, and γ-interferon gene or admixed with conventional adjuvants. Cancer Res 1994; 54:6022-6.
29. Cavallo F, Signorelli P, Giovarelli M, et al. Antitumor efficacy of adenocarcinoma cells engineered to produce interleukin 12 (IL-12) or other cytokines compared with exogenous IL-12. J Natl Cancer I 1997; 89:1049-58.
30. Cavallo F, Di Carlo E, Butera M, et al. Immune events associated with the cure of established tumors and spontaneous metastases by local and systemic interleukin 12. Cancer Res 1999; 59:414-21.
31. Janeway CA Jr, Bottomly K. Signals and signs for lymphocyte responses. Cell 1994; 76:275-85.
32. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998; 392:245-52.
33. Kitamura H, Iwakabe K, Yahata T, et al. The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. J Exp Med 1999; 189:1121-8.
34. Cayeux S, Richter G, Becker C, Pezzutto A, Dorken B, Blankenstein T. Direct and indirect T cell priming by dendritic cell vaccines. Eur J Immunol 1999; 29:225-34.
35. Sallusto F, Lanzavecchia A, Mackay CR. Chemokines and chemokine receptors in T-cell priming and Th1/Th2-mediated responses. Immunol Today 1998; 19:568-74.
36. Forni G, Cavallo F, Consalvo M, et al. Molecular approaches to cancer immunotherapy. Cytokines Mol Ther 1995; 1:225-48.
37. Peron JM, Esche C, Subbotin VM, Maliszewski C, Lotze MT, Shurin MR. FLT3-ligand administration inhibits liver metastases: role of NK cells. J Immunol 1998; 161:6164-70.
38. Simons JW, Jaffee EM, Weber CE, et al. Bioactivity of autologous irradiated renal cell carcinoma vaccines generated by ex vivo granulocyte-macrophage colony-stimulating factor gene transfer. Cancer Res 1997; 57:1537-46.
39. Soiffer R, Lynch T, Mihm M, et al. Vaccination with irradiated autologous melanoma cells engineered to secrete human granulocyte-macrophage colony-stimulating factor generates potent antitumor immunity in patients with metastatic melanoma. Proc Natl Acad Sci USA 1998; 95:13141-6.
40. Sun Y, Jurgovsky K, Moller P, et al. Vaccination with IL-12 gene-modified autologous melanoma cells: preclinical results and a first clinical phase I study. Gene Ther 1998; 5:481-90.
41. Kirk AD, Burkly LC, Batty DS, et al. Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates. Nat Med 1999; 5:686-93.
42. Larsen CP, Elwood ET, Alexander DZ, et al. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. Nature 1996; 381: 434-8.
43. Blazar BR, Taylor PA, Noelle RJ, Vallera DA. CD4(+) T cells tolerized ex vivo to host alloantigen by anti-CD40 ligand (CD40L:CD154) antibody lose their graft-versus-host disease lethality capacity but retain nominal antigen responses. J Clin Invest 1998; 102:473-82.
44. Diehl L, den Boer AT, Schoenberger SP, van der Voort EI, Schumacher TN, Melief CJ. CD40 activation in vivo overcomes peptide-induced peripheral cytotoxic T-lymphocyte tolerance and augments anti-tumor vaccine efficacy. Nat Med 1999; 5:774-9.
45. Sotomayor EM, Borrello I, Tubb E, et al. Conversion of tumor-specific CD4+ T-cell tolerance to T-cell prim-

ing through in vivo ligation of CD40. Nat Med 1999; 5:780-7.
46. Kato K, Cantwell MJ, Sharma S, Kipps TJ. Gene transfer of CD40-ligand induces autologous immune recognition of chronic lymphocytic leukemia B cells. J Clin Invest 1998; 5:1133-41.
47. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. Science 1996; 271:1734-6.
48. Yang YF, Zou JP, Mu J, et al. Enhanced induction of antitumor T-cell responses by cytotoxic T lymphocyte-associated molecule-4 blockade: the effect is manifested only at the restricted tumor-bearing stages. Cancer Res 1997; 57:4036-41.
49. van Elsas A, Hurwitz AA, Allison JP. Combination immunotherapy of B16 melanoma using anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF)-producing vaccines induces rejection of subcutaneous and metastatic tumors accompanied by autoimmune depigmentation. J Exp Med 1999; 190:355-66.
50. Hurwitz AA, Yu TF, Leach DR, Allison JP. CTLA-4 blockade synergizes with tumor-derived granulocyte-macrophage colony-stimulating factor for treatment of an experimental mammary carcinoma. Proc Natl Acad Sci USA 1998; 95:10067-71.
51. Young JW. Dendritic cells: expansion and differentiation with hematopoietic growth factors. Curr Opin Hematol 1999; 6:135-44.
52. Rescigno M, Granucci F, Citterio S, Foti M, Ricciardi-Castagnoli P. Coordinated events during bacteria-induced DC maturation. Immunol Today 1999; 20:200-3.
53. Kapsenberg ML, Hilkens CM, Wierenga EA, Kalinski P. The paradigm of type 1 and type 2 antigen presenting cells. Implications for atopic allergy. Clin Exp Allergy 1999; 29 (Suppl) 2:33-6.
54. Cella M, Scheidegger D, Palmer-Lehmann K, Lane P, Lanzavecchia A, Alber G. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. J Exp Med 1996; 184:747-52.
55. Rissoan MC, Soumelis V, Kadowaki N, et al. Reciprocal control of T helper cell and dendritic cell differentation. Science 1999; 283:1183-6.
56. Wong BR, Josien R, Lee SY, et al. TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. J Exp Med 1997; 186:2075-80.
57. Anderson DM, Maraskovsky E, Billingsley WL, et al. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. Nature 1997; 390:175-9.
58. Dubois B, Massacrier C, Vanbervliet B, et al. Critical role of IL-12 in dendritic cell-induced differentiation of naive B lymphocytes. J Immunol 1998;161:2223-31.
59. Caux C, Liu YJ, Banchereau J. Recent advances in the study of dendritic cells and follicular dendritic cells. Immunol Today 1995; 16:2-4.
60. Cao L, Kulmburg P, Veelken H, et al. Cytokine gene transfer in cancer therapy. Stem Cells 1998; 16(Suppl 1):251-60.
61. Colombo MP, Lombardi L, Melani C, et al. Hypoxic tumor cell death and modulation of endothelial adhesion molecules in the regression of granulocyte colony-stimulating factor-transduced tumors. Am J Pathol 1996; 148:473-83.
62. Morel S, Lévy F, Burlet-Schiltz O, et al. Processing of some antigens by the standard proteasome but not by the immunoproteasome results in poor presentation by dendritic cells. Immunity 2000; 12:107-17.
63. Groettrup M, Schmidtke G. Selective proteasome inhibitors: modulators of antigen presentation ? Drug Discov Today 1999; 4:63-71.
64. Morton DL, Foshag LJ, Hoon DS, et al. Prolongation of survival in metastatic melanoma after active specific immunotherapy with a new polyvalent melanoma vaccine. Ann Surg 1992; 216:463-82.
65. Livingston PO, Wong GY, Adluri S, et al. Improved survival in stage III melanoma patients with GM2 antibodies: a randomized trial of adjuvant vaccination with GM2 ganglioside. J Clin Oncol 1994; 12:1036-44.
66. Berd D, Maguire HC Jr, Schuchter LM, et al. Autologous hapten-modified melanoma vaccine as postsurgical adjuvant treatment after resection of nodal metastases. J Clin Oncol 1997; 15:2359-70.
67. Sensi M, Farina C, Maccalli C, et al. Clonal expansion of T lymphocytes in human melanoma metastases after treatment with a hapten-modified autologous tumor vaccine. J Clin Invest 1997; 99:710-7.
68. Boon T, van der Bruggen P. Human tumor antigens recognized by T lymphocytes. J Exp Med 1996; 183: 725-9.
69. Huang YC, Golumbeck P, Ahmadzadeh M, Jaffee E, Pardoll D, Levitsky H. Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. Science 1994; 264:961-5.
70. Fujiwara H, Aoki H, Yoshioka T, Tomita S, Ikegami R, Hamaoka T. Establishment of a tumor-specific immunotherapy model utilizing TNP-reactive helper T cell activity and its application to the autochthonous tumor system. J Immunol 1984; 133:509-14.
71. Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α. J Exp Med 1994; 179:1109-18.
72. van Kooten C, Banchereau J. Functions of CD40 on B cells, dendritic cells and other cells. Curr Opin Immunol 1997; 9:330-7.
73. Chiodoni C, Paglia P, Stoppacciaro A, Rodolfo M, Parenza M, Colombo MP. Dendritic cells infiltrating tumors cotransduced with granulocyte-macrophage colony-stimulating factor (GM-CSF) and CD40 ligand genes take up and present endogenous tumor-associated antigens, and prime naive mice for a cytotoxic T lymphocyte response. J Exp Med 1999; 190:125-33.
74. Albert ML, Bhardwaj N. Resurrecting the dead: DCs cross-present antigen derived from apoptotic cells on MHC I. The Immunologist 1998; 5:194-8.
75. Melcher A, Todryk S, Hardwick N, Ford M, Jacobson M, Vile RG. Tumor immunogenicity is determined by the mechanism of cell death via induction of heat shock protein expression. Nat Med 1998; 4:581-7.
76. Srivastava PK, Menoret A, Basu S, Binder RJ, McQuade KL. Heat shock proteins come of age: primitive functions acquire new roles in an adaptive world. Immunity 1998; 8:657-65.
77. Bergenbrant S, Yi Q, Osterborg A, Bjorkholm M, et al. Modulation of anti-idiotypic immune response by immunization with the autologous M-component protein in multiple myeloma patients. Br J Haematol 1996; 92:840-6.
78. Adluri S, Gilewski T, Zhang S, Ramnath V, Ragupathi G, Livingston P. Specificity analysis of sera from breast cancer patients vaccinated with MUC1-KLH plus QS-21. Br J Cancer 1999; 79:1806-12.
79. Kwak LW, Pennington R, Boni L, Ochoa AC, Robb RJ, Popescu MC. Liposomal formulation of a self lym-

phoma antigen induces protective antitumor immunity. J Immunol 1998; 160:3637-41.
80. Kwak LW, Campbell MJ, Czerwinski DK, Hart S, Miller RA, Levy R. Induction of immune responses in patients with B-cell lymphoma against the surface-immunoglobulin idiotype expressed by their tumors. N Engl J Med 1992; 327:1209-15.
81. Byars NE, Allison AC. Adjuvant formulation for use in vaccines to elicit both cell-mediated and humoral immunity. Vaccine 1987; 5:223-8.
82. Hsu FJ, Caspar CB, Czerwinski D, et al. Tumor-specific idiotype vaccines in the treatment of patients with B-cell lymphoma: long-term results of a clinical trial. Blood 1997; 89:3129-35.
83. Johnson AG, Tomai M, Solem L, Beck L, Ribi E. Characterization of a nontoxic monophosphoryl lipid A. Rev Infect Dis 1987; 9:S512-6.
84. Maitre N, Brown JM, Demcheva M, et al. Primary T-cell and activated macrophage response associated with tumor protection using peptide/poly-N-acetyl glucosamine vaccination. Clin Cancer Res 1999; 5:1173-82.
85. Yewdell JW, Bennink JR. Cell biology of antigen processing and presentation to major histocompatibility complex class I molecule-restricted T-lymphocytes. Adv Immunol 1992; 52:1-123.
86. Unanue ER, Cerottini JC. Antigen presentation. FASEB J 1989; 3:2496-502.
87. Howard JC. Supply and transport of peptides presented by class I MHC molecules. Curr Opin Immunol 1995; 7:69-76.
88. Rammensee HG. Chemistry of peptides associated with MHC class I and class II molecules. Curr Opin Immunol 1995; 7:85-96.
89. Braciale TJ, Braciale VL. Antigen presentation: structural themes and functional variations. Immunol Today 1991; 12:124-9.
90. Germain RN. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. Cell 1994; 76:287-99.
91. Accolla RS, Adorini L, Santoris S, Sinigaglia F, Guardiola J. MHC: orchestrating the immune response. Immunol Today 1995; 16:8-11.
92. Rammensee HG, Friede T, Stevanovic S. MHC ligands and peptide motifs: first listing. Immunogenetics 1995; 1:178-228.
93. Falk K, Rotzschke O, Stevanovic S, Jung G, Rammensee HG. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature 1991; 351:290-6.
94. Stuber G, Modrow S, Hoglund P, et al. Assessment of major histocompatibility complex class I interaction with Epstein-Barr virus and human immunodeficiency virus peptides by elevation of membrane H-2 and HLA in peptide loading-deficient cells. Eur J Immunol 1992; 22:2697-703.
95. Sette A, Vitiello A, Reherman B, et al. The relationship between class I binding affinity and the immunogenicity of potential cytotoxic T cell epitopes. J Immunol 1994; 153:5586-92.
96. Kawakami Y, Eliyahu S, Sakaguchi K, et al. Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. J Exp Med 1994; 180:347-52.
97. Parkhurst MR, Salgaller ML, Southwood S, et al. Improved induction of melanoma-reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A*0201-binding residues. J Immunol 1996; 157:2539-48.
98. Valmori D, Gervois N, Rimoldi D, et al. Diversity of the fine specificity displayed by HLA-A*0201-restricted CTL specific for the immunodominant Melan-A/MART-1 antigenic peptide. J Immunol 1998; 161:6956-62.
99. Clay TM, Custer MC, McKee MD, et al. Changes in the fine specificity of gp100(209-217)-reactive T cells in patients following vaccination with a peptide modified at an HLA-A2.1 anchor residue. J Immunol 1999; 162:1749-55.
100. Khleif SN, Abrams SI, Hamilton JM, et al. A phase I vaccine trial with peptides reflecting ras oncogene mutations of solid tumors. J Immunother 1999; 22:155-65.
101. Manici S, Sturniolo T, Imro MA, et al. Melanoma cells present a MAGE-3 epitope to CD4(+) cytotoxic T cells in association with histocompatibility leukocyte antigen DR11. J Exp Med 1999; 189:871-6.
102. Correale P, Walmsley K, Nieroda C, et al. In vitro generation of human cytotoxic T lymphocytes specific for peptides derived from prostate-specific antigen. J Natl Cancer I 1997; 89:293-300.
103. Bocchia M, Wentworth PA, Southwood S, et al. Specific binding of leukemia oncogene fusion protein peptides to HLA class I molecules. Blood 1995; 85:2680-4.
104. Greco G, Fruci D, Accapezzato D, et al. Two bcr-abl junction peptides bind to HLA-A3 molecules and allow specific induction of human cytotoxic T lymphocytes. Leukemia 1996; 10:693-9.
105. Bocchia M, Korontsvit T, Xu Q, et al. Specific human cellular immunity to BCR-ABL oncogene-derived peptides. Blood 1996; 87:3587-92.
106. Bosh GJ, Joosten AM, Kessler JH, Melief CJ, Leeksma OC. Recognition of BCR-ABL positive leukemia blasts by human CD4+ T cells elicited by primary in vitro immunization with a BCR-ABL breakpoint peptide. Blood 1996; 88:3522-7.
107. Mannering SI, McKenzie JL, Fearnley DB, Hart DN. HLA-DR1-restricted bcr-abl (b3a2)-specific CD4+ T lymphocytes respond to dendritic cells pulsed with b3a2 peptide and antigen-presenting cells exposed to b3a2 containing cell lysates. Blood 1997; 90:290-7.
108. Demotz S, Grey HM, Appella E, Sette A. Characterization of a naturally processed MHC class II-restricted T-cell determinant of hen egg lysozyme. Nature 1989; 342:682-4.
109. Storkus WJ, Zeh HD, Salter RD, Lotze MT. Identification of T-cell epitopes: rapid isolation of class-I presented peptides from viable cells by mild acid elution. J Immunother 1993; 14:94-103.
110. Papadopoulos KP, Hesdorffer CS, Suciu-Foca N, Hibshoosh H, Harris PE. Wild-type p53 epitope naturally processed and presented by an HLA-B haplotype on human breast carcinoma cells. Clin Cancer Res 1999; 5:2089-93.
111. Skipper JC, Gulden PH, Hendrickson RC, et al. Mass-spectometric evaluation of HLA-A*0201-associated peptides identifies dominant naturally processed forms of CTL epitopes from MART-1 and gp100. Int J Cancer 1999; 82:669-77.
112. Ostankovitch M, Buzyn A, Bonhomme D, et al. Antileukemic HLA-restricted T-cell clones generated with naturally processed peptides eluted from acute myeloblastic leukemia blasts. Blood 1998; 92:19-24.
113. Papadopoulos KP, Suciu-Foca N, Hesdorffer CS, et al. Naturally processed tissue-and differentiation stage-specific autologous peptides bound by HLA class I and II molecules of chronic myeloid leukemia blasts. Blood 1997; 90:4938-46.
114. Bellone M, Iezzi G, Imro MA, Protti MP. Cancer immunotherapy: synthetic and natural peptides in the balance. Immunol Today 1999; 20:457-62.
115. Kawakami Y, Nishimura MI, Restifo NP, et al. T-cell

recognition of human melanoma antigens. J Immunother 1993; 14:88-93.
116. van der Bruggen P, Traversari C, Chomez P, et al. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 1991; 254:1643-7.
117. Kawakami Y, Eliyahu S, Jennings C, et al. Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with in vivo tumor regression. J Immunol 1995; 154:3961-8.
118. Kang X, Kawakami Y, el-Gamil M, et al. Identification of a tyrosinase epitope recognized by HLA-A24-restricted, tumor-infiltrating lymphocytes. J Immunol 1995; 155:1343-8.
119. Wang RF, Wang X, Rosenberg SA. Identification of a novel major histocompatibility complex class II-restricted tumor antigen resulting from a chromosomal rearrangement recognized by CD4+ T cells. J Exp Med 1999; 189:1659-68.
120. Pawelec G, Max H, Halder T, et al. BCR/ABL leukemia oncogene fusion peptides selectively bind to certain HLA-DR alleles and can be recognized by T cells found at low frequency in the repertoire of normal donors. Blood 1996; 88:2118-24.
121. Yotnda P, Firat H, Garcia-Pons F, et al. Cytotoxic T cell response against the chimeric p210 BCR-ABL protein in patients with chronic myelogenous leukemia. J Clin Invest 1998; 101:2290-6.
122. Goydos JS, Elder E, Whiteside TL, Finn OJ, Lotze MT. A phase I trial of a synthetic mucin peptide vaccine. Induction of specific immune reactivity in patients with adenocarcinoma. J Surg Res 1996; 63:298-304.
123. Marchand M, van Baren N, Weynants P, et al. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. Int J Cancer 1999; 80:219-30.
124. Pinilla-Ibarz J, Cathcart K, Korontsvit T, et al. Vaccination of patients with chronic myelogenous leukemia with bcr-abl oncogene breakpoint fusion peptides generates specific immune responses. Blood 2000; 95: 1781-7.
125. Steller MA, Gurski KJ, Murakami M, et al. Cell-mediated immunological responses in cervical and vaginal cancer patients immunized with a lipidated epitope of human papillomavirus type 16 E7. Clin Cancer Res 1998; 4:2103-9.
126. Mukherji B, Chakraborty NG, Yamasaki S, et al. Induction of antigen-specific cytolytic T cells in situ in human melanoma by immunization with synthetic peptide-pulsed autologous antigen presenting cells. Proc Natl Acad Sci USA 1995; 92:8078-82.
127. Shirai M, Pendleton CD, Ahlers J, et al. Helper-cytotoxic T lymphocytes (CTL) determinant linkage required for priming of anti-HIV CD8+CTL in vivo with peptide vaccine constructs. J Immunol 1994; 152:549-56.
128. Bachmann MF, Zinkernagel RM, Oxenius A. Immune responses in the absence of costimulation: viruses know the trick. J Immunol 1998; 161:5791-4.
129. Moss B, Carroll MW, Wyatt LS, et al. Host range restricted, non-replicating vaccinia virus vectors as vaccine candidates. Adv Exp Med Biol 1996; 397:7-13.
130. Hodge JW, Schlom J, Donohue SJ, et al. A recombinant vaccinia virus expressing human prostate-specific antigen (PSA): safety and immunogenicity in a non-human primate. Int J Cancer 1995; 63:231-7.
131. Bronte V, Tsung K, Rao JB, et al. IL-2 enhances the function of recombinant poxvirus-based vaccines in the treatment of established pulmonary metastases. J Immunol 1995; 154:5282-92.
132. Kantor J, Irvine K, Abrams S, Kaufman H, DiPietro J, Schlom J. Antitumor activity and immune responses induced by a recombinant carcinoembryonic antigen-vaccinia virus vaccine. J Natl Cancer I 1992; 84:1084-91.
133. Bronte V, Carroll MW, Goletz TJ, et al. Antigen expression by dendritic cells correlates with the therapeutic effectiveness of a model recombinant poxvirus tumor vaccine. Proc Natl Acad Sci USA 1997; 94:3183-8.
134. Chamberlain RS, Carroll MW, Bronte V, et al. Costimulation enhances the active immunotherapy effect of recombinant anticancer vaccines. Cancer Res 1996; 56:2832-6.
135. Rao JB, Chamberlain RS, Bronte V, et al. IL-12 is an effective adjuvant to recombinant vaccinia virus-based tumor vaccines: enhancement by simultaneous B7-1 expression. J Immunol 1996; 156:3357-65.
136. Carroll MW, Overwijk WW, Surman DR, Tsung K, Moss B, Restifo NP. Construction and characterization of a triple-recombinant vaccinia virus encoding B7-1, interleukin 12, and a model tumor antigen. J Natl Cancer I 1998; 90:1881-7.
137. Irvine KR, Parkhurst MR, Shulman EP, et al. Recombinant virus vaccination against "self" antigens using anchor-fixed immunogens. Cancer Res 1999; 59: 2536-40.
138. Overwijk WW, Lee DS, Surman DR, et al. Vaccination with a recombinant vaccinia virus encoding a "self" antigen induces autoimmune vitiligo and tumor cell destruction in mice: requirement for CD4(+) T lymphocytes. Proc Natl Acad Sci USA 1999; 96:2982-7.
139. Tsang KY, Zaremba S, Nieroda CA, Zhu MZ, Hamilton JM, Schlom J. Generation of human cytotoxic T cells specific for human carcinoembryonic antigen epitopes from patients immunized with recombinant vaccinia-CEA vaccine. J Natl Cancer I 1995; 87:982-90.
140. Chen PW, Wang M, Bronte V, Zhai Y, Rosenberg SA, Restifo NP. Therapeutic antitumor response after immunization with a recombinant adenovirus encoding a model tumor-associated antigen. J Immunol 1996; 156:224-31.
141. O'Neal WK, Zhou H, Morral N, et al. Toxicological comparison of E2a-deleted and first-generation adenoviral vectors expressing alpha1-antitrypsin after systemic delivery. Hum Gene Ther 1998; 9:1587-98.
142. Rosenberg SA, Zhai Y, Yang JC, et al. Immunizing patients with metastatic melanoma using recombinant adenoviruses encoding MART-1 or gp100 melanoma antigens. J Natl Cancer I 1998; 90:1894-900.
143. Baxby D, Paoletti E. Potential use of non-replicating vectors as recombinant vaccines. Vaccine 1992; 10: 8-9.
144. Wang M, Bronte V, Chen PW, et al. Active immunotherapy of cancer with a nonreplicating recombinant fowlpox virus encoding a model tumor-associated antigen. J Immunol 1995; 154:4685-92.
145. Cox WI, Tartaglia J, Paoletti E. Induction of cytotoxic T lymphocytes by recombinant canarypox (ALVAC) and attenuated vaccinia (NYVAC) viruses expressing the HIV-1 envelope glycoprotein. Virology 1993; 195: 845-50.
146. Perkus ME, Tartaglia J, Paoletti E. Poxvirus-based vaccine candidates for cancer, AIDS, and other infectious diseases. J Leukocyte Biol 1995; 58:1-13.
147. Mahnel H, Mayr A. Experiences with immunization against orthopox viruses of humans and animals using vaccine strain MVA. Berl Munch Tierarztl 1994; 107:253-6.
148. Carroll MW, Overwijk WW, Chamberlain RS, Rosenberg SA, Moss B, Restifo NP. Highly attenuated modified vaccinia virus Ankara (MVA) as an effective

recombinant vector: a murine tumor model. Vaccine 1997; 15:387-94.
149. Schneider J, Gilbert SC, Blanchard TJ, et al. Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara. Nat Med 1998; 4:397-402.
150. Belyakov IM, Moss B, Strober W, Berzofsky JA. Mucosal vaccination overcomes the barrier to recombinant vaccinia immunization caused by preexisting poxvirus immunity. Proc Natl Acad Sci USA 1999; 96:4512-7.
151. Brossart P, Goldrath AW, Butz EA, Martin S, Bevan MJ. Virus-mediated delivery of antigenic epitopes into dendritic cells as a means to induce CTL. J Immunol 1997; 158:3270-6.
152. Kaplan JM, Yu Q, Piraino ST, et al. Induction of antitumor immunity with dendritic cells transduced with adenovirus vector-encoding endogenous tumor-associated antigens. J Immunol 1999; 163:699-707.
153. Di Nicola M, Siena S, Bregni M, et al. Gene transfer into human dendritic antigen-presenting cells by vaccinia virus and adenovirus vectors. Cancer Gene Ther 1998; 5:350-6.
154. Ulmer JB, Donnelly JJ, Parker SE, et al. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 1993; 259:1745-9.
155. Donnelly JJ, Ulmer JB, Shiver JW, Liu MA. DNA vaccines. Annu Rev Immunol 1997; 15:617-48.
156. Wang R, Doolan DL, Le TP, et al. Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. Science 1998; 282:476-80.
157. Calarota S, Bratt G, Nordlund S, et al. Cellular cytotoxic response induced by DNA vaccination in HIV-1-infected patients. Lancet 1998; 351:1320-5.
158. Medina E, Guzman CA, Staendner LH, Colombo MP, Paglia P. Salmonella vaccine carrier strains: effective delivery system to trigger anti-tumor immunity by oral route. Eur J Immunol 1999; 29:693-9.
159. Paglia P, Medina E, Arioli I, Guzman CA, Colombo MP. Gene transfer in dendritic cells, induced by oral DNA vaccination with Salmonella typhimurium, results in protective immunity against a murine fibrosarcoma. Blood 1998; 92:3172-6.
160. Ikonomidis G, Paterson Y, Kos FJ, Portnoy DA. Delivery of a viral antigen to the class I processing and presentation pathway by Listeria monocytogenes. J Exp Med 1994; 180:2209-18.
161. Anwer K, Earle KA, Shi M, et al. Synergistic effect of formulated plasmid and needle-free injection for genetic vaccines. Pharm Res 1999; 16:889-95.
162. Pertmer TM, Roberts TR, Haynes JR. Influenza virus nucleoprotein-specific immunoglobulin G subclass and cytokine responses elicited by DNA vaccination are dependent on the route of vector DNA delivery. J Virol 1996; 70:6119-25.
163. Feltquate DM, Heaney S, Webster RG, Robinson HL. Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. J Immunol 1997; 158:2278-84.
164. Krieg AM, Yi AK, Matson S, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 1995; 374:546-9.
165. Klinman DM, Yi AK, Beaucage SL, Conover J, Krieg AM. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. Proc Natl Acad Sci USA 1996; 93:2879-83.
166. Halpern MD, Kurlander RJ, Pisetsky DS. Bacterial DNA induces murine interferon-gamma production by stimulation of interleukin-12 and tumor necrosis factor-alpha. Cell Immunol 1996; 167:72-8.
167. Klinman DM, Barnhart KM, Conover J. CpG motifs as immune adjuvants. Vaccine 1999; 17:19-25.
168. Sato Y, Roman M, Tighe H, et al. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. Science 1996; 273:352-4.
169. Hartmann G, Weiner GJ, Krieg AM. CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. Proc Natl Acad Sci USA 1999; 96:9305-10.
170. Torres CA, Iwasaki A, Barber BH, Robinson HL. Differential dependence on target site tissue for gene gun and intramuscular DNA immunizations. J Immunol 1997; 158:4529-32.
171. Iwasaki A, Torres CA, Ohashi PS, Robinson HL, Barber BH. The dominant role of bone marrow-derived cells in CTL induction following plasmid DNA immunization at different sites. J Immunol 1997; 159:11-4.
172. Porgador A, Irvine KR, Iwasaki A, Barber BH, Restifo NP, Germain RN. Predominant role for directly transfected dendritic cells in antigen presentation to CD8+ T cells after gene gun immunization. J Exp Med 1998; 188:1075-82.
173. Akbari O, Panjwani N, Garcia S, Tascon R, Lowrie D, Stockinger B. DNA vaccination: transfection and activation of dendritic cells as key events for immunity. J Exp Med 1999; 189:169-78.
174. Irvine KR, Rao JB, Rosenberg SA, Restifo NP. Cytokine enhancement of DNA immunization leads to effective treatment of established pulmonary metastases. J Immunol 1996; 156:238-45.
175. Tuting T, Gambotto A, DeLeo A, Lotze MT, Robbins PD, Storkus WJ. Induction of tumor antigen-specific immunity using plasmid DNA immunization in mice. Cancer Gene Ther 1999; 6:73-80.
176. Rosato A, Zambon A, Milan G, et al. CTL response and protection against P815 tumor challenge in mice immunized with DNA expressing the tumor-specific antigen P815A. Hum Gene Ther 1997; 8:1451-8.
177. Chen Y, Hu D, Eling DJ, Robbins J, Kipps TJ. DNA vaccines encoding full-length or truncated Neu induce protective immunity against Neu-expressing mammary tumors. Cancer Res 1998; 58:1965-71.
178. Bowne WB, Srinivasan R, Wolchok JD, et al. Coupling and uncoupling of tumor immunity and autoimmunity. J Exp Med 1999; 190:1717-22.
179. Weber LW, Bowne WB, Wolchok JD, et al. Tumor immunity and autoimmunity induced by immunization with homologous DNA. J Clin Invest 1998; 102: 1258-64.
180. Bronte V, Apolloni E, Ronca R, et al. Genetic vaccination with "self" tyrosinase-related protein 2 causes melanoma eradication but not vitiligo. Cancer Res 2000; 60:253-8.
181. Liljestrom P. Alphavirus expression systems. Curr Opin Biotechnol 1994; 5:495-500.
182. Ying H, Zaks TZ, Wang RF, et al. Cancer therapy using a self-replicating RNA vaccine. Nat Med 1999; 5:823-7.
183. O'Doherty U, Steinman RM, Peng M, et al. Dendritic cells freshly isolated from human blood express CD4 and mature into typical immunostimulatory dendritic cells after culture in monocyte-conditioned medium. J Exp Med 1993; 178:1067-76.
184. Zhou LJ, Schwarting R, Smith HM, Tedder TF. A novel cell-surface molecule expressed by human interdigitating reticulum cells, Langerhans cells, and activated lymphocytes is a new member of the IG superfamily. J Immunol 1992; 149:735-42.
185. Mosialos G, Birkenbach M, Ayehunie S, et al. Circulating human dendritic cells differentially express high levels of a 55-kd actin-bundling protein. Am J Pathol 1996; 148:593-600.

186. Reid CD, Stackpoole A, Meager A, Tikerpae J. Interactions of tumor necrosis factor with granulocyte-macrophage colony-stimulating factor and other cytokines in the regulation of dendritic cell growth in vitro from early bipotent CD34+ progenitors in human bone marrow. J Immunol 1992; 149:2681-8.
187. Szabolcs P, Moore MA, Young JW. Expansion of immunostimulatory dendritic cells among the myeloid progeny of human CD34+ bone marrow precursors cultured with c-kit ligand, granulocyte-macrophage colony-stimulating factor, and TNF-alpha. J Immunol 1995; 154:5851-61.
188. Caux C, Vanbervliet B, Massacrier C, et al. CD34+ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF+TNF alpha. J Exp Med 1996; 184:695-706.
189. Siena S, Di Nicola M, Bregni M, et al. Massive ex vivo generation of functional dendritic cells from mobilized CD34+ blood progenitors for anticancer therapy. Exp Hematol 1995; 23:1463-71.
190. Staquet MJ, Jacquet C, Dezutter-Dambuyant C, Schmitt D. Fibronectin upregulates in vitro generation of dendritic Langerhans cells from human cord blood CD34+ progenitors. J Invest Dermatol 1997; 109: 738-43.
191. Maraskovsky E, Brasel K, Teepe M, et al. Drammatic increase in the numberS of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. J Exp Med 1996; 184:1953-62.
192. Lebsack ME, Maraskovsky E, Roux E, et al. Increased circulating dendritic cells in healthy human volunteers following administration of FLT3 ligand alone or in combination with GM-CSF or G-CSF. Blood 1998; 92 (Suppl.1):2086a.
193. Lynch DH, Andreasen A, Maraskovsky E, Whitmore J, Miller RE, Schuh JC. Flt3 ligand induces tumor regression and antitumor immune responses in vivo. Nat Med 1997; 3:625-31.
194. Esche C, Subbotin VM, Maliszewski C, Lotze MT, Shurin MR. FLT3 ligand administration inhibits tumor growth in murine melanoma and lymphoma. Cancer Res 1998; 58:380-3.
195. Nestle FO, Alijagic S, Gilliet M, et al. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med 1998; 4:328-32.
196. Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature 1998; 392:86-9.
197. Zitvogel L, Regnault A, Lozier A, et al. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. Nat Med 1998; 4:594-600.
198. Boczkowski D, Nair SK, Snyder D, Gilboa E. Dendritic cells pulsed with RNA are potent antigen-presenting cells in vitro and in vivo. J Exp Med 1996; 184:465-72.
199. Reeves ME, Royal RE, Lam JS, Rosenberg SA, Hwu P. Retroviral transduction of human dendritic cells with a tumor-associated antigen gene. Cancer Res 1996; 56:5672-7.
200. Dietz AB, Vuk-Pavlovic S. High efficiency adenovirus-mediated gene transfer to human dendritic cells. Blood 1998; 91:392-8.
201. Di Nicola M, Carlo-Stella C, Milanesi M, et al. Large-scale feasibility of gene transduction into human CD34+ cell derived-dendritic cells by polication/adenoviral complex. Br J Haematol 2000; (In Press).
202. Zhong L, Granelli-Piperno A, Choi Y, Steinman RM. Recombinant adenovirus is an efficient and non-perturbing genetic vector for human dendritic cells. Eur J Immunol 1999; 29:964-72.
203. Moss B. Vaccinia virus: a tool for research and vaccine development. Science 1991; 252:1662-7.
204. Choudhury BA, Liang JC, Thomas EK, et al. Dendritic cells derived in vitro from acute myelogenous leukemia cells stimulate autologous, antileukemic T-cell responses. Blood 1999; 93:780-6.
205. Cignetti A, Bryant E, Allione B, Vitale A, Foa R, Cheever MA. CD34(+) acute myeloid and lymphoid leukemic blasts can be induced to differentiate into dendritic cells. Blood 1999; 94:2048-55.
206. Choudhury A, Gajewski JL, Liang JC, et al. Use of leukemic dendritic cells for the generation of antileukemic cellular cytotoxicity against Philadelphia chromosome-positive chronic myelogenous leukemia. Blood 1997; 89:1133-42.
207. Carlo-Stella C, Regazzi E, Garau D, et al. Ex vivo generation of bcr/abl positive dendritic cells from CD34+ chronic myeloid leukemia cells. Haematologica 1998; 83 (Meeting Supplement):78-9.
208. Balch CM, Reintgen DS, Kirkwood JM, et al.Cutaneous melanoma. Cancer Principles & Practice of Oncology, 5th Edition, De Vita VT, Hellman S, Rosenberg SA (Eds.), Lippincot-Raven, 1997, p. 1947-94.
209. Linehan DC, Goedegebuure PS, Eberlein TJ. Vaccine therapy for cancer. Ann Surg Oncol 1996; 3:219-28.
210. Arienti F, Sule-Suso J, Belli F, et al. Limited antitumor T cell response in melanoma patients vaccinated with interleukin-2 gene-transduced allogeneic melanoma cells. Hum Gene Ther 1996; 7:1955-63.
211. Moller P, Sun Y, Dorbic T, et al. Vaccination with IL-7 gene-modified autologous melanoma cells can enhance the anti-melanoma lytic activity in peripheral blood of patients with a good clinical performance status: a clinical phase I study. Br J Cancer 1998; 77:1907-16.
212. Jager E, Ringhoffer M, Dienes HP, et al. Granulocyte-macrophage-colony-stimulating factor enhances immune responses to melanoma-associated peptides in vivo. Int J Cancer 1996; 67:54-62.
213. Rosenberg SA, Yang JC, Schwartzentruber DJ, et al. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat Med 1998; 4:321-7.
214. Chakraborty NG, Sporn JR, Tortora AF, et al. Immunization with a tumor-cell-lysate-loaded autologous-antigen-presenting-cell-based vaccine in melanoma. Cancer Immunol Immun 1998; 47:58-64.
215. Lotze MT, Hellerstedt B, Stolinski L, et al. The role of interleukin-2, interleukin-12, and dendritic cells in cancer therapy. Cancer J Sci Am 1997; Suppl 1:S109-14.
216. Blieberg H, Rougier P, Wilke HJ. Management of colorectal cancer. Martin Dunitz Publisher, London, 1998.
217. Vermorken JB, Claessen AM, van Tinteren H, et al. Active specific immunotherapy for stage II and stage III human colon cancer: a randomised trial. Lancet 1999; 353:345-50.
218. Foon KA, John WJ, Chakraborty M, et al. Clinical and immune responses in resected colon cancer patients treated with anti-idiotype monoclonal antibody vaccine that mimics the carcinoembryonic antigen. J Clin Oncol 1999; 17:2889-95.
219. Tjoa B, Boynton A, Kenny G, Ragde H, Misrock SL, Murphy G. Presentation of prostate tumor antigens by dendritic cells stimulates T-cell proliferation and cytotoxicity. Prostate 1996; 28:65-9.
220. Correale P, Walmsley K, Nieroda C, et al. In vitro generation of human cytotoxic T lymphocytes specific for peptides derived from prostate-specific antigen. J Natl Cancer I 1997; 89:293-300.
221. Valone F, Small E, Peshwa MV, et al. Phase I trial of dendritic cell-based immunotherapy with APC8015 for hormone-refractory prostate cancer (HRPC). Proc

Am Soc Cancer Res 1998; 39:173(Abstr).
222. Salgallar M, Lodge PA, Tjoa BA, et al. Monitoring of prostate-specific membrane antigen (PSMA)-specific immune response and prostate markers in a phase II clinical trial with patients infused with dendritic cells pulsed with PSMA-derived peptides. Proc Am Soc Cancer Res 1998; 39:(Abstr).
223. Murphy G, Tjoa B, Ragde H, Kenny G, Boynton A. Phase I clinical trial: T-cell therapy for prostate cancer using autologous dendritic cells pulsed with HLA-A0201-specific peptides from prostate-specific membrane antigen. Prostate 1996 ;29:371-80.
224. Rosenberg SA, Lotze MT, Muul LM, et al. A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. N Engl J Med 1987; 316:889-97.
225. Vogelzang NJ, Lipton A, Figlin RA. Subcutaneous interleukin-2 plus interferon alfa-2a in metastatic renal cancer: an outpatient multicenter trial. J Clin Oncol 1993; 11:1809-16.
226. Figlin RA, Pierce WC, Kaboo R, et al. Treatment of metastatic renal cell carcinoma with nephrectomy, interleukin-2 and cytokine-primed or CD8(+) selected tumor infiltrating lymphocytes from primary tumor. J Urol 1997; 158:740-5.
227. Holtl L, Rieser C, Papesh C, et al. Cellular and humoral immune responses in patients with metastatic renal cell carcinoma after vaccination with antigen pulsed dendritic cells. J Urol 1999; 161:777-82.
228. Kugler A, Stuhler G, Walden P, et al. Regression of human metastatic renal cell carcinoma after vaccination with tumor cell-dendritic cell hybrids. Nat Med 2000; 6:332-6.
229. Dianzani U, Pileri A, Boccadoro M, et al. Activated idiotype-reactive cells in suppressor/cytotoxic subpopulations of monoclonal gammopathies: correlation with diagnosis and disease status. Blood 1988; 72:1064-8.
230. Osterborg A, Masucci M, Bergenbrant S, Holm G, Lefvert AK, Mellstedt H. Generation of T cell clones binding F(ab')2 fragments of the idiotypic immunoglobulin in patients with monoclonal gammopathy. Cancer Immunol Immun 1991; 34:157-62.
231. Yi Q, Osterborg A, Bergenbrant S, Mellstedt H, Holm G, Lefvert AK. Idiotype-reactive T-cell subsets and tumor load in monoclonal gammopathies. Blood 1995; 86:3043-9.
232. Yi Q, Eriksson I, He W, Holm G, Mellstedt H, Osterborg A. Idiotype-specific T lymphocytes in monoclonal gammopathies: evidence for the presence of CD4+ and CD8+ subsets. Br J Haematol 1997; 96:338-45.
233. Wen YJ, Lim SH. T cells recognize the VH complementarity-determining region 3 of the idiotypic protein of B cell non-Hodgkin's lymphoma. Eur J Immunol 1997; 27:1043-7.
234. Wen YJ, Ling M, Lim SH. Immunogenicity and cross-reactivity with idiotypic IgA of VH CDR3 peptide in multiple myeloma. Br J Haematol 1998; 100:464-8.
235. Fagerberg J, Yi Q, Gigliotti D, et al. T-cell-epitope mapping of the idiotypic monoclonal IgG heavy and light chains in multiple myeloma. Int J Cancer 1999; 80: 671-80.
236. Bianchi A, Massaia M. Idiotypic vaccination in B-cell malignancies. Mol Med Today 1997; 3:435-41.
237. Nelson EL, Li X, Hsu FJ, et al. Tumor-specific, cytotoxic T-lymphocyte response after idiotype vaccination for B-cell, non-Hodgkin's lymphoma. Blood 1996; 88: 580-9.
238. Bendandi M, Gocke CD, Kobrin CB, et al. Complete molecular remissions induced by patient-specific vaccination plus granulocyte-monocyte colony-stimulating factor against lymphoma. Nat Med 1999; 5:1171-7.
239. Corradini P, Voena C, Astolfi M, et al. High-dose sequential chemoradiotherapy in multiple myeloma: residual tumor cells are detectable in bone marrow and peripheral blood cell harvests and after autografting. Blood 1995; 85:1596-602.
240. Shtil A, Turner JG, Durfee J, Dalton WS, Yu H. Cytokine-based tumor cell vaccine is equally effective against parental and isogenic multidrug-resistant myeloma cells: the role of cytotoxic T lymphocytes. Blood 1999; 93:1831-7.
241. Osterborg A, Yi Q, Henriksson L, et al. Idiotype immunization combined with granulocyte-macrophage colony-stimulating factor in myeloma patients induced type I, major histocompatibility complex-restricted, CD8- and CD4-specific T-cell responses. Blood 1998; 91:2459-66.
242. Massaia M, Borrione P, Battaglio S, et al. Idiotype vaccination in human myeloma: generation of tumor-specific immune responses after high-dose chemotherapy. Blood 1999; 94:673-83.
243. Dhodapkar MV, Steinman RM, Sapp M, et al. Rapid generation of broad T-cell immunity in humans after a single injection of mature dendritic cells. J Clin Invest 1999; 104:173-80.
244. Hsu FJ, Benike C, Fagnoni F, et al. Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. Nat Med 1996; 2:52-8.
245. Reichardt VL, Okada CY, Liso A, et al. Idiotype vaccination using dendritic cells after autologous peripheral blood stem cell transplantation for multiple myeloma-a feasibility study. Blood 1999; 93:2411-9.
246. Wen YJ, Ling M, Bailey-Wood R, Lim SH. Idiotypic protein-pulsed adherent peripheral blood mononuclear cell-derived dendritic cells prime the immune system in multiple myeloma. Clin Cancer Res 1998; 4: 957-62.
247. Cull G, Durrant L, Stainer C, Haynes A, Russell N. Generation of anti-idiotype immune responses following vaccination with idiotype-protein pulsed dendritic cells in myeloma. Br J Haematol 1999; 107:648-55.
248. Lim SH, Bailey-Wood R. Idiotypic protein-pulsed dendritic cell vaccination in multiple myeloma. Int J Cancer 1999; 83:215-22.
249. Titzer S, Christensen O, Manzke O, et al. Vaccination of multiple myeloma patients with idiotype-pulsed dendritic cells: immunological and clinical aspects. Br J Haematol 2000; 108:805-16.
250. Gong J, Chen D, Kashiwaba M, Kufe D. Induction of antitumor activity by immunization with fusions of dendritic and carcinoma cells. Nat Med 1997; 3:558-61.
251. Schultze JL, Cardoso AA, Freeman GJ, et al. Follicular lymphomas can be induced to present alloantigen efficiently: a conceptual model to improve their tumor immunogenicity. Proc Natl Acad Sci USA 1995; 92:8200-4.
252. Schultze JL, Michalak S, Seamon MJ, et al. CD40-activated human B cells: an alternative source of highly efficient antigen presenting cells to generate autologous antigen-specific T cells for adoptive immunotherapy. J Clin Invest 1997; 100:2757-65.
253. Teoh G, Chen L, Urashima M, et al. Adenovirus vector-based purging of multiple myeloma cells. Blood 1998; 92:4591-601.
254. Kwak LW, Taub DD, Duffey PL, et al. Transfer of myeloma idiotype-specific immunity from an actively immunised marrow donor. Lancet 1995; 345:1016-20.
255. Takahashi T, Makiguchi Y, Hinoda Y, et al. Expression

of MUC1 on myeloma cells and induction of HLA-unrestricted CTL against MUC1 from a multiple myeloma patient. J Immunol 1994 ;153:2102-9.
256. Treon SP, Mollick JA, Urashima M, et al. Muc-1 core protein is expressed on multiple myeloma cells and is induced by dexamethasone. Blood 1999; 93:1287-98.
257. Van Baren N, Brasseur F, Godelaine D, et al. Genes encoding tumor-specific antigens are expressed in human myeloma cells. Blood 1999; 94:1156-64.
258. Rao PH, Cigudosa JC, Ning Y, et al. Multicolor spectral karyotyping identifies new recurring breakpoints and translocations in multiple myeloma. Blood 1998; 92:1743-8.
259. Chesi M, Nardini E, Brents LA, et al. Frequent translocation t(4;14)(p16.3;q32.3) in multiple myeloma is associated with increased expression and activating mutations of fibroblast growth factor receptor 3. Nat Genet 1997; 16:260-4.
260. Stevenson FK, Zhu D, King CA, Ashworth LJ, Kumar S, Hawkins RE. Idiotypic DNA vaccines against B-cell lymphoma. Immunol Rev 1995; 145:211-28.
261. Tao MH, Levy R. Idiotype/granulocyte-macrophage colony-stimulating factor fusion protein as a vaccine for B-cell lymphoma. Nature 1993; 362:755-8.
262. Syrengelas AD, Chen TT, Levy R. DNA immunization induces protective immunity against B-cell lymphoma. Nat Med 1996; 2:1038-41.
263. King CA, Spellerberg MB, Zhu D, et al. DNA vaccines with single-chain Fv fused to fragment C of tetanus toxin induce protective immunity against lymphoma and myeloma. Nat Med 1998; 4:1281-6.
264. Biragyn A, Tani K, Grimm MC, Weeks S, Kwak LW. Genetic fusion of chemokines to a self tumor antigen induces protective, T-cell dependent antitumor immunity. Nat Biotechnol 1999; 17:253-8.
265. Kolb HJ, Mittermuller J, Clemm CH, et al. Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients. Blood 1990; 76:2462-5.
266. Mackinnon S, Papadopoulos EB, Carabasi MH, et al. Adoptive immunotherapy evaluating escalating doses of donor leukocytes for relapse or chronic myeloid leukemia after bone marrow transplantation: separation of graft-versus-leukemia responses from graft-versus-host disease. Blood 1995; 86:1261-8.
267. Slavin S, Naparstek E, Nagler A, et al. Allogeneic cell therapy with donor peripheral blood cells and recombinant human interleukin-2 to treat leukemia relapse after allogeneic bone marrow transplantation. Blood 1996; 87:2195-204.
268. Nieda M, Nicol A, Kicuchi A, et al. Dendritic cells stimulate the expansion of bcr-abl specific CD8+ T cells with cytotoxic activity against leukemic cells from patients with chronic myeloid leukemia. Blood 1998; 91:977-83.
269. Schadendorf D. Cancer Vaccines. Once more unto the breach. The Economist 1994; 12:78-9.
270. Molldrem JJ, Lee PP, Wang C, Champlin RE, Davis MM. A PR1-human leukocyte antigen-A2 tetramer can be used to isolate low-frequency cytotoxic T lymphocytes from healthy donors that selectively lyse chronic myelogenous leukemia. Cancer Res 1999; 59: 2675-81.
271. Pass HA, Schwarz SL, Wunderlich JR, Rosenberg SA. Immunization of patients with melanoma peptide vaccines: immunologic assessment using the ELISPOT assay. Cancer J Sci Am 1998; 4:316-23.
272. Wolfel T, Hauer M, Schneider J, et al. A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. Science 1995; 269:1281-4.
273. Vonderheide RH, Hahn WC, Schultze JL, Nadler LM. The telomerase catalytic subunit is a widely expressed tumor-associated antigen recognized by cytotoxic T lymphocytes. Immunity 1999; 10:673-9.
274. Jonsen AR, Durfy SJ, Burke W, Motulsky AG. The advent of the 'unpatients'. Nat Med 1996; 2: 622-4.
275. Nanni P, Forni G, Lollini PL. Cytokine gene therapy: hopes and pitfalls. Ann Oncol 1999; 10:261-6.
276. Pardoll DM. Inducing autoimmune disease to treat cancer. Proc Natl Acad Sci USA 1999; 96: 5340-2.
277. Prevention of cancer in the next millenium: Report of the Chemoprevention Working Group to the American Association for Cancer Research. Cancer Res 1999; 59: 4743-58.
278. Ehrlich P. Ueber den jetzigen Stand der Karzinomforschung. Ned Tijdschr Geneeskd 1909; 5:273-90.