Acta Biochimica Polonica

Vol. 50 No. 3/2003

613–624

QUARTERLY

Review

Cancer immunotherapy using cells modified with cytokine genes★✪

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Received: 11 August, 2003; revised: 06 September, 2003; accepted: 10 September, 2003

Key words: cancer, cytokines, gene therapy, dendritic cells, cancer vaccines

The ability of various cytokines to hamper tumor growth or to induce anti-tumor immune response has resulted in their study as antitumor agents in gene therapy approaches.

In this review we will concentrate on the costimulation of antitumor immune responses using modification of various cell types by cytokine genes. Several strategies have emerged such as (i) modification of tumor cells with cytokine genes *ex vivo* (whole tumor cell vaccines), (ii) *ex vivo* modification of other cell types for cytokine gene delivery, (iii) delivery of cytokine genes into tumor microenvironment *in vivo*, (iv) modification of dendritic cells with cytokine genes *ex vivo*. Originally single cytokine genes were used. Subsequently, multiple cytokine genes were applied simultaneously, or in combination with other factors such as chemokines, membrane bound co-stimulatory molecules, or tumor associated antigens. In this review we discuss these strategies and their use in cancer treatment as well as the promises and limitations of cytokine based cancer gene therapy. Clinical trials, including our own experience, employing the above strategies are discussed.

★Presented at the XXX Winter School of Faculty of Biotechnology, Jagiellonian University, Kościelisko, Poland, 28th February–4th March, 2003.

✪This work was supported by the State Committee for Scientific Research (KBN, Poland) grant PBZ/KBN/K032/P05/2001.

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Abbreviations: CCR, chemokine receptor; CD, cluster of differentiation; DC, dendritic cells; DTH, delayed-type hypersensitivity; GM-CSF, granulocyte-macrophage colony stimulating factor; IFN, interferon; IL, interleukin; MHC, major histocompatibility complex; TAA, tumor associated antigens; Tc, cytotoxic T-cells; Th, T helper cells; TIL, tumor infiltrating lymphocytes; TNF, tumor necrosis factor.

It has long been assumed that the immune system may play a role in controlling tumor growth and development (for a recent review see Smyth *et al.,* 2001). However, the immune interaction between tumor and host appears to be much more complex and difficult than the original immune surveillance theories suggested (Burnet, 1978). A number of very rigorous conditions must be met for effective anti-tumor immunity to be induced that is capable of destroying a tumor.

The successful anti-tumor response involves two processes which must be entirely and properly executed. These include induction of a strong and appropriate immune reaction followed by carrying out effector functions. Failure of either one will result in an inadequate response and eventual tumor growth.

A fundamental point in the generation of such an effective immune response seems to be the context in which tumor antigens are exposed to the immune system. It has been shown in model systems that growing tumor cells do not induce any measurable local or systemic immune responses (He *et al.,* 2000; Kowalczyk *et al.,* 2000; 2001). On the other hand, an immune reaction for given tumor antigens can be triggered off by a number of ways including genetic vaccination with recombinant vaccinia or adenoviral vectors expressing the relevant antigen (Wu *et al.,* 1995; He *et al.,* 2000; Kowalczyk *et al.,* 2000; 2001). It appears that, in contrast to tumor cells, antigen delivery by a viral vector supplies the necessary "danger" signal (Matzinger, 1994; Matzinger *et al.,* 2002; Kowalczyk, 2002). Dendritic cells are so far the only known sensors of such danger signals and respond by a process of antigen uptake, maturation and migration to lymphoid tissues where antigen presentation to T cells occurs. During antigen presentation in lymph nodes dendritic cells secrete cytokines which promote a particular type of immune response. Antigen specific cytotoxic and helper T cells are then generated which are competent to attack tumor cells directly or by inducing other effector mechanisms.

For many years it was assumed that tumor cells may directly prime naïve T cells. Numerous studies have then focused on approaches aimed at improving direct priming conditions by delivery of genes encoding either co stimulatory and major histocompatibility complex (MHC) molecules or cytokines (Tepper & Mule, 1994; Huang *et al.,* 1996; Mackiewicz & Rose-John, 1998; Parmiani *et al.,* 2000; Dranoff, 2002). These genetic modifications were to support development and expansion of antigen specific T-cells after tumor encounter. However, studies with MHC class I negative tumors have shown that MHC restricted response toward tumor antigens may develop despite the lack of their direct presentation by the tumors used for vaccination (Huang *et al.,* 1994; Levitsky *et al.,* 1994). Instead, bone marrow derived cells matching host MHC are required for efficient priming and immune response initiation (Huang *et al.,* 1994; Levitsky *et al.,* 1994). Now, it is generally accepted that these bone marrow derived cells necessary for the induction of immune response are dendritic cells which capture exogenous antigens and migrate to local lymph nodes where they present them together with MHC class II as well as class I molecules to T cells, hence the process of presentation of foreign peptides in the context of MHC class I by dendritic cells is termed cross-priming (Huang *et al.,* 1994a; 1994b; Corr *et al.,* 1996; Doe *et al.,* 1996; Albert *et al.,* 1998)

GENETIC IMMUNIZATION APPROACHES

It is believed that tumors do not provide necessary signals for maturation of dendritic cells and their antigens are not presented in the right (danger) context (Matzinger, 1994; Matzinger, 2002; Kowalczyk, 2002). Instead, despite their foreignness they are ignored or

lead to antigen specific anergy (Ochsenbein *et al.,* 1999; Staveley-O'Carroll *et al.,* 1998). Modification of tumor cells in such a way that they could deliver the danger signal for dendritic cells should increase their immunogenic potential. It seems that anything that is able to disrupt local homeostatic balance and "irritate" dendritic cells should work. Thus it is not surprising that numerous cytokines have demonstrated the ability to increase tumor immunogenicity when expressed by tumor cells. These include IL-1, -2, -3, -4, -6, -7, -12, IFN-γ, TNF-α and GM-CSF (for a recent review see Schadendorf, 2002). Translating these findings into cancer treatment strategy appears pretty straightforward: one has to introduce a proper cytokine gene or genes into tumor cells.

Tumor genetic modification can take place both in *in vitro* and *in vivo* conditions. Needless to say the success of gene therapy as a treatment for cancer will depend on delivering genes specifically to tumor cells *in vivo* (Verma & Somia, 1997; Peng & Vile, 1999; Vile *et al.,* 2000). To date, the majority of studies using both viral and non-viral vectors have used direct intratumoral injection of the vector. A true systemic delivery of genes to tumor sites distant from the site of injection has been shown for only a few trails (Vile *et al.,* 1994; Hurford *et al.,* 1995; Reynolds *et al.,* 2000). Moreover, even in these experiments vector administration was usually highly loco-regional rather than truly systemic (Hurford *et al.,* 1995).

CYTOKINE GENES

In this review we will concentrate on the costimulation of antitumor immune responses using modification of various cell types by cytokine genes. This concept has been tested in a number of animal models and clinical trials. Four major strategies are applied:

I. Modification of tumor cells with cytokine genes *ex vivo* (whole tumor cell vaccines):

A. autologous tumor cells

B. allogeneic tumor cell lines

C. admixed autologous and allogeneic cells

II. Modification of other cell types with cytokine genes *ex vivo* (mixed vaccines):

A. autologous tumor cells admixed with modified autologous fibroblasts

III. Delivery of cytokine genes into tumor microenvironment *in vivo* by:

A. vehicle cells such as TIL

B. direct injection into tumor

IV. Modification of dendritic cells with cytokine genes *ex vivo*.

Various cytokines have been tested using the above strategies. Clinical trials employing cytokine genes in various settings and various diseases are summarized in Table 1 (Jager *et al.,* 2002). Each cytokine displays different biological activity and activates the immune system by a unique mechanism. Originally single cytokine genes were used. Subsequently, multiple cytokine genes (Mackiewicz & Rose-John, 1998) were applied simultaneously, or in combination with other factors such as chemokines, membrane bound co-stimulatory molecules, or tumor associated antigens (TAA) (Nawrocki & Mackiewicz, 1999). Here we will not concentrate on a detailed review of the available literature but rather discuss the issues/problems related to the listed strategies (Nawrocki *et al.,* 2001b).

IN VITRO TRANSDUCTION

Ex vivo cell modification has numerous advantages such as simplicity, avoidance of neutralizing antibodies against vector, and possibility to control transduction efficiency in terms of the number of modified cells as well as the level of transgen expression. However, the major drawback lies in applying this approach for autologous tumor. Primary tumor cell cultures are difficult and sometimes problematic to propagate and expand to large number *in vitro*. Often tumor cell cultures are overgrown by non-cancerous cells such as fibro-

Table 1. Clinical trials employing cytokine genes as immunostimulators

	IL-2	IL-4	IL-7	IL-12	IFNα	IFNβ	IFNγ	GM-CSF	TNF	TGFβ	Multi-cytokine	TuAg or costimulatory + cytokine	Total
Melanoma	5	2		3			3	7	1		9	5	35
Renal cell carcinoma		1						2				1	4
Prostate Ca				1				6			1		8
Ovarian Ca								1				1	2
Lung Ca	2		1					3		1	1		8
Various cancers	2			3				1	2		1		9
Brain tumors	1	2			1	2	1	1		1			9
Leukemia, lymphoma								2				1	3
Pancreas Ca								1					1
Head + neck Ca	1			1	1						1		4
Angioendotelioma					1								1
Mesotelioma	1												1
Sarcoma									1				1
Colon Ca	1												1
Total	13	5	1	8	3	2	4	24	4	2	13	8	87

blasts and thus it is difficult to precisely define the true number of transduced tumor cells. Moreover, prolonged *in vitro* culture may lead to significant changes in their antigenic profile which may not correspond with that of the tumor cells to be treated.

Although some clinical responses have been observed with *ex vivo* transduced autologous tumor cells, from our experience as well from published experimental data it appears that cancer immunotherapy requires repeated immunizations utilizing large numbers of cells (Ochsenbein *et al.,* 1999). The numbers of autologous tumor cells necessary for multiple immunizations will be extremely difficult to achieve. It should be also pointed out that such patient tailored treatment is not only very expensive but also limited exclusively to academic institutions equipped with adequate stuff and instrumentation.

Having in mind that primary immune response takes place only in lymph nodes where T-cells recognize antigens on dendritic cells it would be logically correct to deliver tumor antigens on "carrier cells" which would activate and carry-over at the same time the antigens to dendritic cells. Indeed, several studies have used well-characterized, allogeneic tumor cell lines or fibroblasts as a mean for antigen delivery.

Gene modified allogenic vaccines induce potent response towards alloantigens. The responding T-cells release cytokines which may augment the response against tumor antigens. It has been shown that in a murine melanoma model vaccination of B6 mice with allogeneic K1735 melanoma cells provides significant protection against challenge with syngenic B16 cells. This protection could not be improved by cytokine gene modification of the vaccine (Todryk *et al.,* 2001). On the other hand, vaccination with K1735 cells in syngenic (C3H) mice gave no protection unless the cells were transfected with GM-CSF.

However, opposite results were obtained in other systems with the well defined human

papillomavirus oncoprotein E7 as a model tumor antigen. GM-CSF expression in allogeneic tumor cells used for vaccination in those studies was absolutely necessary for complete protection against BL-1 tumor cells expressing the E7 antigen (Chang *et al.,* 2000).

The allogeneic immunization approach seems very promising for wide clinical practice. Established cell lines can be easily produced in large quantities, and their antigen or cytokine profile may easily be determined. Such cell lines may be thus uniformly standardized and meet all the criteria necessary to compare their effectiveness in clinical trials.

We have developed an original clinical protocol based on the admixture of irradiated autologous melanoma cells and allogeneic cell lines modified with IL-6 and soluble IL-6 receptor (sIL-6R) (Mackiewicz *et al.,* 1995; Mackiewicz & Rose-John, 1998). The trial was initiated in January 1995 and involved grade III and IV melanoma patients with non-resected and resected metastases (Nawrocki *et al.,* 2000). Until now about 300 patients have been treated and the trial reached phase III. The clinical results obtained demonstrated a very significant increase of overall survival and disease free survival (Mackiewicz *et al.,* unpublished). However, the original protocol was modified towards application of allogeneic cell lines instead of the mixture of autologous and allogeneic cells.

Despite all the advantages and simplicity in mass scale production for allogeneic vaccines this approach may not be optimal in every case. Allogeneic vaccines deliver only shared antigens and are devoid of the unique antigens expressed by autologous tumors. Since *ex vivo* transduction of autologous tumor cells is problematic a substantial amount of work has recently focused on direct *in vivo* gene transfer. Similarly to the *ex vivo* approaches tumors are modified with single cytokines, their combinations or costimulatory molecules.

DIRECT INTRA-TUMOR GENE DELIVERY

Direct *in vivo* tumor cells modification with cytokine genes has two major aims: local tumor destruction and induction of systemic anti-tumor immunity capable of destroying occult metastases. In other words gene delivery and its expression in a tumor is supposed to cause, through the immune cells, eradication of all transformed cells scattered all over the body.

Several vectors, including retroviruses, adenoviruses, adeno-associated virus, vaccinia, and HSV, have been genetically modified to express cytokines and costimulatory molecules and used as vectors for intra-tumor cancer therapy.

In experimental models, injection of vectors encoding various cytokines into established tumors was shown to inhibit primary tumor growth, inducing tumor regression, and to prevent the development of distant lung metastasis and subcutaneous tumors. Moreover, mice that had undergone tumor regression following intra-tumor cytokine gene therapy remained immune to a rechallenge with live tumor cells that indicates a strong and tumor specific immune response.

Intra-tumor cytokine gene delivery may also cause tumor destruction without induction of a specific anti-tumor response. For example, IFN-γ secretion through intra-tumor adenovirus injection in mouse hepatoma model led to the regression of primary tumor. The lack of T-cell infiltrates in the liver upon treatment excluded a role of a specific immune response (Bartin *et al.,* 2001). Similar results have been obtained in P-815 tumor model, where intra-tumor injection of adenovirus encoding IFN did not cause any increase in tumor specific CD8+ T-cells. However, in the same model IFN replacement with IL-12 was correlated with clonal tumor-specific T cell expansions *in situ* and in the periphery (Fernandez *et al.,* 1999).

Thus, the mechanisms responsible for tumor destruction after intra-tumor gene delivery may involve induction of specific immune responses, direct cytotoxic or cytostatic effect on tumor cells, activation of innate immune cells such as macrophages or inhibition of angiogenesis (Fathallah-Shaykh *et al.,* 2000; Baratin *et al.,* 2001; Wilczyńska *et al.,* 2001; Yu & Thomas-Tikhonenko, 2001; Liu *et al.,* 2002).

The major limitation of direct intratumoral gene delivery is the strong anti-vector immune response which may severely reduce transduction efficiency. Adenoviral vectors are challenged with pre-existing immunity developed during natural exposure to the virus. Other vectors, on the other hand, induce specific neutralizing antibodies that prevent repetitive administration. The only solution for such difficulties is to use in consecutive gene deliveries different vectors or vectors based on different viral serotypes (Peng & Vile, 1999; Vile *et al.,* 2000; Yu & Restifo, 2002). Alternatively, targeted gene expression in tumor tissue might be obtained by cellular vectors such as T-cells (Ostrand-Rosenberg *et al.,* 1999; Harrington *et al.,* 2002).

T-cell circulate through the body to search for foreign antigens or transformed cells. After receiving appropriate chemokine signal activated T-cells extravasate and infiltrate the site where the pathology occurs (Kjaergaard & Shu, 1999). Many studies have, therefore, sought to use activated T-cells for specific gene delivery into tumors (Rosenberg *et al.,*1990; Melief, 1992; Basse *et al.,* 2000). Most of them used only marker genes to show specific tumor localization. Rosenberg's group tried to use gene modified tumor infiltrating lymphocytes (TIL) for targeted tumor tissue TNF gene expression. At high concentrations, TNF-α was found effective in causing regression of murine tumors. However, due to dose-limiting toxicity therapeutic levels in humans cannot be obtained systemically. Thus, in an attempt to deliver the local high concentrations of TNF to the tumor site without dose-limiting systemic toxicity, the Rosenberg's group used gene modified, *in vitro* expanded TIL. Despite successful TNF gene delivery into T-cells they observed partial expression blockade that prevented maximal cytokine production.

In another study, TNF-gene-transduced TIL showed a higher level of TNF production and higher cytotoxic activity against K562 and Daudi tumor cells. In spite of the fact that autologous tumor cells showed resistance to soluble TNF, the TNF-TIL clearly demonstrated enhanced cytotoxicity against them as compared with neo-TIL. The enhanced cytotoxicity was attributed to autocrine effects of secreted TNF on TIL, which included augmentation of adhesion molecules and interleukin-2 receptor expression, and elevation of production of interferon gamma, lymphotoxin and granulocyte/macrophage-colony-stimulating factor and its paracrine effect on target cells to make them more susceptible to TIL. Although this work showed superiority of gene modified TIL over unmodified cells in *in vitro* assays there were no data concerning their *in vivo* activity (Itoh *et al.,* 1995).

DENDRITIC CELLS MODIFIED WITH CYTOKINE GENES

Induction of antigen-specific T cell-mediated immune responses by DCs depends not only on presentation of antigens in the context of MHC complexes, but also on two further signals i.e. costimulatory molecules (CD40, CD80, CD86) and cytokines (IL-1β, -2, -6, -10, -12, TNF-α). Multiple studies have been carried out in order to evaluate the effectivity of DCs modified with genes encoding immunostimulatory proteins in induction of tumor-specific immune response (Wysocki *et al.,* 2002).

DCs modified to express IL-12 have been shown to induce a potent antitumor immune response (Furumoto *et al.,* 2000; Nishioka *et al.,* 1999; Shimizu *et al.,* 2001). In a murine

model of colon cancer intratumoral administration of DCs adenovirally transduced with IL-12 gene inhibited growth of subcutaneous tumors. This effect was associated with an increased infiltration of the tumors with CD4+ and CD8+ T cells and with polarization of immune response toward Th1/Tc1 cells (Furumoto *et al.,* 2000). In another study in three weakly immunogenic tumor models, intratumoral administration of bone marrow-derived IL-12-expressing DCs resulted in a complete regression of established tumors. The induced immune response was associated with production of IFN-γ by T helper cells (Nishioka *et al.,* 1999). Also in a murine model of neuroblastoma, the intratumoral injection of DCs modified to express IL-12 induced a complete regression of tumors within three weeks. This effect was also associated with decreased apoptosis of tumor infiltrating cells (Shimizu *et al.,* 2001).

Transfection of immature DCs with the GM-CSF gene did not increase the immunostimulatory capacity of DCs as demonstrated by analysis of expression of MHC and costimulatory molecules as well as antigen presentation to T cells *in vitro*. However, when administered *in vivo*, GM-CSF-expressing DCs demonstrated an enhanced migration into lymph nodes and induced a more potent immune response against different antigens tested as compared with unmodified DCs (Curiel-Lewandowski *et al.,* 1999).

DCs transduced with a retroviral vector carrying IL-7 gene increase T cell proliferation by a factor of 2 in an autologous mixed lymphocyte reaction (MLR) and by a factor of 2.7 in allogenic MLR as compared with unmodified DCs (Westermann *et al.,* 1998). Miller and colleagues analyzed the effect of intratumoral administration of DCs modified to express IL-7 (Miller *et al.,* 2000). In two murine lung cancer models DCs transduced with an adenovirus carrying IL-7 gene induced an extremely potent and long-lasting antitumor immune response. Mice that rejected tumors after administration of the genetically modified DCs were totally protected from a subsequent tumor rechallenge. However, only 20–25% of animals treated with adenovirus encoding IL-7 survived the rechallenge.

In a few studies, DCs modified simultaneously with genes encoding TAA and cytokines have been shown to induce potent antigen-specific anti-tumor immune response. In a study of Nakamura and colleagues, DCs transduced with an adenoviral vector carrying GM-CSF and gp70 (murine colon carcinoma antigen) genes were shown to be superior in induction of anti-tumor immune response to DCs expressing exclusively the gp70 gene (Nakamura *et al.,* 2002). Secretion of GM-CSF by transduced DCs enhanced the expression of CCR7 on the DCs which is crucial for effective trafficking of DCs towards secondary lymphoid organs. Human DCs simultaneously expressing IL-2 and the MUC-1 antigen have also been shown to effectively stimulate proliferation of autologous lymphocytes in MLRs (Trevor *et al.,* 2001). Similarly, human monocyte-derived DCs cotransduced with genes encoding malignant melanoma antigens and genes encoding IL-12 or IFN-α resulted in a strong activation of antigen-specific CTLs and polarization of immune response toward Th1 type (Tuting *et al.,* 1998).

DENDRITIC CELLS MODIFIED WITH GENES ENCODING COSTIMULATORY MOLECULES

Another strategy of modifying DCs with genes encoding immunostimulatory molecules was tested by Kikuchi and colleagues (Kikuchi *et al.,* 2000). Murine bone marrow-derived DCs were transduced with an adenoviral vector encoding CD40L protein. The CD40L molecule, usually expressed by CD4+ T cells, interacts with CD40 located on the surface of DC (Kikuchi *et al.,* 2000). Several recent reports suggest that DCs cannot stimulate cytotoxic T cells directly unless they are first stimulated *via* CD40 (Toes *et al.,*

1999; Bennet *et al.*, 1998; Ridge *et al.,* 1998). CD40 triggering by CD4+ T cells results in an upregulation of adhesion and costimulatory molecules and production of several inflammatory cytokines and chemokines, e.g. IL-12, MIP-1 (Schoenberger *et al.,* 1998; Caux *et al.,* 1994; Cella *et al.,* 1996).

Intratumoral injection of CD40L-expressing DCs into B16 and CT26 tumors induced a strong inhibition of tumor growth and significantly extended survival of treated animals as compared with unmodified DCs. Moreover, splenocytes obtained from mice treated with CD40L-expressing DCs were able to transfer tumor-specific immunity to naïve recipients (Kikuchi *et al.,* 2000).

SEARCHING FOR SIGNS OF IMMUNE RESPONSE

The obvious ultimate goal of anti-cancer vaccines is to cure or extend patients' survival. Thus, the most important endpoints in clinical trials are measures of clinical response such as tumor regression, symptoms relief and disease free or overall survival. However, a failure of a vaccine clinical effectiveness could be due to its inability to induce a proper immune response or stem from tumor resistance to immune attack. In order to resolve these issues it is necessary to establish laboratory tests which would be able to correlate tumor regression with vaccine induced immune responses. For clinicians such tests would be very useful for comparison of different vaccination modalities or early prediction of treatment efficacy. Thus, it is reasonable to evaluate not only the primary but also secondary endpoints such as the quality of immune responses elicited by the vaccine. Unfortunately, there are no standard laboratory tests which could be successfully employed for such measurements. Moreover, it is not known which quantitative or functional immunological requirements are truly and unequivocally connected with tumor regression.

The currently employed monitoring methods in the vast majority of trials include DTH skin reaction and laboratory tests such as ELISPOT and MHC tetramer staining assays (for a review see Coulie & van der Bruggen, 2003). The easiest way for antigen specific T-cell quantitative analysis is so called tetramer staining. Soluble recombinant MHC molecules are folded *in vitro* in the presence of antigenic peptides to form tetrameric complexes (hence the name) which bind the specific T-cell receptors. An alternative method measures cytokine producing cells by flow cytometry or in ELISPOT assay. In the two latter assays T-cells are first stimulated *in vitro* with a peptide, live tumor cells or tumor lysates followed by cytokine detection. Whereas in flow cytometry methods cytokines are detected within the antigen specific T-cells allowing their enumeration, in the ELISPOT assay antibodies are used to capture and detect cytokines produced by individual antigen specific T-cells. Every cytokine producing cell leaves thus a spot on the culture dish bottom which can be more or less easily enumerated.

In our melanoma vaccine trials we perform two types of tests: i) Immune response against the vaccine cells which are allogeneic tumor cells. The test is based on the flow cytometric SAAL assay (Kowalczyk *et al.,* 2000). These tests may be used for monitoring patients' ability to mount an immune response. ii) Other sets of tests include assays measuring immune responses against defined and undefined tumor antigens. CD8 + T-cells' response to melanoma antigens such as Mart, gp100, and NY-ESO is assessed in HLA selected patients by peptide stimulation followed by intracellular cytokine staining. CD4 response is measured by T-cell stimulation with tumor cell lysates in the presence of autologous monocytes as a source for antigen processing and presentation.

So far, with a few rare exceptions, no convincing correlations have been shown between the magnitude of vaccine induced immune response measured by laboratory tests

and clinical outcome. In many cases even very sensitive approaches are unable to detect any signs of vaccine induced specific immune responses despite fairly good clinical effectiveness (Nawrocki *et al.,* 2001a).

With the exception of the NY-ESO antigen, peptide stimulation or tetramer staining in these assays provide insights only for CD8+ mediated response. Moreover these analyses are only restricted to a very limited number of antigens leaving other responses undetected. For reasons unknown to us analysis of CD4+ T-cell response towards tumor antigens has not been performed in most clinical trials. It is also surprising that in order to make such measurements it is necessary to use very sensitive approaches including PCR enabling detection of a single specific cell among 10^6 others. It is often forgotten that an immune response in order to be successful must be very strong in both qualitative and quantitative ways and thus should be easily detected. In animal models complete protection against tumor is achieved when about 1.5% of the total CD8+ T-cell pool in peripheral blood and over 30% among tumor infiltrating lymphocytes are tumor specific (Kowalczyk *et al.,* 2001). In current clinical trials the number of tumor-antigen specific T-cells is only a fraction of that or is undetectable. Thus, either all these vaccines simply do not work in most cases for many reasons or we are overlooking the response in our tests.

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