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Gene-based vaccines for immunotherapy of prostate cancer - lessons from the past

Review Article

Milcho Mincheff* and Serguei Zoubak

The George Washington University Medical Center

***Correspondence**: Milcho Mincheff, Head, Tumor Immunology Laboratory, Department of Medicine, The George Washington Medical Center, 2300 Eye Street, N.W., Ross Hall 705, Washington, DC 20037; Tel: 202 994 7765; fax: 202 994 0465; e-mail: mcamsm@gwumc.edu

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Abbreviations: activation-inducible TNF receptor, (AITR); antigen-presenting cells, (APCs); cytotoxic T lymphocyte antigen 4, (CTLA-4); delayed type hypersensitivity, (DTH); glucocorticoid-induced tumor necrosis factor receptor, (GITR); GITR ligand, (GITR-L); prostate acidic phosphatase, (PAP); prostate-specific membrane antigen, (PSMA); "secreted" prostate-specific membrane antigen, (sPMSA); T cell receptor, (TCR); truncated prostate-specific membrane antigen, (tPSMA); tumor-infiltrating lymphocytes, (TILs); tyrosinase-related protein-1, (TRP-1)

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Summary

Gene-based vaccination in its current mode of application is effective in breaking tolerance to a self- or tumor-associated antigen, but the response is narrow and restricted to few of the potential epitopes due to immunodominance. In cancer, immunodominance carries the risk of inefficient immune surveillance due to loss of MHC alleles or point mutations in the recognized sequences. We have found that a T cell response to sub-dominant epitopes can be primed with transfected dendritic cells in which the newly expressed antigen is purposefully targeted for proteasomal degradation. Beginning in May 1998, we performed a phase I/II clinical trial for immunotherapy of prostate cancer that targeted the prostate-specific membrane antigen (PSMA). The primary objective of the study was to determine the safety of the described vaccines after repeated intradermal injections (Mincheff et al., 2000a; Mincheff et al., 2000b), since using PSMA as a target could be seriously offset by the development of autoimmunity (Gilboa, 1999b; Overwijk and Restifo, 2000). So far, six years since the study has begun, no patient has experienced any short- or long-term side effects, including anti-DNA antibody. Twenty-nine patients from this random population were treated solely by immunotherapy. Eighteen of them had biochemical recurrence following radical prostatectomy and eleven responded to the therapy with a PSA drop exceeding 50% of pre-therapy value. Patients with advanced disease and distant metastases were not influenced by the immunotherapy despite the fact that they all showed signs of T cell immunity towards PSMA. We found, however, that the post-vaccination T cell response was directed against only two of the potential 4 PSMA epitopes that had high affinity for binding. At least in vitro, priming with one of our vaccines led to a poly-epitope response. Unfortunately, even in such instances, consequent exposure to poly-epitope expressing dendritic cells during re-immunization led to selection of an immunodominant clone. To alleviate immunodominance and decrease tumor evasion due to loss of antigenic determinants, a poly-epitope T cell response would need to be maintained. Ensuring

such a cytotoxic T cell response, therefore, would require either construction of separate epitope encoding vectors for boosting, an approach with limited therapeutic application, or identifying conditions during boosting that would restrict immunodominance. CD4 T cell depletion, GITR-L signaling or CTLA-4 all show promise in achieving this goal.

I. Introduction

A. Tumor antigen recognition

Evidence that the immune system recognizes tumor antigens is supported by the existence of tumor infiltrating lymphocytes but, since cancer cells fail to establish and support an effective immune milieu, tumors often prevail and survive. Worsening the problem is the fact that recognition of cancer antigens on tumor cells seems to evoke a tolerant state by induction of anergy in antigen-reactive T cells. In the past few years it has become increasingly evident that induction of tissue-specific autoimmunity can lead to tumor destruction. Initially Coulie and colleagues (Coulie et al, 1994) discovered that the target for a melanoma-specific CD8+ T cell clone grown from a melanoma patient was wild-type tyrosinase, a melanosomal enzyme selectively expressed in melanocytes. Subsequently, a number of investigators found that their melanoma-specific CD8+ T cells indeed recognized melanocyte-specific antigens rather than melanoma-specific antigens (Bakker et al, 1994; Cox et al, 1994; Kawakami et al, 1994). Most of these antigens appear to be normal melanosomal proteins, and a number of them, including tyrosinase, tyrosinase-related protein-1 (TRP-1), TRP-2, and glycoprotein 100 (gp100), are involved in melanin biosynthesis. Other melanosomal proteins such as MART1/Melan A have no known function but are nonetheless melanocyte-specific tissue differentiation antigens. As time progressed, evidence accumulated that the dominant targets of immune responses against tumors were tissue-specific or differentiation antigens. In contrast, recognition of peptides derived from unique tumor-specific mutations represented infrequent reactivities (Coulie et al, 1995; Wolfel et al, 1995; Robbins et al, 1996). Similar analysis of the specificities of tumor-infiltrating lymphocytes (TILs) in prostate cancer biopsies also revealed responses against tissue-specific antigens (McNeel and Disis, 2000). Possible targets included the prostate-specific membrane antigen (PSMA) (Murphy et al, 1996; Eder et al, 2000), the prostate-specific antigen (PSA) (Kim et al, 1998; Sanda et al, 1999) and prostate acidic phosphatase (PAP) (Fong et al, 2001).

The findings that the existing anti-tumor immune responses are predominantly targeting tissue-specific antigens open a new venue for cancer immunotherapy. In practical terms, however, harnessing autoimmunity for cancer therapy presents several problems:

i. Identification of a target antigen or a combination thereof that will confer protection. In a recent study performed in mice, anti-TRP-1 but not anti-TRP-2 or anti-gp-100 specific T cells induced vitiligo and anti-tumor immunity (Overwijk et al, 1999). This may have been true for the particular mouse strain in that study but it does show that targeting a single antigen based on analysis of T cell responses from tumor bearing patients or animals may be misleading. It also shows the shortcomings of using a single peptide derived from a tissue specific antigen for raising sustained autoimmunity sufficient to eradicate tumor. A cancer vaccine against a multitude of peptides against a tissue-specific antigen will definitely offer some advantages. This approach is strengthened by the discovery that, as is the case with different animal strains, particular autoantigen in different people may manifest different ability to break tolerance and induce autoimmunity (Hammer et al, 1997).

ii. The prostate-specific membrane antigen (PSMA) is a type II integral membrane glycoprotein with a molecular weight of ~100 kDa (Israeli et al, 1993). It has a folate hydrolase, as well as neuropeptidase activity. PSMA is highly expressed in benign prostate secretory-acinar epithelium, prostatic intraepithelial neoplasia and prostate adenocarcinoma (Murphy et al, 1998). There is good evidence that PSMA expression is increased in high Gleason score tumors and in hormone-refractory tumor cells (Troyer et al, 1995), which makes it an excellent target for immunotherapy. More recently, weak expression has been described in several normal tissues such as a subset of proximal renal tubules, duodenal and colonic mucosa. A shorter, alternatively spliced cytosolic form of PSMA, named PSM', is the predominant form expressed in benign prostate epithelium (Grauer et al, 1998). Recently PSMA expression has been detected in tumor neovasculature (Chang et al, 1999), as well as in other healthy tissues both in human (Renneberg et al, 1999) and in mice (Bacich et al, 2001).

II. Clinical trial

Breaking of tolerance to tissue-specific antigens requires presentation of antigen to T cells by specialized, antigen presenting cells: the dendritic cells. This can be performed by a procedure known as naked DNA immunization. We have already performed a clinical trial on immunotherapy of prostate cancer using this approach and we have demonstrated its safety. Beginning in May 1998, we performed in Sofia, Bulgaria, a phase I/II clinical trial for immunotherapy of prostate cancer that targeted the prostate-specific membrane antigen (PSMA). The primary objective of the study was to determine the safety of the described vaccines after repeated intradermal injections (Mincheff et al, 2000a, b, 2001), since using PSMA as a target could be seriously offset by the development of autoimmunity (Overwijk and Restifo, 2000; Gilboa, 2001).

Sixty-five patients were accessed into the study and were repeatedly immunized. Fifty-nine of them were in the study for a period between 2.5 and 3 years. No patient experienced short or long-term side effects including the development of anti-DNA antibody (Mincheff et al,

2000a). We also found that repeated local intradermal injection of rHuGM-CSF (Sargramostim) was a safe procedure and was well tolerated. Heterologous immunization regimen that consisted of two initial intradermal immunizations at 3-week intervals with a cocktail consisting of 200 μg plasmid DNA and 9 IU/m^2 b.s.a., followed by a recombinant adenoviral boost (5x108 PFUs of Ad5PSMA) led to uniform immunization as judged by the development of delayed type hypersensitivity reaction (DTH) to PSMA. DTH was measured 24 and 48 hours following intradermal injection of the plasmid immunization cocktail and was compared to reactions developing after intradermal injection at two separate sites of plasmid cocktail that contained the empty plasmid backbone instead, or of GM-CSF only. The patients were heterogeneous with regard to local advancement of disease, presence of distant metastases, or hormone treatment and refractoriness, which does not permit unequivocal interpretation of the results. Nevertheless, several responders to the immunotherapy could be identified. Twenty-nine patients from this random population were treated solely by immunotherapy (**Table 1**). Eighteen of them had biochemical recurrence following radical prostatectomy and eleven responded to the therapy with a PSA drop exceeding 50% of pre-therapy value (**Table 1**). In contrast, only one of the 11 patients with advanced metastatic disease was influenced by IT with the PSA remaining flat at 10-13 ng/ml and a decrease in bone pains. The remaining 10 patients experienced disease progression despite immunizations.

The PSA curve of a typical responder to immunotherapy is shown on **Figure 3**. The patient was prostatectomized in January, 1996, Gleason score 5, negative margins. Biochemical recurrence was first detected in February, 1999. Immunotherapy, consisting of two plasmid immunizations followed by a recombinant adenoviral boost was initiated in March, 1999. Regular boosts were performed at 3-4 month intervals alternating between the plasmid DNA and the adenoviral vector.

Patients with advanced disease and distant metastases were not influenced by the immunotherapy despite the fact that they all showed signs of T cell immunity towards PSMA. Anti-PSMA immunity was assayed by the presence of PSMA-reactive, γIFN-producing T cells in their peripheral blood (**Figure 2**).

The escape of tumor cells from immune surveillance despite presence of anti-PSMA T cell immunity in those patients could be mediated through a number of mechanisms:

III. Tumor evasion

A. Tumor evasion

Especially in advanced disease with a big tumor load, can be mediated through multiple pathways (Gilboa, 1999a; Ohm et al, 1999; Shah and Lee, 2000; Beck et al, 2001; Cefai et al, 2001; Garrido and Algarra, 2001; Pasche, 2001; Smyth et al, 2001; Carbone and Ohm, 2002; Dunn et al, 2002; Koyama et al, 2002; Ng et al, 2002; Schreiber et al, 2002). Tumor cells secrete lymphokines such as TGF-β and VEGF which suppress dendritic cell and T cell function (Ohm et al, 1999; Shah and Lee, 2000; Beck et al, 2001; Pasche, 2001; Dunn et al, 2002; Koyama et al, 2002). Fas-L and other apoptosis inducing agents are expressed on tumor cells and induce programmed cell death in infiltrating lymphocytes (Cefai et al, 2001; Koyama et al, 2002).

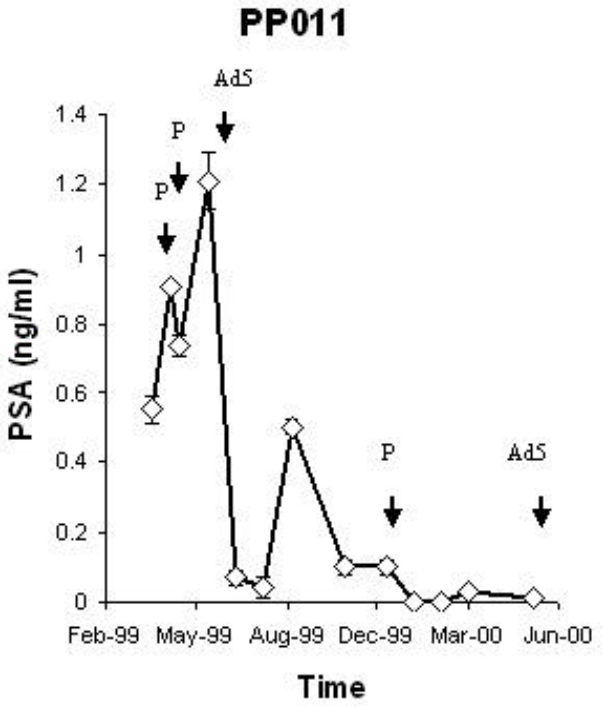

Figure 1. Serum PSA of a patient following radical prostatectomy (1996), biochemical recurrence (January, 1999) and immunotherapy (March 1999 – August 2000). SDs represent three separate determination of PSA in serum derived from three venipunctures on three consecutive days.
(P-thPSMA plasmid; Ad5 – Ad5PSMA)

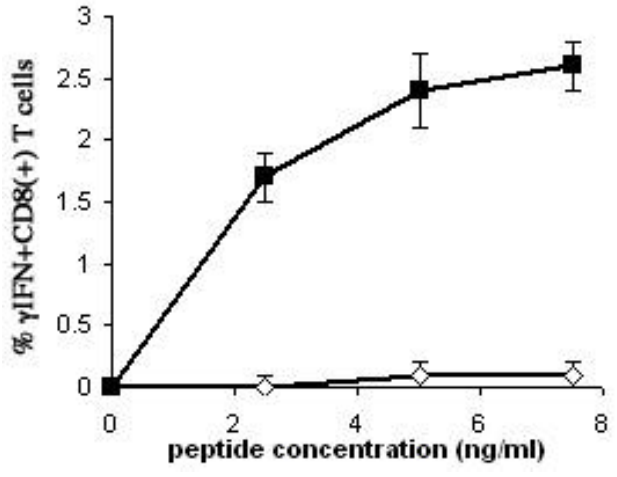

Figure 2. . γ-interferon-positive CD8+T cells following 6-hour stimulation of peripheral blood from HLA-A2+ cancer patients with HLA-A2-specific, PSMA-derived peptide (MMNDQLMFL). Cells were stained using the FastImmune CD8 intracellular cytokine detection kit. Diamonds – prior to immunization (control), squares – post immunization. Data are from five different experiments involving five patients.

B. Immunodominance

The response of the host immune system to only a few of the many possible epitopes in an antigen, additionally exacerbates the problem (Zinkernagel and Doherty, 1979; Yin et al, 1993; Yewdell and Bennink, 1999; Wherry et al, 1999; Belz et al, 2000; Chen et al, 2000; Hislop et al, 2002; Palmowski et al, 2002; Rodriguez et al, 2002). We find gene-based vaccination in its current mode of application effective in breaking tolerance to a self-antigen, but the boosting narrows and restricts the response to few of the potential epitopes (Mincheff et al, 2003). For example, the post-vaccination T cell response of some of the HLA A2 patients from the clinical trial performed by us was directed against only two of the potential 4 PSMA peptide motifs that had high affinity for binding (**Figure 3**).

Table 1. Results from a clinical trial on DNA immunization for immunotherapy of prostate cancer

Outcome	Immunotherapy only	
	Post-Prostatectomy	Distant metastases
Disease Progression	7	10
Improvement (Responders* to Therapy)	11	1
Total Number of Patients	18	11

Responders* – Decrease of PSA exceeding 50% of initial value, decrease in bone pains (where applicable).

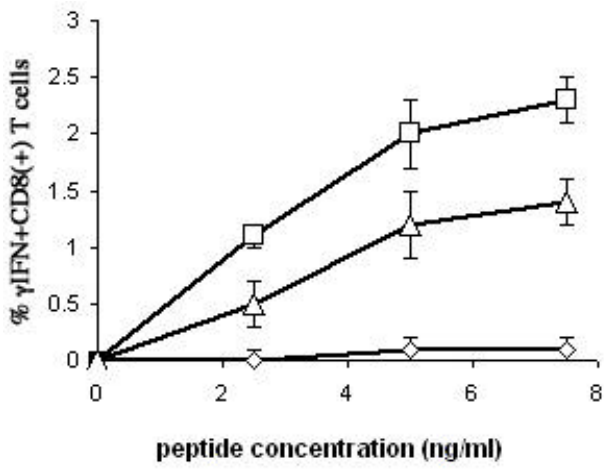

Figure 3. γ-IFN-positive CD3+ T cells following 6-hour stimulation of peripheral blood of HLA-A2+ prostate cancer patients. The following PSMA peptides were identified by BIMAS to bind with high affinity to HLA A2, synthesized and tested in an in vitro assay: MMNDQLMFL ($PSMA_{663}$), ALFDIESKV ($PSMA_{711}$), LMFLERAFI ($PSMA_{668}$) and GIUDALFDI ($PSMA_{707}$). Legend: Stimulation was performed by a) squares – $PSMA_{663}$, b) diamonds – $PSMA_{711}$, c) triangles – $PSMA_{668}$. Results with $PSMA_{707}$ are not shown but are comparable to pre-immunization values (see Figure 1). Data are from three separate experiments with blood from one patient. Cells were stained using the FastImmune CD8 intracellular cytokine detection kit.

Immunodominance ensures the tight specificity of the immune reaction and prevents untoward autoimmunity (Yewdell and Bennink, 1999; Rodriguez et al, 2002). However, it carries the risk of inefficient immune surveillance in cases such as cancer in which mutations of the epitope or downregulation of MHC alleles occur (Hicklin et al, 1998; Hiraki et al, 1999; Dunn et al, 2002; Schreiber et al, 2002). Malignant transformation and tumor progression are frequently associated with loss of HLA class I antigens. For example, a recent review of the literature (Ferrone and Marincola, 1995) reported that ~15% and ~55% of surgically removed primary and metastatic melanoma lesions, respectively, were not stained in immunohistochemical reactions by monoclonal antibodies to monomorphic determinants of HLA class I antigens. Loss or reduced HLA class I antigen expression enables tumor cells to evade the host's immune response (Cordon-Cardo et al, 1991; Rivoltini et al, 1995; Hicklin et al, 1998; de la Salle et al, 1999; Hiraki et al, 1999) and downregulation of HLA class I antigens in metastases from patients with malignant melanoma is associated with poorer prognosis (van Duinen et al, 1988).

Numerous factors combine to establish an immunodominance hierarchy (Yewdell and Bennink, 1999). They include among others:

1. Lack of T cells that are responsive to a sub-dominant epitope (Baldwin et al, 1999)
2. Low affinity of the epitope for binding to MHC (Ma and Kapp, 2001)
3. Ineffective generation and transport of sub-dominant epitopes by APCs (Mo et al, 2000)
4. Intrinsic control of CD8 T cells to respond to sub-dominant epitopes (Noel et al, 1996; Boise and Thompson, 1996; Rabinowitz et al, 1996; Kersh et al, 1998; Schwartz et al, 2001; Guntermann and Alexander, 2002)
5. Extrinsic regulatory networks (T regulatory cells) (Suri-Payer et al, 1998; Thornton and Shevach, 1998; Thornton and Shevach, 2000; Levings et al, 2001; Piccirillo and Shevach, 2001; Shevach, 2001; Sanchez-Fueyo et al, 2002; Sakaguchi, 2003).

We concentrated our efforts on studying the effects of the extrinsic regulatory networks, particularly CTLA-4 and GITR-L signaling and T regulatory cell influence on the establishment of immunodominance during priming and boosting with a gene-based vaccine.

1. Immunodominance and CTLA-4 inhibition

A homologue of CD28, CTLA-4 also binds to the B-7 family members (Greene et al, 1996; Sanchez-Fueyo et al, 2002) but inhibits T cell activation (van der Merwe et al, 1997). Mice lacking CTLA-4 reveal a striking phenotype of polyclonal T cell activation and tissue infiltration which results in death by 3-4 weeks of age, indicating a powerful regulatory role for CTLA-4 (Thompson and Allison, 1997; Waterhouse et al, 1995). Weak signals through the T cell receptor (TCR) are prompt to inhibition (Manzotti et al, 2002) and, at least in vitro, no CTL stimulation to subdominant epitopes occurs if CTLA-4 is not inhibited (Mincheff et al, 2004). Alternatively, CTLA-4 may act as a non-signaling "decoy" receptor reducing the available ligand for CD28 costimulation (Masteller et al, 2000;

Doyle et al, 2001; Mincheff et al, 2004). No matter what the mechanism is, inhibition of CTLA-4 may alleviate immunodominance and thus improve the efficacy of anti-tumor vaccines.

2. CD4+CD25+ T cell depletion and cancer immunodominance

Enhanced priming to sub-dominant epitopes by CTLA-4 inhibition is at least partially mediated through the inhibition of CD4+CD25+ T cell function (Mincheff et al, 2004). These CD4+ T cells are a minor subpopulation (10%) that co-expresses the IL-2 receptor α-chain (CD25) (Sakaguchi et al, 1995) and they can prevent both the induction and effector function of autoreactive T cells (Suri-Payer et al, 1998; Shevach, 2001; Levings et al, 2001). Additionally, they suppress polyclonal T cell activation in vitro by inhibiting IL-2 production (Thornton and Shevach, 1998). Based on these data, we speculate that immunodominance that develops after re-immunization may be reduced by CD4+CD25+ T cell depletion prior to boosting.

3. CD4+CD25+ T cell regulation

Very little is known of the physiologic regulation of CD4+CD25+ T cells in vivo (McHugh et al, 2002). Recent reports suggest that glucocorticoid-induced tumor necrosis factor receptor (GITR), also known as TNFRSF18 – a member of the TNF-nerve growth factor receptor gene superfamily – is predominantly expressed on CD4+CD25+ T cells (McHugh et al, 2002; Shimizu et al, 2002) and stimulation of GITR abrogates CD4+CD25+ T cell-mediated suppression (Shimizu et al, 2002). The gene encoding the natural ligand of murine GITR has been cloned and characterized. The putative GITR ligand (GITR-L) is composed of 173 amino acids with features resembling those of type II membrane proteins and is 51% identical to the human activation-inducible TNF receptor (AITR) ligand, TL6. Expression of the GITR-L is restricted to immature and mature splenic dendritic cells. GITR-L binds GITR expressed on HEK 293 cells and triggers NF-κB activation. Functional studies reveal that soluble CD8-GITR-L prevents CD4+CD25+ regulatory T-cell-mediated suppressive activities (Kim JD et al, 2003). Stimulation through this receptor has been shown to break immunologic tolerance (Shimizu et al, 2002), i.e. it acts similarly to CD4+CD25+ T cell depletion (Kwon et al, 2003).

IV. Immunodominance during priming and boosting

A. "Truncated" vs. secreted vaccines (tVacs vs. sVacs). Dendritic cells transfected with truncated vaccines primes to both dominant and subdominant epitopes of the target antigen

To enhance priming to sub-dominant epitopes, we designed a vaccine (hPSMAT; truncated (tPSMA); tVac)(Mincheff et al, 2003) whose product encoded for only the extracellular domain of PSMA. The product, expressed following transfection with this vector, is retained in the cytosol and is degraded by the proteasomes. For the "secreted" (sPMSA) vaccine, a signal peptide sequence was added to the expression cassette. The expressed protein following transfection with such vaccines is glycosylated and directed to the secretory pathway. Dendritic cells transfected in vitro with tVacs primed T cells to both dominant and subdominant epitopes (Mincheff et al, 2003). Subsequent boosting with antigen-presenting cells (APCs) that expressed both dominant and sub-dominant epitopes, however, narrowed the immune response to the dominant ones (Mincheff et al, 2003). Research from other groups has gained similar results (Firat et al, 1999; Mateo et al, 1999; Loirat et al, 2000; Smith et al, 2001; Palmowski et al, 2002). In all these instances, boosting with polyepitope encoding constructs resulted in failure to expand polyepitope CTLs. A likely explanation is that competition between T cells for antigen on individual APC leads to obscuring of responses to sub-dominant epitopes when both the dominant and subdominant epitopes are present on the same APC (Palmowski et al, 2002; Kedl et al, 2003).

New vaccines (separate DNA vaccines encoding isolated dominant and subdominant epitopes (Barouch et al, 2001) might maximize epitope dispersal among APCs thus inducing broad immunity against numerous epitopes, dominant and subdominant. Due to the HLA polymorphism of the human population, however, construction of such separate vaccines is mainly of academic interest and will have limited therapeutic application. Different approaches for the maintenance of a poly-epitope CTL response following repeated boosting, therefore, are necessary. Some of those are listed below:

B. CTLA-4 inhibition and immunodominance. Addition of anti-CTLA-4 antibodies during priming alleviates immunodominance

We find that in vitro priming to subdominant responses is enhanced by CTLA-4 inhibition (Mincheff et al, 2003). Will similar CTLA-4 inhibition during in vivo re-immunization (boosting) preserve a poly-epitope CTL response (Mincheff et al, 2004)? What will be the cytokine production profile of the sub-dominant T cell clones? T1/T2 polarization (γ-IFN vs. IL-4 secretion) has been shown to depend on the amount of the antigen and on the affinity of the peptide for MHC (Kumar et al, 1995), with weaker signals promoting IL-4 secretion. CTLA-4 inhibition may promote T cell activation at instances of weak T cell receptor engagement (Manzotti et al, 2002). Will there be a difference in the cytokine profile of the sub-dominant clones raised by either minigene re-immunization or CTLA-4 inhibition? Will sub-dominant clones be cytotoxic to tumor cells?

C. CD4+CD25+ T cell prior to priming reduces immunodominance

Results from our laboratory show that the enhanced priming to sub-dominant epitopes by CTLA-4 inhibition is at least partially mediated through the inhibition of

CD4+CD25+ T cell function (Mincheff et al, 2004). For obvious reasons, CD4+CD25+ T cell depletion prior to in vivo boosting may lead to serious side effects (Sakaguchi et al, 2001). Could alleviation of immunodominance be achieved by means other than CD4+CD25+ T cell depletion?

D. In some cases, GITR-signaling during priming reduces immunodominance

We find that while CD4+CD25+ T cell depletion prior to in vitro priming with sVacDCs alleviates immunodominance, co-transfection of dendritic cells with GITR-L does so in some but not all cases(Mincheff et al, 2004). Could immunodominance in vivo be restricted by GITR signaling? Could this be achieved by the co-administration of anti-GITR antibodies or by enhanced GITR-L co-expression during re-immunization? Preliminary results from our laboratory (Mincheff et al, 2004) suggest that in some cases in vitro, co-transfection of dendritic cells with GITR-L alleviate immunodominance.

V. Conclusion

Immunotherapy is a safe, non-invasive, relatively inexpensive procedure that can avoid side effects that often result from surgical, cryosurgical or radiation therapy. Gene based vaccination is effective in breaking tolerance to tumor-associated antigens, but the response is directed towards few of the potential epitopes due to immunodominance. Tumor cells that have lost the immunodominant epitope due to mutations are no-longer recognized and evade immune surveillance. Designing a protocol for immunotherapy, therefore, necessitates stimulation of an immune response directed against a multitude of epitopes. Increasing the number of epitopes available for presentation to T cells is the initial step. It mandates increased degradation of the antigen following DNA immunization and we have already initiated experimentation directed at this (Mincheff et al, 2003). A logical continuation to the current work involves manipulation of the intimate mechanisms controlling the processes of stimulation and/or suppression of T cells recognizing the “sub-dominant” epitopes.

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Milcho Mincheff Serguei Zoubak