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Advances in Brief

Enhancement of Cytolytic T Lymphocyte Precursor Frequency in Melanoma Patients following Immunization with the MAGE-1 Peptide Loaded Antigen Presenting Cell-based Vaccine[1]

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Abstract

Identification of human melanoma-associated peptide antigens for CTLs has opened unprecedented opportunities for active specific immunotherapy for melanoma with synthetic peptide. We have shown that immunization with a *MAGE-1* gene encoded nonapeptide (EADPTGHSY)-pulsed autologous antigen presenting cell-based vaccine induces autologous melanoma-reactive and peptide-specific CTL response, *in situ*, at the vaccination site and at distant tumor deposits in patients who are HLA-A1+ and whose melanoma cells express the MAGE-1 mRNA. Here, we show that such immunization is also capable of increasing the frequency of autologous melanoma-reactive CTL precursors in the circulation. We further show that *in vitro* stimulation of the postimmunization peripheral blood lymphocytes with the MAGE-1 nonapeptide-loaded antigen presenting cell and interleukin-2 leads to significant expansion of peptide-specific and autologous melanoma-reactive CTL response.

Introduction

As a model, human melanoma has served studies of T cell-mediated immune response against human tumors and studies of human tumor antigen remarkably well. In this model, the evidence of T cell-mediated immune response against autologous melanoma cells has been firmly established, and a number of genes encoding melanoma-associated antigens and their peptide products that are recognized by CTLs have been structurally defined (1–10). These developments have provided a valuable reaffirmation of the *raison d'être* for tumor immunology, in general, and have renewed interest in active specific immunotherapy in this model, in particular. Interestingly, with the exception of the recently described melanoma-specific antigen, CDK4 mutant (10), all the other CTL-determined melanoma antigens are "self" antigens (*i.e.*, there is no structural alteration in the encoding genes or in the peptides serving as the antigenic epitope). Although recent studies have proven that CTLs capable of recognizing peptides derived from these self antigens can often be recovered from melanoma patients, neither the question of whether these epitopes are truly immunogenic, *in vivo*, nor the question of how tolerance for these potentially immunogenic epitopes is induced has been fully resolved. We have recently shown that *in vivo* immunization of patients who are HLA-A1 positive and whose melanoma cells express the *MAGE-1* gene with cultured autologous APCs[3] pulsed with *MAGE-1* gene-coded nonapeptide, EADPTGHSY (11), can induce a peptide-specific and autologous melanoma reactive CTL response, *in situ*, at the vaccination site and at distant tumor sites (12). Here, we report that this type of immunization is capable of inducing a peptide-specific CTL response in the circulation as well.

Materials and Methods

Patients. Patients with advanced metastatic melanoma who were HLA-A1 positive and whose melanoma cells expressed the MAGE-1 nonapeptide, EADPTGHSY, were studied with informed consent and with Institutional Review Board approval.

Peptides. Details of peptide synthesis have been published (12). Briefly, the MAGE-1.A1 nonapeptide, EADPTGHSY (11), was synthesized in the Biotechnology Center (University of Connecticut) using the Applied Biosystems peptide synthesizer model 431 A and Fast Moc chemistry. The synthetic peptide was purified by reverse-phase chromatography on a Perkin Elmer Cetus BioLc high-performance liquid chromatography system. The CTL epitope function of the peptide was checked (13) in an *in vitro* stimulation assay against the relevant MAGE-1-specific CTL clone MZ 82/30 (gift of Thierry Boon, Brussels).

Antigen Presenting Cells. Monocytes/macrophages, derived from Ficoll-Hypaque gradient-separated PBLs and grown as adherent populations on plastic dishes, were cultured in AIM-V medium (Life Technologies, Inc.) without any exogenous serum and supplemented with 1000 units/ml of GM-CSF (Immunex) for 2 weeks. Afterward, the cultured cells were harvested, washed, and loaded with the peptides as described previously (12). A cell line exhibiting predominant lymphoblastoid B-cell features (CD3− and B1+) and expressing the characteristic accessory molecules of an APC (MHC class I and II+, ICAM-1+, and B7.1+) was found to grow out of the APC culture from one of our study patients, H. S. This cell line, HS-APC-L, was grown in continuous culture for months in the same culture medium, and these cells expressed the same MHC class I molecules as those of the patient. Because the HS-APC-L cells were found capable of presenting the MAGE-1 nonapeptide to the MAGE-1-specific CTL quite well, they were used in this study for the purpose of *in vitro* stimulation with the relevant peptide-pulsed APCs.

Peptide Pulsing of APCs and Immunization Procedures. The procedure for peptide pulsing and the immunization protocol have been described previously (12). Briefly, the peptide, dissolved in PBS, was loaded on to the APCs at a concentration of 2 $\mu g/10^6$ cells in 1 ml of medium for 2 h at 37°C. After incubation, the unbound peptide was washed off in PBS, and the requisite number of peptide-pulsed APCs were suspended in physiological saline for immunization. The immunization protocol consisted of four monthly intradermal injections of increasing numbers of peptide-pulsed APCs (10^5, 5×10^5, 10^6, and 10^7 cells) for the four consecutive injections. Ten days after the first intradermal injection, each patient was given a single i.v. injection of 10^8 nonapeptide-pulsed autologous APCs, as described previously (12).

***In Vitro* Stimulation.** PBLs isolated on Ficoll-Hypaque gradient, before and after immunization, were stimulated in *in vitro* coculture (1×10^6 cells/ml) in 48-well cluster plates (Costar, Cambridge, MA) with the peptide-loaded, irradiated (100 Gy) stimulatory APC line (HS-APC-L) in IMDM (Life Technologies, Inc.) supplemented with 10% human serum and 50 units/ml of rIL-2 (Collaborative Research). The cocultures were fed every other day, washed, and split as necessary. They were restimulated every 7–10 days.

Phenotypic Analysis. Details have been described previously (14).

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[3] The abbreviations used are: APC, antigen presenting cell; PBL, peripheral blood lymphocyte; IL, interleukin; LDA, limiting dilution analysis; CTLp, CTL precursor; TNF, tumor necrosis factor; DC, dendritic cell.

Microcytotoxicity Assay. The ^{51}Cr-release microcytotoxicity assay has been described previously (14).

LDA of Autologous Melanoma-specific CTLp Frequency. The LDA of CTLp frequency was carried out according to the procedure of Lefkovits and Waldmann (15). Briefly, cryopreserved PBLs obtained before and after immunization were seeded in round-bottom 96-well plates (Costar), at concentrations ranging from 8000 to 62 cells/well using a limiting dilution factor of 1/1 in the presence of a constant number (10,000 cells/well) of the MAGE-1 nonapeptide-pulsed APCs and IL-2 (50 units/ml) in a total volume of 0.2 ml. Forty-eight replicate wells of each PBL dilution were set. The culture medium was partially replaced every 3 days, and the cultures were restimulated with the same number of stimulator cells on day 7. After 14 days of culture, one-half of the contents of each well was removed and tested for the presence of autologous melanoma reactive cytolytic effector cells in the 4-h microcytotoxicity assay in the presence and absence of anti-MHC class I monoclonal antibody W6–32 (a gift of Soldano Ferrone, Valhalla, NY). The cytotoxicity assay was performed in the presence of a 50-fold excess of K-562 cells to eliminate nonspecific lysis. A positive test was defined as one showing MHC class I restricted (*i.e.*, cytolysis inhibitable, equal to or in excess of 50%, by the anti-MCH class I monoclonal antibody) chromium release in test wells above 3 SD of the mean of the spontaneous release. This usually amounted to specific lysis of 15% or higher. The fractions of negative wells were plotted against the number of cells seeded, and a linear regression analysis of best fit was carried out using Statworks (Cricket Software, Philadelphia, PA). On the basis of the Poisson distribution, the frequency of precursors was determined by the slope of the regression lines as per Lefkovits and Waldmann (15).

Cytokine Synthesis Assay. Details of cytokine synthesis assay have been described (12, 13).

Results and Discussion

The freshly isolated and cryopreserved PBLs obtained before and after immunization showed no cytolytic activities against the autologous targets at effector:target ratios as high as 100:1; nor did they synthesize TNF-β upon *in vitro* stimulation with the peptide-loaded APCs (cumulative data not shown). Considering that the absence of direct cytolytic activity or the absence of cytokine synthetic capacity in the unfractionated PBLs might not be a true reflection of the effect of the immunization *in vivo*, we undertook a CTLp frequency analysis in LDA with the pre- and postimmunization PBLs simultaneously. Fig. 1 shows the result of LDA of the autologous melanoma-reactive CTLp frequency analysis of in the PBLs before and after immunization in two of the three patients from whom melanoma cells were available. As shown, the frequency of CTLp in the PBLs of both patients was substantially improved upon immunization. In patient H. S. (Fig. 1*A*), the preimmunization frequency of CTLp was estimated to be approximately 1 in 56,000. The frequency of autologous melanoma-reactive CTLp postimmunization was estimated to have risen to 1 in 3,200. The pre- and postimmunization CTLp frequencies in patient RM (Fig. 1*B*) were found to be approximately 1 in 82,000 and 1 in 4,000, respectively. The frequency of autologous melanoma reactive CTLp in the other patient could not be performed because we were unable to establish a good melanoma cell line from this patient.

Thus, although direct CTL and TNF synthesis assays revealed no evidence of induction of peptide-specific CTL activity in circulation after immunization, we decided to examine whether or not such activity could be induced upon further *in vitro* stimulation and expansion of the stimulated T cells in the presence of IL-2. To follow a standard protocol of *in vitro* stimulation, we used an APC line, HS-APC-L, that was established from the leucopheresed blood from one of the study patients. This APC line was grown in continuous culture in AIM V medium (Life Technologies, Inc.) supplemented with 1000 units/ml of GM-CSF (Immunex) without any exogenous serum. These cultured cells exhibited many characteristic features of professional APC [MHC class I and II+, ICAM-1+, B-7.1 (CD80)+], and they presented the MAGE-1 nonapeptide, EADPTGHSY, efficiently to the MAGE-1 CTL 82/30 (cumulative data not shown). Thus, for each experiment, *in vitro* cocultures were set up with the cryopreserved pre- and postimmunized PBLs derived from each patient, followed by functional assays on day 7 through 21 (cytolysis, cytokine synthesis, specificity of function).

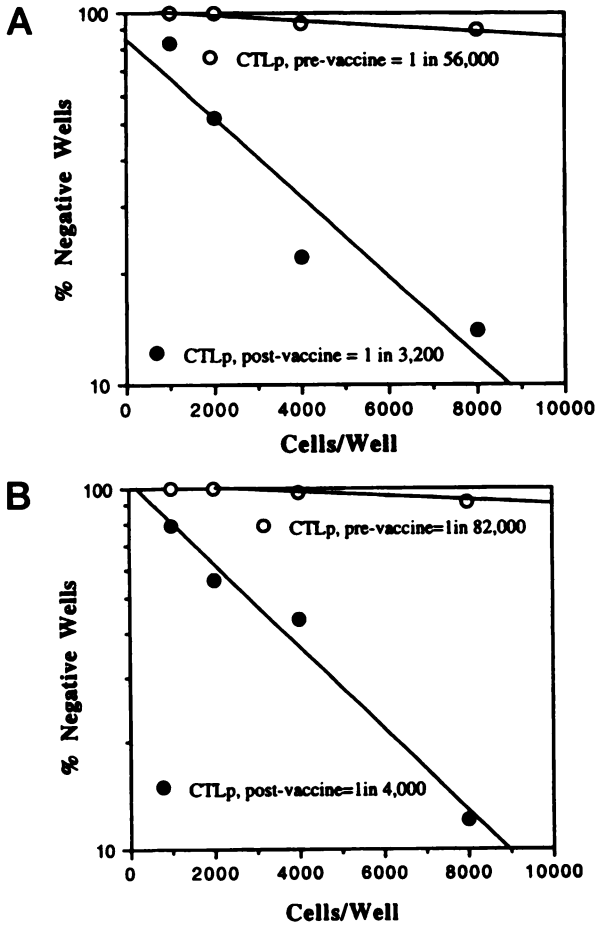

Fig. 1. Frequencies of CTLp directed to the autologous melanoma cells after immunization with the peptide-loaded APCs. The frequency of CTLp was determined by the slope of the regression lines (for details, see "Results and Discussion"). *A*, LDA of CTLp frequency analysis in patient H. S. *B*, LDA of CTLp frequency analysis in patient R. M.

Table 1 shows a representative cytotoxicity assay with the *in vitro* stimulated pre- and postimmunization PBLs. As shown, the preimmunization PBLs showed no evidence of peptide-specific or autoreactive CTL activity. In contrast, the *in vitro* stimulation and expansion of the effector cells in IL-2 generated a remarkable degree of MAGE-1 nonapeptide-specific CTL activity in the postimmunized PBLs from all three patients. In addition, the *in vitro* stimulated postimmunization PBLs also made TNF-β when stimulated by the autologous melanoma cells and/or by the peptide-loaded HLA-A1+ APCs (Table 2). Again, under identical culture conditions, no cytokine synthesis was observed with the preimmunized PBLs from all three patients. Only the MAGE-1.A1 nonapeptide, EADPTGHSY, effectively stimulated the postimmunization PBLs *in vitro* (Tables 1 and 2). It is noteworthy that although the patients were simultaneously immunized with autologous APC loaded with the octapeptide (ADPTGHSY), cocultures with the octapeptide-loaded APCs did not induce either an octapeptide-specific or the nonapeptide-specific CTL response in the postimmunization PBLs (Tables 1 and 2). The inducibility of CTL activity against the autologous melanoma targets could not be tested in all three patients because we could not establish a good melanoma line from the third patient. However, the *in vitro*

Table 1 *Peptide-specific CTL induction in PBLs upon in vitro peptide presentation and expansion*

	% cytotoxicity by the *in vitro* stimulated PBLs[a]																	
	HS system						RM system						RL system					
	Preimmune			Postimmune[b]			Preimmune			Postimmune			Preimmune			Postimmune		
	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10
MZ 3.1[c]			4	33	29	36			3	18	38	35	9	9	17	10	25	29
MZ 2.2			0	1	5	3			2	0	2	3	4	1	6	4	7	11
MZ 2.2 + EADPTGHSY			0	29	37	49			4	11	16	22	8	12	12	16	30	37
MZ 2.2 + ADPTGHSY			0	1	2	8			3	2	2	5	1	6	2	4	7	8
HS-M	2	3	3	49	60	63			4	14	17	25	8	12	16	34	44	59
RM-M									5	12	15	24			10			34
RL-F + EADPTGHSY															8			50
K-562			7			10			9	8	11	11			9			11

[a] Cryopreserved pre- and postimmune PBLs were stimulated by the MAGE-1 nonapeptide-pulsed APCs in coculture. Cytotoxicity was tested on day 7 or 8 of *in vitro* stimulation.
[b] PBLs were isolated 7 days after the fourth intradermal immunization.
[c] MZ 3.1, MAGE-1+/HLA-A1+ allogeneic target line; MZ 2.2, MAGE-1−/HLA-A1+ variant line; HS-M, melanoma line derived from patient H. S.; RM-M, melanoma line derived from patient R. M.; RL-F, fibroblasts derived from patient R. L.

stimulated postimmunized PBLs from this patient were able to lyse the relevant peptide-loaded autologous fibroblasts (Table 1, RL system). The phenotypic analyses of the *in vitro* stimulated preimmunized PBLs showed mixed phenotypes (both CD4+ and CD8+ T cells). In contrast, CD8+ cells were more selectively expanded in the postimmunized PBLs after *in vitro* stimulation with the MAGE-1 nonapeptide-loaded APCs (Fig. 2).

The emergence of peptide-specific effector function in the *in vitro* coculture could be observed in the *in vitro* stimulated postimmunized PBLs as early as the 7th day. Continued growth and function of the effector cells could be maintained up to 4–5 weeks, after which, consistent growth and function of the effector cells could not be maintained, despite a number of different manipulations (collective data not shown).

Of all the CTL-determined melanoma antigens that have thus far been structurally defined, the antigens coded by the *MAGE* family of genes and their respective peptides are particularly attractive from an immunotherapeutic perspective because, other than in the testes, these genes are not expressed in any adult tissues. Accordingly, the question of whether or not any one of these peptides could be used, in one formulation or another, to generate a peptide-specific and autologous melanoma reactive CTL response, *in vivo*, is crucially important. In this context, the results of our pilot study, communicated earlier (12) and presented here, strongly suggest that the MAGE-1 nonapeptide, EADPTGHSY, loaded on to cultured autologous APCs, is immunogenic *in vivo*. That is, the vaccine recruits a CTL response *in situ* in the vaccination site, in distant tumor sites, and in circulation. It should, however, be pointed out that it is by no means certain that the peptide is immunogenic only when administered onto APCs. Nevertheless, the theoretical rationale for using APCs to present a CTL-determined peptide *in vivo* is understandable.

The potential usefulness of "APCs" in general, and of DCs in particular, in the design of cancer vaccine has generated much interest lately (16). The superiority of DCs as APCs and as initiators of T cell-dependent immune response has been shown in a number of experimental systems (17). Accordingly, it has been generally believed that DCs are likely to be better APCs than are monocyte/macrophages in cancer vaccine design (18). Because isolation of "pure DCs" in humans is not an easy task, and although we have not compared the monocyte/macrophage-derived APCs with DCs in our model, it should be noted that the APCs used in our study share many of the characteristics of DCs and that they present peptide antigens and provide co-stimulation quite well, *in vitro* as well as *in vivo* (12, 13). Thus, until a satisfactory method for large-scale isolation of DCs is worked out, monocyte/macrophage-derived APCs remain a reasonable option in cancer vaccine design in humans. Further methodological manipulations can also be introduced to make them a better vehicle for delivery of antigenic peptide(s) and co-stimulatory signals. A number of such manipulations are presently under study in our laboratory.

LDA of precursor frequency has been a powerful technique for assessing the clonal basis of immune response. This particular assay

Table 2 *TNF synthesis by pre- and postimmunized PBLs upon peptide presentation*

	TNF (pg/ml) synthesized by 3×10^4 PBLs[a]					
	HS System		RM System		RL System	
Stimulated by:	Preimmune	Postimmune	Preimmune	Postimmune	Preimmune	Postimmune
None	1 (0)[b]	3 (2)	4 (6)	5 (6)	1 (0)	3 (2)
HS-APC	14 (7)	8 (6)	4 (12)	6 (6)	14 (7)	8 (6)
HS-APC + EADPTGHSY	5 (6)	64 (7)	4 (10)	35 (6)	5 (6)	64 (7)
HS-APC + ADPTGHSY	6 (6)	6 (5)	7 (4)	4 (5)	6 (6)	6 (5)
MZ 3.1[c]	3 (4)	54 (7)	3 (0)	32 (5)	4 (3)	54 (7)
MZ 2.2	3 (3)	2 (3)	1 (16)	1 (6)	3 (3)	2 (3)
MZ 2.2 + EADPTGHSY	4 (4)	28 (5)	5 (7)	36 (6)	4 (4)	28 (5)
MZ 2.2 + ADPTGHSY	6 (3)	0 (4)	5 (15)	5 (6)	6 (3)	0 (4)
HS-M	0 0	40 (0)			0 0	40 (0)
RM-M			3 (9)	65 (6)		65 (6)
RL-F					4	8
RL-F + EADPTGHSY					3	120
RL-F + ADPTGHSY					4	20

[a] TNF synthesis by the PBLs, when stimulated by MAGE-1 nonapeptide (EADPTGHSY)-pulsed APCs, was examined around day 21 of coculture.
[b] Numbers in parentheses represent TNF synthesis by the PBL stimulated by the HS-APC pulsed with the control octapeptide ADPTGHSY.
[c] MZ 3.1, MAGE-1+/HLA-A1+ allogeneic target line; MZ 2.2, MAGE-1-/HLA-A1+ variant line; HS-M, melanoma line derived from patient H. S.; RM-M, melanoma line derived from patient R. M.; RL-F, fibroblasts derived from patient R. L.

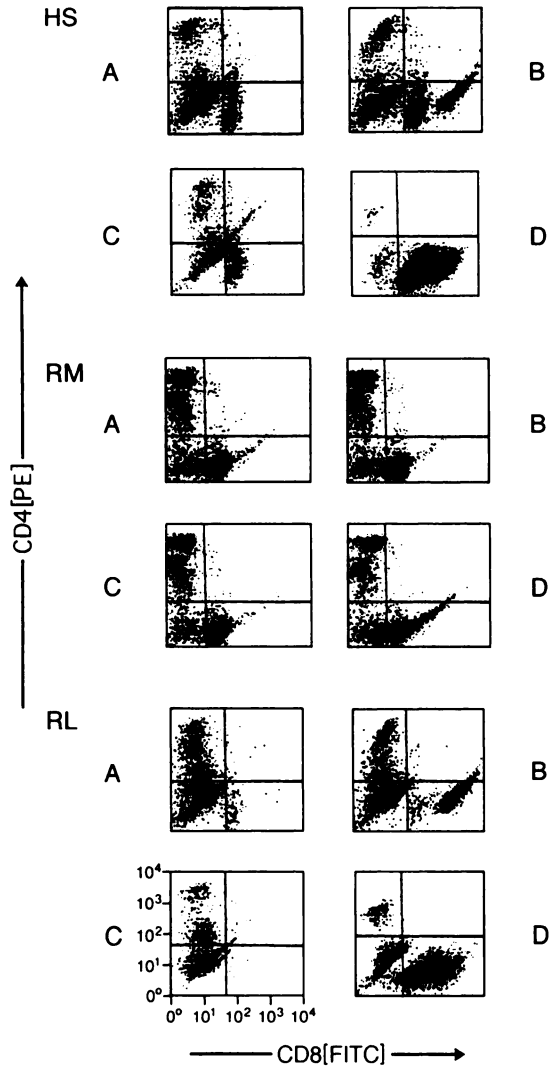

Fig. 2. Phenotype of the PBLs in coculture with the MAGE-1 nonapeptide pulsed HS-APC-L cells performed in two-color cytofluorometric analysis [CD4.PE (*Y* axis)/ CD8.FITC (*X* axis)]. In each panel, *A* and *B* represent the preimmunization PBLs stimulated with control peptide (ADPTGHSY)-pulsed APCs and with the MAGE-1 nonapeptide (EADPTGHSY)-pulsed APCs, respectively; whereas *C* and *D* represent the postimmunization PBLs stimulated in identical fashion. *C*, control peptide-pulsed APCs; *D*, MAGE-1 nonapeptide-pulsed APCs.

has allowed a critical examination of the crucial question of whether or not a given vaccine can improve the respective antigen-specific precursor frequency. In this context, the result of our study is noteworthy. The data reveal that the immunization substantially improved the precursor frequency of autologous melanoma-specific CTL and show that peptide-specific and autologous melanoma-reactive CTL could be expanded from the postimmunized PBLs after *in vitro* stimulation with the peptide-pulsed APCs and IL-2. This provides a strong affirmation of our underlying hypothesis that the immunological unresponsiveness toward autologous cancer cells that express a CTL-determined epitope might be broken through immunization with a vaccine made of autologous APCs made to deliver the antigenic epitope(s) and the accessory co-stimulatory signal(s) simultaneously. The results reveal an interesting paradox, however: despite the fact that the immunization induced an autologous melanoma-reactive CTL response, *in situ* and in circulation, no major protective antitumor response was seen in these cases. We have considered a number of explanations (inadequate dose or duration of immunization, wrong time to immunize, too much disease, inadequate peptide presentation by the melanoma cells, *in vivo*, and so forth) for the absence of major therapeutic response (12). Although these explanations are theoretically valid, it is possible that the absence of protective response might have resulted from insufficient amplification of fully activated and functional effector cells. An increase in the CTLp frequency, unless accompanied by concomitant amplification of fully activated and functional effector cells, might not be enough. After all, an effective protective or rejection response will require clonal expansion of a sufficient number of fully activated CTL to participate, directly or indirectly, in the rejection response. Presently, other than measuring function in relatively crude assays, we have no satisfactory way of evaluating whether or not immunization amplifies fully activated and antigen-specific functional CTLs. Thus, it is by no means clear whether an increase in the precursor frequency can be taken as a reflection of clonal expansion of those precursors to their functionally activated effector cell state. We believe that this issue is relevant, especially in the context of developing successful active specific cancer immunotherapy with antigens that are essentially self antigens. Formidable constraints exist in the way for self antigen-specific CTLs to undergo substantial amplification *in vivo*. We have encountered one such peripheral constraint imposed by the subsequent emergence of Th2-type CD4+T cells in our model.[4] Regardless of this or other constraints, when the results of our pilot study are taken collectively, the evidence of induction of autologous tumor-reactive and peptide-specific CTL response, *in situ* and in circulation, provides strong encouragement to the search for ways to enhance the efficacy of specific peptide- and APC-based vaccine in melanoma *in vivo*.

Acknowledgments

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