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Melanoma vaccines: the paradox of T cell activation without clinical response

Abstract In recent years significant progress in the understanding of the immune biology of melanoma has evolved from the identification of melanoma antigens (MAs) recognized by T cells. MAs consist of intracellular proteins that are expressed on the surface of cancer cells in association with human leukocyte antigen (HLA) class I molecules and therefore are suitable targets for cytotoxic T lymphocytes (CTLs). Several new monitoring strategies have been implemented to evaluate the status of activation and localization of vaccine-induced T cells in the peripheral circulation as well as the tumor site, including limiting dilution, in vitro sensitization, and ELISPOT. Previous studies aimed at monitoring patients receiving vaccination have utilized mainly those three methods. These methods have demonstrated that antigen-specific vaccination can elicit immune responses detectable in the peripheral blood of immunized patients. These assays, however, have been faulted by their requirement for in vitro expansion of T cells (limiting dilution or in vitro sensitization) or for limited sensitivity (ELISPOT). More recently, the use of soluble HLA/peptide complex tetramers, intracellular fluorescence-activated cell sorting (FACS) analysis, and real-time polymerase chain reaction (PCR) has been proposed for the monitoring of vaccine trials. These methods have the appeal of allowing direct enumeration of T cells specific for a particular epitope within relevant samples such as peripheral blood lymphocytes, lymph nodes, and tumors. We are evaluating whether utilizing a combination of HLA/peptide tetramer (tHLA) together with Taqman-based real-time reverse-transcription (RT)-PCR and intracellular FACS analysis could establish a direct and comprehensive strategy for the assessment of epitope-specific immune response in vivo. In conditions close to those of the tumor microenvironment or in peripheral blood lymphocytes, however, a different status of T cell activation can be expected due to a direct stimulation of T cells by tumor or antigen-presenting cells. We observed that activated T cells can easily be detected in the peripheral blood of patients who have received MA-specific vaccines. However, when T cells are stimulated with their relevant epitope, a high level of T cell receptor downregulation occurs that does not allow identification of vaccine-specific T cells directly with tHLA. Thus evaluation of epitope-specific T cells at the tumor site, where they might be exposed to stimulation by interaction with tumor cells and/or in bulk peripheral blood mononuclear cells, might be more efficiently analyzed with functional methods such as intracellular FACS and Taqman-based real time RT-PCR.

Key words Tumor immunity · Vaccination · Cytotoxic T lymphocytes · Localization · Human leukocyte antigen tetramers

Introduction

In recent years significant progress in the understanding of the immune biology of melanoma has evolved from the identification of melanoma antigens (MAs) recognized by T cells [3, 22]. MAs consist of intracellular proteins that are expressed on the surface of cancer cells in association with human leukocyte antigen (HLA) class I molecules and therefore are suitable targets for cytotoxic T lymphocytes (CTLs). The peptide sequences (epitopes) from MAs responsible for recognition by CTLs in association with different HLA class I alleles have been identified and used for the vaccination of

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patients with advanced melanoma [23]. These studies have demonstrated that most MAs are expressed similarly by tumors from different patients and therefore vaccines can be developed that could be used to immunize patients with advanced melanoma.

Clinical trials performed at the US National Cancer Institute have shown that epitope-specific vaccination is effective in eliciting expansion of melanoma-specific CTL reactivity in vivo [20, 24]. As previously reported, the treatment of metastatic melanoma patients with a synthetically modified gp100 peptide (209-2M) resulted in clinical tumor regression in 13 of 31 patients (42%), but only three of 19 (16%) patients demonstrated peripheral blood reactivity in the in vitro sensitization (IVS) assay [23]. However, inexplicably the amplification of immune responses caused by these vaccines is in most cases not sufficient to cause cancer regression unless the systemic administration of interleukin (IL)-2 is added to the treatment. The paradoxical coexistence within the same organism of anticancer CTLs with cancer cells remains an enigma [17]. It is not presently clear whether the immune response induced by the vaccine is quantitatively insufficient or lacks some unknown qualities that could sustain it when started.

Understanding the induced immune response equals understanding the induced CTL response. Should this response be described by CTL function (status of activation), antigen binding properties (precursor frequency), or both? This is a common question in the viral model when effector T cell responses are being evaluated. The same question can be raised when monitoring the response of cancer patients to vaccination. Several monitoring strategies have been implemented to evaluate the status of activation and localization of vaccine-induced T cells in the peripheral circulation as well as the tumor site. Among those are in vitro sensitization [20, 24], limiting dilution assays [5], and ELISPOT [25]. The ELISPOT assay has enjoyed notable popularity because of its simplicity, relative accuracy, and sensitivity [25].

Analysis of postvaccination peripheral blood mononuclear cells (PBMCs) from patients treated with g209-2M peptide in incomplete Freund adjuvant identified T cells specific for the altered epitope in four of six patients [21]. In the four patients, the estimated frequencies ranged between 1/1,000 and 1/2,000 epitope-specific T cells [21]. In no patient was it possible to identify T cells recognizing the natural epitope (g209) by the ELISPOT assay, while limiting dilution assays estimated precursor frequencies for g209-reactive T cells to range between 1/3,000 and 1/6,000 [23]. Because of the dependency upon cytokine secretion/proliferation, these assays may underestimate the actual frequency of CTL precursors by not identifying T cells with a threshold for cytokine expression/proliferation above the stimulus applied in the assay [26]. Moreover, naive T cells, which are less responsive to epitope-specific stimulation, might be missed by these functional assays [6]. Recently the use of HLA/epitope tetramers [1] has offered a tool to measure the frequency of CTL precursors directly, presumably independently of their functional state. Measurements by this assay demonstrated CTL precursor frequencies considerably higher than those suggested by ELISPOT or limiting dilution assays [26].

The use of soluble HLA/peptide complex tetramers has been also complemented by other techniques that allow direct measurement of T cell reactivity in tested samples such as intracellular fluorescence-activated cell sorting (FACS) analysis [11] and measurement of cytokine gene expression by real-time polymerase chain reaction (PCR) [9, 12]. Although in vitro sensitization assays have suggested increased MA-specific CTL reactivity in response to epitope-based vaccination, direct quantitative assessment of the extent of these immune responses in terms of the number of precursors and their status of activation cannot be performed.

The recent introduction of more sensitive quantitation methods to analyze the level of response has provided a new insight into T cells in different models. However, the major difference between the viral system and tumor model is the precursor frequency of antigen-specific T cells. Frequently, the viral system provides a substantial amount of effector cells, as measured by tetramers [2, 4, 8, 13, 18, 26]. This is in contrast to the tumor model where the results so far describe a very low but still promising number of effector cells raised after vaccination [16].

Gallimore et al. have shown in viral models that in some situations where there is excessive antigen, antigen-specific T cells can be detectable using tetramers, although these cells exhibit a diminished capacity to produce interferon (IFN)-γ and lytic activity in vitro [7]. Data presented by Zajac et al. include a critical finding that would have been missed had tetramers not been available [28]. Their data suggest that antiviral CTLs can persist in vivo in a nonfunctional state. Lee et al. have reported similar findings in a melanoma model [15]. They identified antigen-specific $CD8^+$ T cells in the peripheral circulation in six of 11 patients with metastatic melanoma using peptide-specific tetramers. The CTLs from one patient were found to be in an anergic state since they did not directly lyse melanoma target cells or produce cytokines, although these cells expressed surface marker belonging to the group of effector markers [15]. This does not agree with data obtained by Lee et al., who detected tetramer-positive CTLs that responded to peptide stimulation by production of IFN-γ, as measured by intracellular FACS [16]. Furthermore these cells expressed surface receptors belonging to the activated/memory category.

What is the explanation for the silenced phenotype of some CTLs? Utilizing a combination of HLA/peptide tetramer (tHLA) together with Taqman-based real-time reverse transcription (RT)-PCR and intracellular FACS analysis as a direct and comprehensive method for the assessment of epitope-specific immune response in vivo might give further insight into the functional level of the antigen-specific T cells and answer that question.

Pre- and postvaccination samples from both the peripheral circulation and the tumor site from patients treated with epitope-specific vaccination are helpful when these questions are raised.

We have analyzed specific T cell precursor frequencies in pre- and postvaccination PBMCs from 12 patients vaccinated with g209-2M peptide emulsified in incomplete Freund adjuvant; five of the patients received a combination of peptide and IL-12 (Table 1). Epitope-specific T cells were analyzed using HLA-A2 tetramers. Phycoerythrin-tHLA-A*0201 complexes (tHLA) were synthesized as described previously [1]. tHLA reagents were generated for the following epitopes: gp100:209–217 (ITDQVTCPFSV, g209); gp100:209-217 (210 M) (IMDQVTCPFSV, g209-2M); and FluM1: 58–66 (GILGFVFTL, Flu). Table 1 lists the ratio (pre/postvaccination) of the calculated precursor frequencies of peptide-specific $CD8^+$ cells in PBMCs on day 1. All patients were found to have a ratio above 1, correlating with an induced response after vaccination. This also shows that peptide-based vaccination can increase the amount of antigen-specific T cells. P1 and P5 showed the best response, with a ratio of 15 for P1 for g209-2M-peptide-specific cells and a ratio of 42.3 for P5 for g209-peptide-specific cells. The addition of IL-12 had no affect on the calculated ratio.

As seen in other models, peptide vaccination increases the number of antigen-specific CTLs. This enhancement can be detected with tHLA complexes as a new tool to measure very low numbers of specific cells. The low number of T cell precursors in the melanoma model has led to various attempts to increase the number of specific cells. Among these, several involving the sorting of antigen-specific cells by selecting the tetramer-positive cells, followed by in vitro expansion before further analysis, have shown good results [27].

Another method used to increase the number of antigen-specific cells is in vitro sensitization [20, 24]. Antigen-specific T cell reactivity is increased in response to peptide stimulation. Using this method, we have evaluated whether enhancement of T cell precursor frequencies secondary to vaccination (Table 2) corresponds to increased number of peptide-specific T cells after in vitro stimulation with the relevant epitope. PBMCs from the same group of patients as listed in Table 1 were stimulated with 209-peptide on day 1 and thereafter incubated for 10 days with the addition of IL-2 every other day. At day 10 the cells were stained with tHLA against the specific epitope. We evaluated the antigen-specific response and calculated precursor frequencies in terms of the ratio of pre/postvaccination antigen-specific cells. The calculations show that in vitro stimulation with g209 leads to a peptide-specific expansion in the post-vaccination samples.

An important question when monitoring patients is the nature of the response in postvaccination samples after direct stimulation with peptide in vitro. Analyzing the IFN-γ production by intracellular FACS together with tHLA staining determines the nature of the specific responding cells. We analyzed pre- and postvaccination PBMCs from a patient treated with a combination of gp100 and MART-1 vaccination. Nonadherent PBMCs were stimulated for 6 h in vitro with peptide. After 2 h, Brefeldin A was added. After another 4 h, the cells were fixed and stained for analysis. As shown in Fig. 1, the samples before treatment showed only slight staining with g209 tHLA. At the same time, no IFN-γ was produced after stimulation with either g209 or MART-1 peptide. In contrast, the postvaccination samples exhibited increased tHLA staining (0.06–0.20), and after stimulation with g209 IFN-γ was produced. We found, however, that activated T cells with a high level of TCR downregulation due to activation cannot be assessed directly with tHLA (Fig. 1).

An important question is whether some tetramer-positive cells may not be detected due to TCR downregulation. Thus epitope-specific T cells at the tumor site might be more efficiently analyzed using functional methods such as intracellular FACS and Taqman-based real time RT-PCR. We analyzed the reactivity of

Table 1 Calculated precursor frequencies (ratio pre/postvaccination) of peptide-specific $CD8^+$ cells in PBMCs on day 1 (peptide-specific/10^6 $CD8^+$ cells)

	g209	g209-2M	Flu
Peptide alone			
P1	5.9	15.15	6.30
P2	4.3	2.22	1.41
P3	1.6	2.76	1.10
P4	8.0	9.30	–
P5	42.3	2.40	1.20
P6	1.9	2.30	1.20
P7	4.4	3.80	5.80
Peptide + IL-12			
P8	8.2	11.20	0.60
P9	4.4	4.30	1.40
P10	11.1	14.40	1.70
P11	0.8	1.10	2.00
P12	3.1	10.80	0.80

Table 2 Calculated precursor frequencies (ratio pre/postvaccination) of g209-specific $CD8^+$ cells on day 10 (peptide-specific 10^6 $CD8^+$ cells)

	g209	g209-stim
Peptide alone		
P1	5.9	34.0
P2	4.3	0.8
P3	1.6	290.6
P4	8.0	1037
P5	42.3	22.6
P6	1.9	59.7
P7	4.4	5.5
Peptide + IL-12		
P8	8.2	–
P9	4.4	6.1
P10	11.1	4.0
P11	0.8	36.0
P12	3.1	–

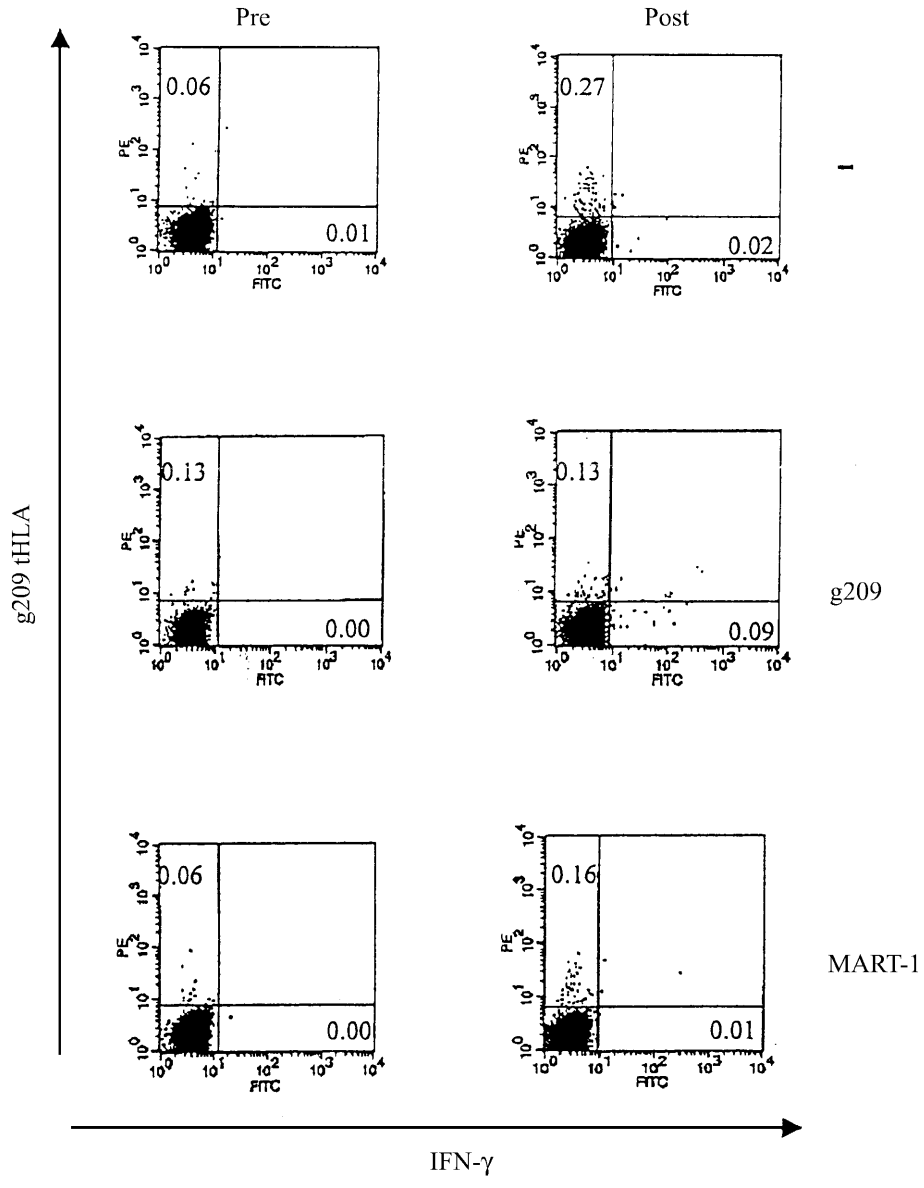

Fig. 1 FACS analysis of intracellular IFN-γ expression in pre- and postvaccination PBMCs. The PBMCs were obtained after two vaccinations and were stimulated with g209 or MART-1 peptide 1 μM. $CD3^+/CD8^+$ cells were gated for analysis and numbers indicate the percentage of cells in the quadrant over total gated cells. g209 tHLA staining decreased upon stimulation due to downregulation of TCR, as previously noted in the analysis of epitope-specific clonal populations (data not shown)

PBMCs after peptide stimulation in vitro utilizing real-time quantitative RT-PCR. Measurement of gene expression was performed using the ABI Prism 7700 Sequence Detection System (ABI, Foster City, CA, USA), as previously described [10]. As shown in Table 3, the reactivity of PBMCs from patients immunized with 209-2M peptide in incomplete Freund adjuvant showed a ratio (fold increase; pre/postimmunization) >1 in seven of nine patients. Correlating the production of mRNA with that of IFN-γ using IC FACS analysis allows for both functional and epitope-specific analysis of PBMCs from peptide-treated patients.

Since the frequency of specific T cells at the tumor site might be more relevant, it should be noted that in conditions close to the tumor microenvironment a combination of T cells with different status of activation can be expected due to direct stimulation of T cells by tumor or antigen-presenting cells. Ideally, comparison should be made of T cell precursor frequency in specimens obtained from the same lesion before and after treatment. We have previously shown that material obtained from fine-needle aspirate biop-

Table 3 Reactivity of PBMCs from patients immunized with 209-2M peptide in incomplete Freund adjuvant (fold increase pre/postvaccination)

209-2M peptide	g209-2M
P1	6.1
P2	95.9
P3	8.1
P4	4.2
P5	3.5
P6	2.2
P7	1.5
P8	1.1
P9	0.9

sies can be used for serial evaluation of identical lesions [14, 19]. Thus we are collecting specimens from fine-needle aspirate biopsies from patients undergoing various vaccination protocols to be studied with the combination of methods that we have previously described.

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