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ORIGINAL ARTICLE

**Marieke Griffioen · Martina Borghi · Peter I. Schrier
Susanne Osanto · Dirk Schadendorf**

Analysis of T-cell responses in metastatic melanoma patients vaccinated with dendritic cells pulsed with tumor lysates

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Abstract In melanoma patients, $CD8^+$ cytotoxic T cells have been found recognizing self-proteins of which the expression is restricted to the melanocytic lineage. These melanocyte differentiation antigens are expressed in normal melanocytes as well as in 80–100% of primary and metastatic melanoma. In this report, six HLA-A*0201–subtyped metastatic melanoma patients vaccinated with dendritic cells (DCs) pulsed with autologous tumor lysates and keyhole limpet hemocyanin (KLH) were screened for the presence of $CD8^+$ T cells specific for three HLA-A*0201–binding peptides derived from the melanosomal antigens MART-1/Melan-A, gp100, and tyrosinase. For this purpose, nonstimulated as well as in vitro peptide-stimulated peripheral blood mononuclear cells (PBMCs) were tested for peptide-specific IFN-γ release by enzyme-linked immunosorbent spot (ELISpot) assays. Furthermore, expression of the melanosomal antigens MART-1/Melan-A, gp100, and tyrosinase in tumor lesions was analyzed by immunohistochemistry before and after vaccination. We also used the ELISpot technique to investigate whether KLH-specific T cells were induced and whether these cells released type 1 (IFN-γ) and/or type 2 (IL-13) cytokines. Our data show induction of $CD8^+$ T cells specific for the melanosomal peptides MART-1/Melan-$A_{27–35}$ or tyrosinase$_{1–9}$, as well as IFN-γ–releasing KLH-specific T cells, in two of six vaccinated melanoma patients, but do not support an association between the induction of these T cells and clinical responses.

Keywords Dendritic cells · Melanoma · T lymphocytes · Vaccination

M. Griffioen · M. Borghi · P. I. Schrier · S. Osanto
Department of Clinical Oncology,
Leiden University Medical Center,
Leiden, The Netherlands

D. Schadendorf (✉)
Skin Cancer Unit, German Cancer Research Center
and Department of Dermatology,
University Hospital Mannheim,
Universität Heidelberg,
Theodor Kutzer Ufer 1,
68167 Mannheim, Germany
E-mail: d.schadendorf@dkfz.de
Tel.: +49-621-3832127
Fax: +49-621-3832163

Introduction

In melanoma patients, $CD8^+$ cytotoxic T cells (CTLs) have been found recognizing self-proteins of which the expression is restricted to the melanocytic lineage (differentiation antigens: MART-1/Melan-A, gp100, and tyrosinase) or testis (cancer-testis antigens: MAGE, BAGE, and GAGE). Melanocyte differentiation antigens are expressed in normal melanocytes as well as in a high proportion of primary and metastatic melanoma (80–100%) [7]. Cancer-testis antigens are expressed in tumors of different histological origins, but not in normal tissues, except for the testis. Most cancer-testis antigens are less frequently expressed in human melanoma than melanocyte differentiation antigens. The *MAGE* genes, for example, are expressed in 30-40% of primary and 70–80% of metastatic melanoma [5].

The isolation of tumor antigens led to the development of new strategies for immunizing patients. Several vaccination trials using HLA class I–binding peptides derived from MAGE-3 [17] or MART-1/Melan-A [14], gp100 [14, 23], and tyrosinase [14] reported complete and partial remissions. Since it became clear that dendritic cells (DCs) are the antigen-presenting cells (APCs) that initiate immune responses in vivo, clinical studies were started using peptide-pulsed DCs as a vaccine. Nestle et al. [18] vaccinated melanoma patients with DCs pulsed with autologous tumor lysates or peptides derived from MART-1/Melan-A, gp100, and tyrosinase or MAGE-1 and MAGE-3. Six of 16

patients showed partial or complete remissions as well as peptide-specific delayed-type hypersensitivity (DTH) reactivity. In another clinical trial, regressions of individual metastases were observed in 6 of 11 patients vaccinated with mature DCs pulsed with the MAGE-3 peptide [33]. Although a clear association between immunological and clinical responses has not yet been demonstrated, the remissions using DC-based approaches for vaccination are encouraging; however, a proof of efficacy is still missing.

To evaluate the efficacy of T cell–based therapies, reliable methods for immunological monitoring of antigen-specific T cells are a prerequisite. Several methods, e.g., fluorescent MHC-tetrameric complexes and enzyme-linked immunosorbent spot (ELISpot) assays, promise a sensitive quantification of antigen-specific T cells directly from peripheral blood samples [22, 24]. MHC-tetrameric complexes not only allow detection and quantification, but also phenotypic characterization, selection, and expansion of peptide-specific T cells. The ELISpot technique visualizes cytokine release by single cells and enables detection of T-cell responses not only to peptides, but also to whole proteins and cells [31].

Although CTLs can act alone when killing target cells, their differentiation from naïve $CD8^+$ T cells often depends on "help" from $CD4^+$ T cells [4, 21, 32]. Newly activated $CD4^+$ T cells commit early to a pathway of differentiation that results in the formation of two functionally distinct subsets: T helper 1 (T_H1) and T_H2 cells, each producing a specific set of cytokines. It is generally believed that T_H1-mediated responses are most beneficial in the context of tumor protection. DCs provide not only antigenic and activating signals that initiate clonal expansion of T cells, but also polarizing signals leading to T_H1 or T_H2 immunity. Upon CD40 ligation or maturation with monocyte-conditioned medium, for example, DCs produce IL-12 that skews differentiation of $CD4^+$ T cells toward T_H1 [15]. Therefore, delivery of tumor antigens by fully mature, T_H1-stimulating DCs are highly promising vaccines against cancer.

In this report, we screened six metastatic melanoma patients vaccinated with DCs pulsed with autologous tumor lysates and keyhole limpet hemocyanin (KLH) for the induction of peptide-specific $CD8^+$ T cells. Since no autologous tumor lysate was available anymore for further analysis, T-cell reactivity specific for three peptides derived from the melanosomal antigens MART-1/Melan-A, gp100, and tyrosinase was measured by ELISpot. Furthermore the induction of type 1 (IFN-γ) and/or type 2 (IL-13) cytokine-releasing T cells specific for KLH was analyzed by the ELISpot technique. Our data show induction of $CD8^+$ T cells specific for the melanosomal peptides MART-1/Melan-$A_{27–35}$ or tyrosinase$_{1–9}$, as well as IFN-γ–releasing KLH-specific T cells, in two of six melanoma patients, but do not support an association between the induction of these T cells and clinical responses.

Materials and methods

Peptides

Peptides were synthesized by solid-phase methods using an automated multiple peptide synthesizer (Abimed AMS 422) and Fmoc chemistry. The purity of the peptides was higher than 90% as determined by reversed phase HPLC. Peptides were dissolved in dimethyl sulfoxide at 100 mg/ml and stored at −70°C. From this stock solution, peptide was dissolved in Iscove's Modified Dulbecco's Medium (IMDM) at 1 mg/ml, adjusted to pH 7.0 and stored at −20°C. The following HLA-A*0201–binding peptides were synthesized: MART-1/Melan-$A_{27–35}$ AAGIGILTV [8, 16], gp100$_{154–162}$ KTWGQYWQV [2], tyrosinase$_{1–9}$ MLLAVLYCL [34], and flu$_{58–66}$ GILGFVFTL [3].

Generation of dendritic cells (DCs) from peripheral blood

Leukocytes were prepared from peripheral blood (100 ml) according to previously published reports [18] using Ficoll density centrifugation. DCs were generated as described previously [18]. Briefly, PBMCs were resuspended in RPMI 1640 plus 10% FCS (Life Technologies, Eggenstein, Germany) and allowed to adhere to plastic dishes (Costar, Cambridge, MA, USA). After 2 h at 37°C, the nonadherent cells were removed, and adherent cells were subsequently cultured for 7 days with GM-CSF (800 U/ml; kindly provided by Dr U. Haus, Novartis, Nürnberg, Germany) and IL-4 (500 U/ml; PharMingen, Hamburg, Germany). Phenotypic changes were monitored by light microscopy and flow cytometry.

Pulsing of in vitro generated DCs

Routinely, 1×10^6 DCs were generated after 7 days of culture in GM-CSF and IL-4. DCs were pulsed with tumor lysates derived from autologous tumor tissue at a concentration of 100 μg/ml for 4 h at RT. In parallel, DCs were pulsed with 50 μg/ml keyhole limpet hemocyanin (KLH) *Megathura crenulata* protein (Calbiochem, Bad Soden, Germany). For injection, DCs were washed three times in sterile PBS and resuspended in a total volume of 0.5-ml PBS.

Preparation of tumor lysate

Tumor tissue was immediately placed into PBS or medium after surgical removal. Subsequently, adjacent nonmalignant tissue was macroscopically removed by scalpel, and tumor cells were dispersed to create create single-cell suspensions. Aliquots were taken for cell counting and viability staining by trypan blue. For lysate preparation, cells were disrupted by repeated (4–5 times) freezing (on liquid nitrogen) and thawing (at RT) cycles. Lysis was monitored by light microscopy. Larger particles were removed by centrifugation (10 min, 600 rpm), supernatants were passed through a 0.2-μm filter, protein contents determined, and aliquots stored at −80°C until use.

Delayed-type hypersensitivity (DTH)

DTH skin tests were done as described [18]. Briefly, tumor-lysate pulsed DCs and nonpulsed DCs were intradermally injected before vaccination and at weeks 6 and 10 after start.

Melanoma patients

Six advanced melanoma patients with American Joint Committee on Cancer (AJCC) stage disease IV were vaccinated with DCs pulsed with autologous tumor lysates and KLH, as described by

Nestle et al. [18]. The vaccine preparation was administered intralymphatically into an uninvolved inguinal lymph node or injected in close proximity to the regional lymph nodes. Eligible melanoma patients received four vaccinations at weekly intervals. The fifth vaccination was administered during week 6, and immunization was subsequently continued at monthly intervals for up to ten vaccinations, depending on clinical response. All six melanoma patients were HLA-typed using standard serological techniques and subtyped as HLA-A*0201 by PCR. Two of the six patients were previously described by Nestle et al. [18] as patient 4 (patient Bo) and patient 7 (patient Fe).

In vitro stimulation of PBMCs

Total PBMCs were isolated by Ficoll Hypaque density gradient centrifugation from heparinized blood. PBMCs were seeded in 24-well plates at 3×10^6 cells/well in IMDM containing 10% human serum (HS), 2-mM glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin, 300 IU/ml human rIL-2 (Chiron, Amsterdam, The Netherlands), and 2 μg/ml of MART-1/Melan-$A_{27–35}$, $gp100_{154–162}$, and $tyrosinase_{1-9}$ or $flu_{58–66}$ at 37°C and 8% CO_2. Fresh medium containing rIL-2 was added every 2 to 3 days. After 12 days, total PBMCs were collected and tested for peptide-specific IFN-γ release in ELISpot assays. In addition, PBMCs were seeded in 24-well plates at 2×10^6 cells/well in RPMI containing 5% fetal calf serum (FCS), 2-mM glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin. PBMCs (2×10^6) were either not stimulated or stimulated with 5 μg/ml KLH (Sigma, Zwijndrecht, The Netherlands) or 10 μg/ml phytohemagglutinin (PHA; Murex Biotech, Dartford, UK). After 48 h, total PBMCs were collected and tested for release of IFN-γ and IL-13 in ELISpot assays. All PBMCs derived from the same patient at different time points were tested simultaneously.

IFN-γ ELISpot assay

Ninety-six-well nylon Silent Screen plates (Nalge Nunc International) were coated with 100 μl of a mouse monoclonal antibody against human IFN-γ (1-D1 K; Mabtech, Nacka, Sweden) diluted to 5 μg/ml in PBS overnight at 4°C. Wells were washed several times with PBS and blocked with 100 μl of IMDM + 5% HS for 1 h at 37°C. The HLA-A*0201-processing-defective T2 cell line [25] was cultured in IMDM + 5% FCS, washed several times, and pulsed with MART-1/Melan-$A_{27–35}$, $gp100_{154–162}$, and $tyrosinase_{1-9}$ or flu_{58-66}, at 5 μg/ml for 2 h in IMDM at 37°C. After several washes, peptide-pulsed T2 cells were seeded at 1×10^5 cells/well together with 1×10^5 nonstimulated PBMCs or 5×10^4 and 1×10^4 peptide-stimulated PBMCs in triplicate in 200 μl of IMDM + 5% HS overnight at 37°C. PBMCs stimulated with KLH were seeded at 1.5×10^5 cells/well and PBMCs stimulated with PHA at 1.5×10^5 and 1.5×10^4 cells/well in triplicate in 200 μl of RPMI + 5% FCS overnight at 37°C. Spots were visualized as described previously [11]. Briefly, wells were washed several times with PBS + 0.05% Tween 20 (PBST) and incubated with 100 μl of a biotinylated mouse antibody against human IFN-γ (7-B6-1-Biotin; Mabtech) diluted at 0.3 μg/ml in PBS for 2 h at RT. After several washes with PBST, wells were incubated with 100 μl of a solution containing complexes between avidin and biotinylated horseradish peroxidase (Vectastain ABC system; Vector Laboratories, Burlingame, CA, USA) for 1 h at RT. Wells were washed several times and the colorimetric reaction was started by adding 100 μl of a substrate solution containing 3-amino-9-ethylcarbazol (Sigma). After 5 min, the colorimetric reaction was stopped under running water and spots were counted by computer-assisted video image analysis (CVIA) using KS ELISpot software release 4.1 (Carl Zeiss Vision, Hallbergmoos, Germany). For statistical analysis, Student's *t*-test for unpaired samples was used.

IL-13 ELISpot assay

Ninety-six-well ELISA plates (Greiner, Alphen aan den Rijn, The Netherlands) were coated with 100 μl of an antibody against human IL-13 (QS-13; U-CyTech, Utrecht, The Netherlands) diluted to 10 μg/ml in PBS overnight at 4°C. Wells were washed several times with PBST and blocked with 200 μl of PBS + 1% BSA for 1 h at 37°C. PBMCs stimulated with KLH were seeded at 1.5×10^5 cells/well and PBMCs stimulated with PHA at 1.5×10^5 and 1.5×10^4 cells/well in triplicate in 200 μl of RPMI + 5% FCS for 4–5 h at 37°C. After removal of the cells, spots were visualized as previously described for the rhesus macaques IFN-γ ELISpot assay by Allen et al. [1], according to the manufacturer's instructions (U-CyTech, Utrecht, The Netherlands). Briefly, wells were incubated with 200 μl of ice-cold deionized water and placed for 10 min on melting ice. After several washes with PBST, wells were incubated with 100 μl of a biotinylated polyclonal antibody against human IL-13 diluted in PBS + 1% BSA overnight at 4°C. Wells were washed several times with PBST and incubated with 50 μl of a gold-labeled goat antibiotin antibody (GABA) diluted in PBS + 1% BSA for 1 h at 37°C. After several washes with PBST, wells were incubated with 30 μl of an activation solution containing activator I and II mixed at 1:1 (v/v). Clear spots appeared between 15 and 30 min, and the reaction was stopped by rinsing the wells with distilled water. Spots were counted by manual light microscopy. For statistical analysis, Student's *t*-test for unpaired samples was used.

Results

Six advanced melanoma patients vaccinated with DCs pulsed with autologous tumor lysates and KLH were screened for the induction of T cells specific for KLH, and $CD8^+$ T cells specific for the broadly-expressed melanocyte differentiation antigens MART-1/Melan-A, gp100, and tyrosinase. Expression of the melanocyte differentiation antigens in tumor lesions was analyzed by immunohistochemistry before and after vaccination. All six melanoma patients were HLA-A*0201–subtyped and had metastatic melanoma AJCC stage disease IV (Table 1).

Table 1 Patient characteristics, status before vaccination, and response to vaccination. *NED*no evident disease, *CR* complete response,*PR* partial response, *PD* progressive disease

[a] Palliative; stage IV (AJCC)
[b]Since entry into the vaccination study

Patient	HLA type	Previous immunotherapy	Metastases before vaccination	Number of vaccinations received	Clinical response
Me	HLA-A*0201	IFN-α/IL-2[a]	None	10	NED 1.8 years[b]
Br	HLA-A*0201	IFN-α/IL-2[a]	None	6	NED 6.5+ years[b]
Bo	HLA-A*0201	None	Skin, lung	9	CR 5.5+ years[b]
Fe	HLA-A*0201	IFN-α[1]	Skin	9	PR
Je	HLA-A*0201	IFN-α/IL-2[1]	LN, skin, parotis	10	PD
Ko	HLA-A*0201	IFN-α[1]	Retropertional LN, lung	5	PD

Clinical response

Among the six patients analyzed, four patients had measurable metastatic lesions at study entry (patients Bo, Fe, Je, and Ko) and thus could be evaluated for clinical response (Table 1). Two of these four patients showed a response: one a complete (patient Bo) and one a partial (patient Fe) response to treatment. Patient Bo had multiple frequently recurring skin metatastases on his right thigh which was not any longer controllable by surgery, laser, kryotherapy, or radiation. In addition, four lung metastases up to 1 cm were present before vaccination. Patient Fe had hundreds of skin metastases covering the entire leg. Two other patients had tumor progression within the first 10 weeks (patient Je; extensive cutaneous and lymph node involvement) and 6 weeks (patient Ko, visceral metastases) of vaccination. Patient Bo has been disease-free for more than 5.5 years since the last vaccination, whereas patient Fe died 2.4 years after the end of immunization of a cause not related to melanoma. Two (patient Me and Br) of the six patients had recurrent skin metastases prior to vaccination, which were resected. Since these patients were macroscopically free of disease at the beginning of vaccination, they could not be evaluated for clinical response. However, their clinical course has been remarkable, since these patients have been disease-free for more than 3.5 years (patient Br) and 1.8 years (patient Me) since the last vaccination.

KLH-specific T cells

Total PBMCs derived from patients before and after vaccination were tested for release of type 1 (IFN-γ) and type 2 (IL-13) cytokines in ELISpot assays after 48 h of preincubation with KLH and, as a positive control, PHA (Table 2). All PBMCs released IFN-γ as well as IL-13 after stimulation with PHA, demonstrating that the PBMCs were not damaged during the freezing and thawing procedure before the assay. KLH-specific IFN-γ–releasing T cells were measured in two (patients Br and Ko) of six melanoma patients at all time points after vaccination, whereas these T cells could not be detected before vaccination. In one (patient Bo) of six melanoma patients, KLH-specific IL-13–releasing T cells might have been induced, although these T cells were only measurable at the eighth, and not at the ninth, vaccination. Therefore, these data show that vaccination with DCs pulsed with autologous tumor lysates and KLH led to the induction of KLH-specific IFN-γ–releasing T cells in two of six melanoma patients.

Melanosomal peptide-specific $CD8^+$ T cells

Total PBMCs were tested for release of IFN-γ in ELISpot assays upon incubation with T2 cells pulsed with HLA-A*0201–binding peptides derived from MART-1/Melan-A (MART-1/Melan-$A_{27–35}$), gp100 ($gp100_{154–162}$), tyrosinase ($tyrosinase_{1–9}$), and, as a control, influenza ($flu_{58–66}$). Total PBMCs derived from patients before and after vaccination were either not stimulated (Table 3) or stimulated in vitro with single peptides for 12 days (Table 4).

$CD8^+$ T cells specific for MART-1/Melan-$A_{27–35}$, $gp100_{154–162}$, and $tyrosinase_{1–9}$ were rarely detected among nonstimulated PBMC (Table 3). Similarly, $CD8^+$ T cells specific for $flu_{58–66}$ were measured among nonstimulated PBMCs in only one of six melanoma patients at the seventh vaccination. However, after in vitro peptide stimulation, $flu_{58–66}$-specific $CD8^+$ T cells could be detected in four of six melanoma patients at almost all time points examined (Table 4), indicating that utilizing the ELISpot assay, peptide-specific $CD8^+$ T cells can be measured among peptide-stimulated, but not noncultured PBMCs.

After in vitro peptide stimulation, $CD8^+$ T cells specific for MART-1/Melan-$A_{27–35}$ could be measured only before, and not after, vaccination in patient Me. $CD8^+$ T cells specific for $gp100_{154–162}$ and $tyrosinase_{1–9}$ were not detected in this patient at any time point

Table 2 Mean numbers of IFN-γ and IL-13 ELISpots produced by 1.5×10^5 peripheral blood mononuclear cells (PBMCs) upon stimulation with keyhole limpet hemocyanin (KLH) and phytohaemagglutinin (PHA)

Patient	Vaccination[a]	IFN-γ			IL-13		
		–	KLH	PHA[b]	–	KLH	PHA
Me	1	2	2	**85**[c]	0	1	**430***
	4	1	3	**73**[c]	0	1	**320c**
	7	3	5	**72**[c]	2	4	**600**[c]
	10	1	3	**90**[c]	0	0	**340**[c]
Br	1	0	2	**199**[c]	2	0	**740**[c]
	5	23	**88**[c]	**217**[c]	44	27	**650**[c]
	6	25	**85**[c]	**261**[c]	36	28	**650**[c]
Bo	1	0	1	**400**[c]	0	0	**570**[c]
	8	4	2	**410**[c]	12	**26**[c]	**570**[c]
	9	2	3	**530**[c]	22	24	**720**[c]
Fe	1	1	1	**450**[c]	0	1	**120**[c]
	6	2	1	**490**[c]	0	0	**140**[c]
Je	1	0	0	**350**[c]	1	1	**490**[c]
	4	1	2	**340**[c]	2	0	**480**[c]
	6	1	0	**250**[c]	0	1	**480**[c]
	10	2	1	**490**[c]	0	0	**420**[c]
Ko	1	0	0	**210**[c]	0	1	**84**[c]
	4	3	**19**[c]	**203**[c]	0	2	**79**[c]
	5	4	**23**[c]	**237**[c]	1	1	**74**[c]

[a]Time point of patients' PBMC collection for ELISpot analysis. PBMCs were collected prior to the planned vaccination as indicated by the numbers (*1* prior to the first vaccination,*4* prior to the fourth vaccination, etc.). The first four vaccinations were administered at weekly intervals, the fifth vaccination during week 6, and immunization continued at monthly intervals for up to ten vaccinations

[b]If the number of spots produced by 1.5×10^5 PHA-stimulated PBMCs could not be accurately measured, the number of spots produced by 1.5×10^4 PBMCs were counted and multiplied by 10

[c]Indicates that the number of spots produced by PBMCs stimulated with KLH or PHA is significantly higher ($p < 0.05$) than the number of spots produced by nonstimulated PBMCs. For statistical analysis, Student's *t*-test for unpaired samples was used

Table 3 Mean numbers of IFN-γ ELISpots produced by 1×10^5 nonstimulated peripheral blood mononuclear cells (PBMCs) upon incubation with (peptide-pulsed) T2 cells

Patient	Vaccination[a]	T2	T2 + $Mart_{27–35}$	T2 + $gp100_{154–162}$	T2 + $tyrosinase_{1–9}$	T2 + $flu_{58–66}$
Me	1	3	4	2	2	5
	4	4	4	2	1	4
	7	9	6	13	11	**20**[b]
	10	3	6	4	**8**[b]	6
Br	1	27	30	42	32	20
	5	40	53	50	44	46
	6	76	88	123	113	75
Bo	1	50	30	36	37	72
	5	36	44	38	43	49
	8	30	27	21	30	26
	9	156	128	107	116	107
Fe	1	13	8	6	8	8
	4	21	32	14	26	39
	6	31	33	45	41	29
	9	23	25	18	16	22
Je	1	16	19	14	22	23
	4	6	10	9	7	7
	6	24	19	17	23	26
	8	3	2	3	6	2
	10	11	21	13	19	16
Ko	1	2	**9**[b]	4	4	3
	4	38	41	29	26	31
	5	27	39	45	**55**[b]	46

[a]Time point of patients' PBMC collection for ELISpot analysis. PBMCs were collected prior to the planned vaccination as indicated by the numbers (*1* prior to the first vaccination,*4* prior to the fourth vaccination, etc.). The first four vaccinations were administered at weekly intervals, the fifth vaccination during week 6, and immunization continued at monthly intervals for up to ten vaccinations

[b]Indicates that the number of spots produced by nonstimulated PBMCs upon incubation with peptide-pulsed T2 cells is significantly higher ($p < 0.05$) than the number of spots produced upon incubation with nonloaded T2 cells. For statistical analysis, Student's *t*-test for unpaired samples was used

examined, demonstrating that vaccination did not induce $CD8^+$ T cells specific for the three melanosomal peptides in patient Me. In patient Br, however, $CD8^+$ T cells specific for MART-1/Melan-$A_{27–35}$ were measured at all time points after vaccination, whereas these T cells could not be detected before vaccination. In this patient, similar numbers of $gp100_{154–162}$-specific $CD8^+$ T cells could be measured before vaccination as well as at the fifth vaccination. Also in patient Bo, similar numbers of $CD8^+$ T cells specific for MART-1/Melan-$A_{27–35}$ could be measured before vaccination as well as at the eighth vaccination. In patient Fe, MART-1/Melan-$A_{27–35}$ and $tyrosinase_{1–9}$-specific $CD8^+$ T cells were measured at two and one of the three time points after vaccination, respectively, whereas these T cells could not be detected before vaccination. In this patient, $gp100_{154–162}$-specific $CD8^+$ T cells were measured only before, and not after, vaccination. Also in patient Je, MART-1/Melan-$A_{27–35}$-specific $CD8^+$ T cells could be measured only before vaccination. Finally, patient Ko had detectable numbers of MART-1/Melan-$A_{27–35}$-specific $CD8^+$ T cells before vaccination, that were markedly increased after vaccination. Similarly, high numbers of $tyrosinase_{1–9}$-specific $CD8^+$ T cells were detected after vaccination, whereas these T cells could not be measured before vaccination. Taken together, these data show that vaccination with DCs pulsed with autologous tumor lysates led to the induction of $CD8^+$ T cells specific MART-1/Melan-$A_{27–35}$ in three (patients Br, Fe, and Ko) of six melanoma patients. $CD8^+$ T cells specific for $tyrosinase_{1–9}$ were induced by vaccination in two (patients Fe and Ko) of six patients. Remarkably, in two of these patients (patients Br and Ko), vaccination also led to the induction of KLH-specific IFN-γ–releasing T cells. Finally, patient Ko, who had high numbers of MART-1/Melan-$A_{27–35}$- and $tyrosinase_{1–9}$-specific $CD8^+$ T cells after vaccination, suffered from rapidly progressive disease (Table 1). Therefore, these data do not demonstrate an association between induction of melanosomal peptide-specific $CD8^+$ T cells and clinical response.

Discussion

Several reports have shown that the high sensitivity of the ELISpot assay allows direct quantitative assessment of antigen-reactive T cells from peripheral blood. By measuring IFN-γ release by nonstimulated PBMCs upon incubation with peptide-pulsed T2 cells, we rarely detected $CD8^+$ T cells specific for influenza and melanosomal peptides, whereas these T cells could readily be measured after 12 days of in vitro peptide stimulation. Similarly, Pass et al. [19], using the same ELISpot technique as described in this study, measured gp100-specific $CD8^+$ T cells among in vitro peptide-stimulated, but not noncultured PBMCs. However, Scheibenbogen

Table 4 Mean numbers of IFN-γ ELISpots produced by 5×10^4 peptide-stimulated peripheral blood mononuclear cells (PBMCs) upon incubation with (peptide-pulsed) T2 cells

Patient	Vaccination[a]	PBMCs stimulated with:							
		$Mart_{27–35}$		$gp100_{154–162}$		$tyrosinase_{1–9}$		$flu_{58–66}$	
		T2	T2 + $Mart_{27–35}$	T2	T2 + $gp100_{154–162}$	T2	T2 + $tyrosinase_{1–9}$	T2	T2 + $flu_{58–66}$
Me	1	13	**32**[b]	11	18	5	9	19	**256**[c]
	4	7	8	7	6	12	15	40	**205**[c]
	7	0	0	1	1	4	3	6	**89**[c]
	10	2	4	3	3	4	8	6	**265**[c]
Br	1	2	2	0	**17**[b]	1	1	2	6
	5	6	**27**[b]	4	**11**[b]	0	0	1	3
	6	3	**18**[b]	1	6	4	4	2	5
Bo	1	4	**10**[b]	1	1	2	1	2	**37**[b]
	5	2	2	1	1	1	2	1	4
	8	2	**18**[b]	9	8	6	4	12	**41**[b]
	9	20	20	21	14	15	21	6	**13**[b]
Fe	1	10	8	9	**41**[b]	8	6	23	**123**[b]
	4	42	**86**[b]	98	78	9	**21**[b]	4	**187**[b]
	6	22	32	26	34	5	3	4	**28**[b]
	9	6	**17**[b]	7	12	3	3	13	**137**[b]
Je	1	1	**7**[b]	0	2	1	2	22	**93**[b]
	4	1	2	0	0	1	2	73	**149**[b]
	6	1	1	0	0	2	1	21	**144**[b]
	8	0	0	0	2	1	0	2	**256**[b]
	10	1	1	0	0	0	0	50	**113**[b]
Ko	1	0	**33**[b]	25	33	39	54	22	30
	4	7	**132**[b]	2	1	1.0	**16**[b]	2	1.5
	5	11	**184**[b]	3	5	6.5	**117**[b]	3	3

[a]Time point of patients' PBMC collection for ELISpot analysis. PBMCs were collected prior to the planned vaccination as indicated by the numbers (*1* prior to the first vaccination,*4* prior to the fourth vaccination, etc.). The first four vaccinations were administered at weekly intervals, the fifth vaccination during week 6, and immunization continued at monthly intervals for up to ten vaccinations

[b]Indicates that the number of spots produced by peptide-stimulated PBMCs upon incubation with peptide-pulsed T2 cells is significantly higher ($p < 0.05$) than the number of spots produced upon incubation with nonloaded T2 cells. For statistical analysis, Student's *t*-test for unpaired samples was used

[c]Number of spots produced by 1×10^4 PBMCs are shown

et al. [26, 27] detected influenza-reactive $CD8^+$ T cells in 9 (36%) of 25 melanoma patients and 18 (75%) of 24 healthy controls by measuring IFN-γ release by non-cultured PBMCs upon stimulation with peptide. These data suggest that PBMCs might be better antigen-presenting cells than T2 cells. It can be speculated that T2 cells may impair the sensitivity of the ELISpot assay by stimulating alloreactive T cells.

Since it has been published that the frequency of influenza-reactive $CD8^+$ T cells remains stable during a 1-year period with low intraindividual variability in healthy persons [29], we assessed the frequency of these T cells as a control for T-cell reactivity during vaccination. After in vitro stimulation, we measured influenza-specific $CD8^+$ T cells in four of six melanoma patients at almost all time points during vaccination, whereas we consistently failed to detect influenza-specific $CD8^+$ T cells in patients Ko and Br. In melanoma patients, a decrease of influenza-reactive $CD8^+$ T cells has been observed at the time of relapse [30]. Although this might explain the lack of influenza-reactive $CD8^+$ T cells in patient Ko, who progressed quite rapidly, $CD8^+$ T cells specific for influenza were also not detectable in patient Bo, who has been free of disease for more than 5.5 years since study entry.

The ELISpot technique has successfully been used to detect tumor-specific $CD8^+$ T cells in peripheral blood of melanoma patients [31]. Herr et al. [12] measured $CD8^+$ T cells specific for MART-1/Melan-A and tyrosinase in all four individuals they examined, including two healthy donors and two melanoma patients. Scheibenbogen et al. [27] measured melanosomal peptide-reactive $CD8^+$ T cells in 5 of 25 melanoma patients. $CD8^+$ T cells specific for tyrosinase are particularly interesting, since these T cells were shown to be present in 4 of 7 melanoma patients who responded to treatment with chemotherapy and IFN-γ ± IL-2 [30]. The ELISpot technique also proved to be useful to monitor tumor-specific $CD8^+$ T cells in peripheral blood of melanoma patients during vaccination [31]. Reynolds et al. [20] showed the induction of $CD8^+$ T cells specific for MAGE-3 and/or MART-1/Melan-A in 9 of 15 patients vaccinated with an extract of four melanoma cell lines. In peptide-based vaccination trials, tyrosinase-reactive $CD8^+$ T cells were shown to be induced in 4 of 15 patients vaccinated with tyrosinase peptides plus GMCSF [28], and gp100-specific $CD8^+$ T cells in almost all patients vaccinated with gp100 peptides [19].

In this report, we show the induction of $CD8^+$ T cells specific for the melanosomal peptides MART-1/Melan-$A_{27–35}$ or $tyrosinase_{1–9}$ in three of six melanoma patients vaccinated with DCs pulsed with autologous tumor

lysates which were not any longer available for immunological analysis. High numbers of MART-1/Melan-$A_{27–35}$-specific and tyrosinase$_{1–9}$-specific $CD8^+$ T cells were induced in patient Ko, whereas low numbers of MART-1/Melan-$A_{27–35}$-specific $CD8^+$ T cells were observed in patient Br and low numbers of MART-1/Melan-$A_{27–35}$-specific and tyrosinase$_{1–9}$-specific $CD8^+$ T cells in patient Fe. Since patient Ko progressed rapidly, these data do not support an association between the induction of melanosomal peptide-specific $CD8^+$ T cells and clinical response.

However, since no metastases of this patient were available for immunohistochemical analysis, it can be speculated that the melanoma cells escaped destruction by the immune system due to loss of expression of MART-1/Melan-A and tyrosinase. In a recently reported study, IFN-γ secretion upon peptide encounter was measured during ALVAC-p53 vaccination in late stage colonic cancer patients. The authors concluded that p53-specific T-cell responses were not accompanied by clinical responses [6].

Thus far, most vaccination trials have failed to demonstrate a clear association between immunological and clinical responses. In patients vaccinated with the MAGE-3 peptide, partial and complete remissions of single metastases were observed, but expansion of MAGE-3–specific CTLs could not be detected [17]. Similarly, clinical responses, but no expansion of gp100-specific CTLs, were observed in patients vaccinated with gp100 peptides plus IL-2, whereas in patients vaccinated with gp100 peptides in the absence of IL-2, expansion of gp100-specific CTLs, but no clinical responses, were measured [23]. Nestle et al. [18] demonstrated partial or complete remissions as well as peptide-specific DTH reactivity in 6 of 16 melanoma patients vaccinated with DCs pulsed with peptides or autologous tumor lysates. However, peptide-specific DTH reactivity was also observed in 5 of 10 patients with progressive disease.

DCs provide antigenic and activating signals that initiate clonal expansion of T cells as well as polarizing signals leading to T_H1 or T_H2 immunity [15]. T_H1 cells stimulate $CD8^+$ T cell–mediated immune responses and are therefore believed to be beneficial for tumor protection. To investigate whether vaccination with DCs pulsed with autologous tumor lysates and KLH leads to the induction of a T_H1- or T_H2-mediated response, we measured release of type 1 (IFN-γ) and type 2 (IL-13) cytokines by total PBMCs upon stimulation with KLH in ELISpot assays. Our data show the induction of KLH-specific IFN-γ–releasing T cells in two of six melanoma patients. Remarkably, in these two patients, melanosomal peptide-specific $CD8^+$ T cells were also induced, suggesting that in these patients, vaccination led to the initiation of a T_H1-mediated response. Interestingly, both patients with clinical response (Bo, Fe) did demonstrate strong DTH reactivity in KLH skin testing [18], however, patients' PBMCs from peripheral blood failed to elicit an IFN-y release upon KLH encounter in ELISpot. Whether that is related to the semimature phenotype of the DCs generated [10] or whether this is a reflection of different T-cell subsets in skin and peripheral blood needs further investigation. Several reports have demonstrated that mature, IL-12–producing DCs induce T_H1-mediated immune responses, whereas T_H2-mediated responses are generated by immature DCs [13, 15]. The requirement of mature DCs for inducing an effective immune response has been further emphasized by Dhodapkar et al. [9], who have shown that vaccination with immature DCs pulsed with influenza matrix peptide led to the specific inhibition of influenza-specific $CD8^+$ T-cell effector function and the appearance of influenza-specific IL-10–producing T cells, whereas these effects were not observed when injecting mature DCs. Although in our study no maturation factor(s) were added during culturing, pulsing DCs with cell lysates has been demonstrated to induce maturation, as shown by increased expression of CD83, CD86, and CD80 (Kropshofer and Schadendorf, unpublished data).

In this study, six HLA-A*0201–subtyped melanoma patients vaccinated with DCs pulsed with autologous tumor lysates and KLH were screened for $CD8^+$ T cells specific for three melanosomal peptides and KLH-specific T cells by the ELISpot technique. $CD8^+$ T cells specific for the melanosomal peptides MART-1/Melan-$A_{27–35}$ or tyrosinase$_{1–9}$ as well as IFN-γ–releasing KLH-specific T cells were shown to be induced in two of six vaccinated melanoma patients. However, a clear association between the induction of these T cells and clinical responses could not be demonstrated.

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