Rapid Induction of Tumor-specific Type 1 T Helper Cells in Metastatic Melanoma Patients by Vaccination with Mature, Cryopreserved, Peptide-loaded Monocyte-derived Dendritic Cells

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Abstract

There is consensus that an optimized cancer vaccine will have to induce not only $CD8^+$ cytotoxic but also $CD4^+$ T helper (Th) cells, particularly interferon (IFN)-γ–producing, type 1 Th cells. The induction of strong, ex vivo detectable type 1 Th cell responses has not been reported to date. We demonstrate now that the subcutaneous injection of cryopreserved, mature, antigen-loaded, monocyte-derived dendritic cells (DCs) rapidly induces unequivocal Th1 responses (ex vivo detectable IFN-γ–producing effectors as well as proliferating precursors) both to the control antigen KLH and to major histocompatibility complex (MHC) class II–restricted tumor peptides (melanoma-antigen [Mage]-3.DP4 and Mage-3.DR13) in the majority of 16 evaluable patients with metastatic melanoma. These Th1 cells recognized not only peptides, but also DCs loaded with Mage-3 protein, and in case of Mage-3DP4–specific Th1 cells IFN-γ was released even after direct recognition of viable, Mage-3–expressing HLA-$DP4^+$ melanoma cells. The capacity of DCs to rapidly induce Th1 cells should be valuable to evaluate whether Th1 cells are instrumental in targeting human cancer and chronic infections.

Key words: dendritic cells • vaccination • $CD4^+$ T cells • T helper cells • tumor immunity

Introduction

Active specific immunotherapy of cancer aims at generating strong tumor-specific T cell immunity. So far the focus has been on inducing $CD8^+$ CTL responses (1, 2). Ample evidence indicates, however, that $CD4^+$ Th cells, particularly IFN-γ–producing Th1 cells, are another critical component of an effective antitumor immune response (3–5) as Th1 cells (i) help to initiate antigen-specific $CD8^+$ T cells (by expressing CD40L and activating dendritic cells [DCs]* via CD40), (ii) amplify and sustain $CD8^+$ T cell function (by secreting cytokines such as IL-2), and (iii) can inhibit tumor growth even in the absence of $CD8^+$ T cells by releasing IFN-γ (a macrophage activating and antiangiogenetic cytokine; reference 6) or exerting direct cytotoxicity independent of MHC class I molecules.

Tumor antigens and epitopes restricted by MHC class II molecules (2–4) have recently been described so that it is now possible to ask whether tumor-specific Th responses can be induced in cancer patients by vaccination. We and others have previously provided the proof of concept that vaccination with mature monocyte-derived, peptide-loaded DCs can expand tumor-specific CTL (7, 8). We now wanted to explore whether such DCs would also be able to induce tumor-specific Th1 cells as the crucial second component of potent antitumor immunity. As in our previous trials (9–11) we have chosen to study melanoma as most MHC class I and class II–restricted tumor antigens have been identified for this tumor.

DC vaccination induced unequivocal, melanoma-antigen (Mage)-3 tumor peptide-specific effector as well as recall Th1 responses. This is to the best of our knowledge the first time that ex vivo detectable Th1 cells specific for hu-

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**Abbreviations used in this paper:* DC, dendritic cell; DTH, delayed type hypersensitivity; Mage, melanoma-associated gene.

man tumor antigens have been reproducibly induced in human cancer patients by vaccination.

Materials and Methods

Clinical Protocol and Study Design. We report here about the first phase of a prolonged clinical trial in which five DC vaccinations were administered subcutaneously at 14 d intervals followed 1 mo later by an evaluation. The study was approved by the Ethics Committees and regulatory authorities, and informed written consent was given by all patients. HLA-A1, -A2.1, and/or -A3$^+$ (as defined by serologic and molecular typing) patients suffering from metastatic cutaneous melanoma (surgically incurable/no longer curable distant or lymph node metastases; progression despite standard surgical, radiation, chemotherapy, and/or immunotherapy) were eligible. Otherwise inclusion and exclusion criteria (systemic chemo-, radio-, or immunotherapy ≤4 wk before, concomitant antitumor or immunomodulatory therapy, etc.) were as described previously (9, 10).

DC Vaccine. Standardized DCs were generated from an initial leukapheresis ($\geq 10^{10}$ nucleated cells) essentially as described previously (12, 13). In short, PBMCs isolated on Lymphoprep™ (Nycomed Pharma) were plated on Nunc Cell factories™ Nunclon™ culture flasks (Nalge Nunc International) in complete medium, i.e., RPMI 1640 (GMP quality; BioWhittaker), 20 μg/ml gentamicin, 2 mM glutamine, and 1% heat-inactivated autologous plasma. Adherent cells were cultured in 800 U/ml GM-CSF (Leukomax™; Novartis) and 500 U/ml IL-4 (GMP quality; CellGenix) for 6 d, then matured by adding 10 ng/ml TNF-α (GMP quality, a gift of Boehringer-Ingelheim), 10 ng/ml IL-1β (GMP quality; Amedak), 100 U/ml IL-6 (GMP quality; a gift from Novartis), and 1 μg/ml prostaglandin E2 (Minprostin™; Amersham Pharmacia Biotech and Upjohn). Mature DCs were harvested on day 7, then frozen and stored in aliquots as described previously (12). Release criteria for the DCs included typical morphology (>95% nonadherent veiled cells), phenotype (>95% HLA-DR^{+++}, CD86^{+++}, CD40$^+$, CD25$^+$, >75% CD83^{++}), and negative microbial tests.

On the day of vaccination a DC aliquot was thawed and loaded with several MHC class I and II–restricted peptides (3 h in RPMI 1640 and 1% HSA), all in pharmaceutical quality (Clinalfa). Each of the individual five HLA-A1 (Mage-1, EADPTGHSY; Mage-3, EVDPIGHLY; Tyrosinase, KSDICTDEY; FluNP, CTELKLSDY; and FluPB1, VSDGGPNLY), eight HLA-A2.1 (Mage-4, GVYDGREHTV; Mage-10, GLYDGMEHL; GnTV, VLPDVFIRCV; Tyrosinase, YMDGTMSQV; gp100, IMDQVPFSV; Melan-A, ELAGIGILTV; FluMP, GILGFVFTL; and FluBNP, KLGEFYNQMM), or three HLA-A3 (Mage-1, SLFRAVITK; gp100, LIYRRRLMK; and FluNP, ILRGSVAHK) restricted peptides was pulsed at 30 μM on separate batches of 4 million DCs to avoid uncontrollable competition at the MHC molecules. Thus, HLA-A1$^+$ patients (15, 16, 27, 28), A2.1$^+$ patients (02, 12, 17, 19, 20, 21, 23) and A.3$^+$ patients (05, 08, 10, 14, 18, 19, 27) received a total of 20, 32, and 12 million DCs, respectively. One group (group A) of the MHC class I–restricted peptides (HLA-A1–restricted FluNP, Mage-3; HLA-A2.1–restricted FluMP, Melan-A, gp100, Mage-3; HLA-A3–restricted FluNP, Mage-1) was pulsed onto DCs in the presence of 100 ng/ml Rankl (reference 14; obtained from Cell Concepts and biosafety tested in analogy to GMP guidelines) in uneven patients, without Rankl in even patients. Vice versa the residual MHC class I peptides (group B) were pulsed in the presence of Rankl in even patients, and without in uneven patients. This design was chosen to allow for a comparison of the influence of Rankl pretreatment of the DCs on the induction of CTL responses with each patient serving as his own control. In case of MHC class II–restricted peptides all DCs in all patients were pulsed with 30 μg/ml Mage-3.DR13 (LLKYRAREPVTKAE; reference 15) and Mage-3.DP4 (KKLLTQHFVQENYLEY) peptides (16), while the two DR4-restricted MHC class II peptides Tyrosinase (SYLQDSVPDSFQD; anchor-modified for high-affinity binding to DR4) (17) and gp100 (WNRQLYPEWTEAQRLD; reference 18) were loaded only onto half of the DCs (in analogy to the group A and B MHC class I–restricted peptides, respectively) in order to avoid competition for the DR4 molecules. Peptide-loaded DCs were then collected, washed, and administered (2×10^6 DCs in 0.5 ml PBS and 1% HSA/site) by superficial subcutaneous injection (i.e., at the dermis/subcutis boundary) close to axillary or inguinal lymph nodes as described previously (9, 10). Additionally, 4 million DCs (without any exposure to Rankl) were loaded with KLH alone (by adding at day 5 of DC culture 10 μg/ml endotoxin-free keyhole limpet hemocyanin; obtained from Calbiochem, and biosafety tested in analogy to GMP guidelines), cryopreserved, thawed, and administered once at vaccination #1.

Measurement of Immune Responses. Immediately before each vaccination and 4 wk after vaccination #5 blood was drawn and the fresh PBMCs were used for serial ex vivo ELISPOT analyses. In addition, cryoconserved PBMCs prepared from the initial and a second, smaller leukapheresis (performed 4 wk after vaccination #5 primarily for immunomonitoring) were thawed and assayed simultaneously. All assays were done and evaluated in a blinded fashion.

Measurement of Cytokine Releasing Effector CD4$^+$ T Cell Responses in Uncultured PBMCs by ELISPOT Analysis. PBMCs (in select cases depleted from CD4$^+$ or CD8$^+$ T cells by MACS®; Miltenyi Biotec) were added in triplicates at 5×10^5 cells per 96 wells precoated with anti–IL-2 (clone 5355.111; R&D Systems), anti–IFN-γ (1-D1K; Mabtech), or anti IL-4 (MP4–25D2; BD PharMingen) mAb. Then KLH or class II peptides were added at 10 μg/ml (peptides in select cases also at 1.0 and 0.1 μg/ml with comparable results), and after 20 h (40 h for IL4) wells were washed and incubated with biotinylated mAb to IL-2 (BAF 202; R&D Systems), IFN-γ (7-B6–1; Mabtech), or IL-4 (12–1; Mabtech) for 2 h. Final staining and computer assisted analysis was done as described previously (9, 10). Background without antigen was less than three spots (except where indicated), and subtracted. Responses were considered significant if a minimum of five spot-forming cells per well were detected, and additionally, this number was at least twice that in negative control wells.

Measurement of Recall CD4$^+$ T Cell Responses by Antigen-specific Proliferation and ELISPOT Analysis. Thawed PBMCs were MACS®-depleted of CD8$^+$ cells (in select examples of CD4$^+$ cells), and plated in triplicate at 2×10^5 cells flat-bottomed, 96-well in medium containing 10% heat inactivated human serum. MHC class II tumor peptides were added at 10 μg/ml without any cytokines. On day 4 ^{3}[H]-thymidine was added for 16 h. KLH- and staphylococcal enterotoxin-proliferation were done as described previously (9, 19). For some subjects CD4$^+$ T cells were isolated simultaneously from thawed pre- and posttherapy (i.e., 4 wk after vaccination no. 5) PBMC aliquots by negative magnetic bead selection (MACS® kit; Miltenyi Biotec), then stimulated once with autologous, peptide-loaded mature DCs (10 μg/ml). On day 8 T cells were stimulated either with peptide alone or with peptide-loaded DCs and IFN-γ spot-forming cells were measured by ELISPOT.

Generation of CD4+ T Cell Clones. CD4+ T lymphocytes (10^6) isolated by MACS® were cocultured with 30×10^3 autologous peptide-pulsed (10 μg/ml, 3 h) and irradiated (100 Gy) mature DCs in 24-wells in 2 ml RPMI 1640 with 10% heat-inactivated human serum. On day 3 IL-2 (50 U/ml) and IL-7 (10 ng/ml) were added. On day 7 peptide-specific T cells were cloned by limiting dilution. Stimulators were irradiated, peptide-pulsed (1 h, 10 μg/ml) autologous EBV-B cells ($5–10 \times 10^3$ cells; round bottomed, 96 well), while irradiated allogeneic LG2-EBV ($5–10 \times 10^3$ cells per well) served as feeders. Weekly restimulations were done with irradiated peptide-pulsed EBV-B cells in the presence of IL-2 (50 U/ml) and feeder cells.

Chromium Release and IFN-γ ELISA Assays. Cr^{51} assays (4 h) using autologous EBV-B cells (plus or minus transduction with

Table I. *Patient's Characteristics, Status Before and Response to DC Vaccination*

				Metastases at study entry								
				Regional		Distant						
Patient code	Sex age	Center	Previous therapy	Skin	LN	Skin	LN	Lung	Liver	Skeleton	Others	Clinical response
				Fully evaluable patients (five vaccinations)								
02	m-48	E	S, ILP, IT, RT, CIT	–	–	–	–	D/5	–	–	Pancreas 2/35	CR
05	f-77	E	S, ILP, IT, RT, CT	6/34	–	–	–	–	–	–		SD
08	f-64	E	S, IT, CIT, CT	–	–	1/3	1/4	–	2/80	–	Muscle 1/60	OP
10	m-41	E	S, IT	–	2/13	–	–	–	–	–		SD
12	f-65	E	S, CT	–	–	–	1/15	D/10	–	–		SD
14	m-58	E	S, IT, CT	–	–	–	–	D/25	–	–		OP
15	f-35	E	S, CIT	–	2/20	–	–	–	–	–		SD
16	m-63	I	S	–	2/30	–	1/25	–	–	1/20		OP
17	f-60	I	S, CIT	–	–	D/10	3/26	1/3	–		Kidney 2/15, suprarenal 2/60	OP
18	m-77	I	S	–	–	–	–	3/30	1/14	3/60		OP
19	m-70	E	S, IT	–	–	–	–	9/25	–	2/25		OP
20	m-64	E	S, IT, RT	D (>50)/5	–	D/5	–	–	–	–		SD
21	m-59	E	S, IT	–	2/9	–	–	–	–	–		SD
23	f-36	E	S, IT, CT	3/14	–	–	–	–	–	–	Intestine D	SD
27	f-44	I	S, IT	–	–	–	1/51	1/16	–	–		SD
28	m-55	E	S, CIT, ILP	–	2/6	–	3/30	–	–	–		OP
				Not fully evaluable patients (less than five vaccinations)								
01	f-29	E	CIT	–	–	–	D/8	–	1/5	–	Spleen 3/35 peritoneum D muscle 2/30	†
03	m-59	E	S, IT, CT	1/70	D/15	–	–	–	–	–	Sinus cavernosus infiltration; bone	†
04	f-55	E	S, IT, CT	–		–	D/30	–	–	–		OP
07	f-48	I	S, CIT, RT	–	1/20	–	D/50	–	–	–		OP
09	m-57	E	S, CIT	1/11	–	–	D	–	D	–	Pancreas, duodenum, gastric D	OP
11	f-71	E	S	D (>50)/5	–	–		–	–	–		OP
13	f-49	I	S	–	–	–	D/35	–	–	–		OP
24	f-37	I	S, CT, IT, RT	–	–	–	1/45	1/20	–	–		OP

Treatment Centers: E, Erlangen; I, Innsbruck.
Pretreatment therapy: IT, immunotherapy; C(I)T, chemo(immuno)therapy; S, surgery; RT, radiotherapy; ILP, isolated limb perfusion.
Metastases at study entry: the number and diameter of the largest metastases present at study entry are listed (number/diameter in mm); D, diffuse.
Clinical Response: CR, complete regression; OP, overall progression; SD, stable disease.
†Deceased.

retro-Ii.MAGE-3, plus or minus peptide loading at 10 μg/ml) as targets were performed as described previously (16). The pMFG retrovirus encoding Ii.MAGE-3 was constructed and used as described previously (16). For IFN-γ assays 20-h supernatants of EBV-B cell (20×10^3)/CD4$^+$ T cell (4×10^3 cells) cocultures (96 well, 25 U/ml IL-2) were analyzed by ELISA (Biosource). To test whether Th1 clones recognized autologous DCs loaded with Mage-3 protein immature monocyte-derived DCs were incubated overnight with 20 μg/ml protein Mage-3 (provided by GlaxoSmithKline) or OVA (as a negative control) in the presence of IL-1β, IL-6, PGE_2, and TNF-α to induce their maturation. CD4$^+$ T cell clones were then cocultured with 15×10^3 protein-loaded DCs in flat-bottomed microwells for 16 h and supernatants analyzed by IFN-γ ELISA. To test whether the Th1 clones can directly recognize tumor cells several MAGE-3–expressing melanoma cell lines (see Fig. 6), either HLA-DP4$^+$ or HLA-DP4-, were seeded at 15×10^3 cells per flat-bottomed microwell and incubated for 48 h to allow the formation of a monolayer. CD4$^+$ T cell clones were then added (4×10^3 cells per well) and supernatants analyzed by IFN-γ ELISA after 16-h coculture.

Delayed type hypersensitivity (DTH) was not part of the protocol but could be assessed in a few patients upon informed written consent by intradermal injection at two sites of either 1.5×10^5 peptide-loaded mature DCs or 50 μg pure peptide in 0.1 ml PBS/1%HSA per site. Negative controls were nonpulsed DCs and vehicle.

Results

Patients and DC Vaccine. We report here about the first phase (five biweekly vaccinations with peptide-loaded DCs) of a prolonged and rather complex DC vaccination trial. This first phase was designed to address the safety and immunological efficacy of DC immunization to MHC class II–restricted melanoma peptides. 28 patients (progressive despite standard surgical, radiation, chemo-, and/or immunotherapy) with surgically incurable metastatic cutaneous melanoma (22 with distant [patients 1–4, 6–9, 12, 13, 14, 16, 17, 18, 19, 20, and 23–28] and six with locoregional metastases [05, 10, 11, 15, 21, 22]) were enrolled (Table I). 16 patients received five vaccinations and were fully evaluable (02, 05, 08, 10, 12, 14–21, 23, 27, and 28). Four patients had to be excluded before vaccination no. 1 as they were not found eligible after prestudy screening (patients 06, 22, 25, and 26), two died before vaccination no. 2 and 3, respectively, (patients 01 and 03) and six patients (04, 07, 09, 11, 13, and 24) dropped out between vaccination no. 2 and 6 (patients 13 and 24 before no. 5; 04, 07, 09, 11 before no. 6) due to death or need for modification of therapy (e.g., chemotherapy) because of progression. All DC preparations fulfilled the release criteria.

Toxicity. No major (>grade II) toxicity was observed in any patient. We noted, however, transient raise in body temperature (38.8°C) in 5/16 fully evaluable patients (patients 08, 12, 15, 19, 23), and in 0/12 patients who were not fully evaluable. In addition, strong (>4 cm induration) DTH reactions (Fig. 1 a) which first developed after the second to fourth vaccination, and later increased to indurations of up to 12 cm were observed at the vaccination sites in the majority of patients (Fig. 1 a) (in all of the 16 fully evaluable patients except 10, 14, 17, 19, and in five [04, 07, 09, 13, 24] of the seven patients who were not fully evaluable but had received at least two vaccinations). DTH reactions to nonpeptide-pulsed DCs were negative or <1 cm. This suggested induction of immunity to MHC class II peptides as DTH assays typically detect primed Th cells. Indeed, we found that unequivocal DTH reactions occurred also in response to intradermally injected Mage-3 MHC class II–restricted peptides (Fig. 1 b), and not only to DCs carrying these peptides. Biopsies indicated a predominant CD4$^+$ T cell infiltrate with only scattered CD8$^+$ T cells (data not shown). DTH to intradermal peptides was not part of the protocol, but in the three patients (positive DTH in patients 12 and 08; Fig. 1 b; negative DTH in patient 04) that could be evaluated appeared to correlate with induction of immunity (see below).

Immune Responses. A single injection of KLH-pulsed, cryoconserved DCs rapidly induces KLH-specific Th1 cells. Each patient received (only) at vaccination no. 1 a single subcutaneous injection of 4 million mature KLH-pulsed monocyte-derived DCs as a positive control for the functioning of the cryopreserved DCs, and to get an estimate of the general competence of the patient's immune system to respond. Ex vivo ELISPOT assays performed 2 wk after the single injection of KLH-pulsed DCs revealed that patients had rapidly developed Th1 type immunity (Fig. 2 b and c), and a KLH-specific memory type response was evident in antigen-specific proliferative assays performed 3 mo after the single vaccination (Fig. 2 a). The Th1 responses although detectable in all patients were variable in strength. KLH-specific responses were mediated by CD4$^+$ T cells, as evidenced by CD4$^+$ T cell depletion (data not shown). The 12 patients who were not fully evaluable (see Table I, and above) like the fully evaluable ones all had a negative ELISPOT at onset, but those seven who received vaccination no. 1 had developed KLH-specific Th1 cells 14 d after the single injection of KLH-loaded DCs (patient 03: 82 IFN-γ/3 IL-4 spot-forming cells [SFC]; 04: 146 IFN-γ/13 IL-4 SFC; 07: 22 IFN-γ/1 IL-4 SFC; 09:

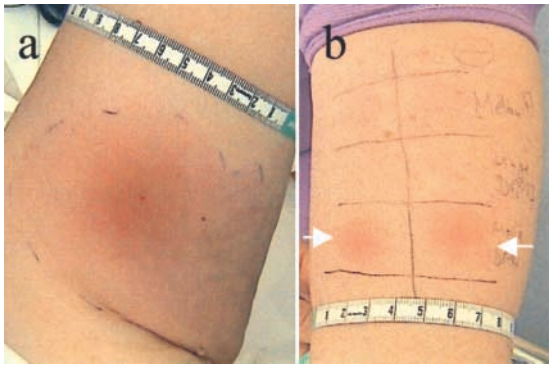


Figure 1. DTH reactions to peptide-loaded DCs and to Mage-3DP4 peptide alone. (a) Massive local reaction at the vaccination site to peptide-loaded mature DCs in patient 12 (>10 cm induration at 48 h), (b) DTH reaction (arrows) to intradermally administered MAGE-3.DP4 peptide. No DTH after injection of vehicle, MelanA.A2, or Mage-3.DR13 peptides.

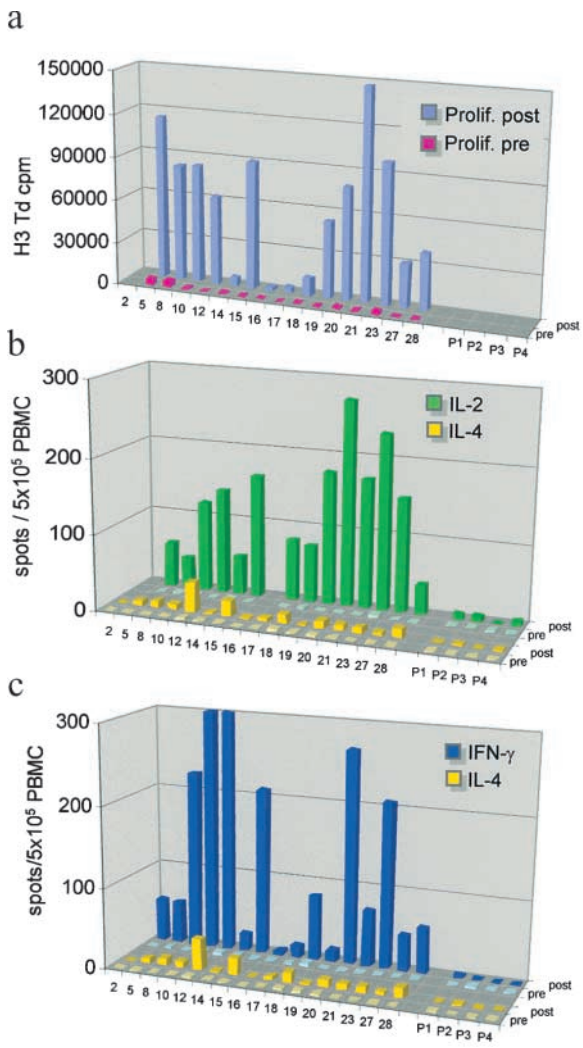


Figure 2. Priming to KLH after a single injection of KLH-pulsed DCs. (a) Proliferation assay. Pre and postvaccination (i.e., before and 12 wk after the single injection of 4 million KLH-loaded DCs) PBMCs were thawed, CD8 depleted, pulsed with KLH and proliferation simultaneously measured on day 5. For P1-P4 only Elispots were performed (see b and c). SEM for measurements was <20%. This assay was performed in parallel with the assay described in Fig. 5. (b and c) Ex vivo Elispot analysis. KLH-specific IL-2 (b), IFN-γ (c), and IL-4 (b and c) producing cells from before and 14 d after DC immunization were quantified in freshly isolated uncultured PBMCs. Patients P1 to P4 received a single subcutaneous injection of KLH (4 or 100 μg) without DCs and were not primed to KLH. SEM for measurements was <20%.

56 IFN-γ/2 IL-4 SFC; 11: 79 IFN-γ/1 IL-4 SFC; 13: 31 IFN-γ/5 IL-4 SFC, and patient 24: 30 IFN-γ/2 IL-4 SFC. Interestingly, four additional melanoma patients who served as controls and received a single subcutaneous injection of KLH without DCs were not immunized to KLH indicating the adjuvant properties of DCs (Fig. 2 b and c).

Vaccination with Mage-3.DP4 or Mage-3.DR13 Tumor Peptide-pulsed DCs Rapidly Induces a Th1 Effector Response. Serial ex vivo ELISPOT analysis of blood freshly drawn before each vaccination (i.e., 2 wk after the preceding vaccination) as well as 4 wk after vaccination no. 5 revealed that a Th1 type immune response was induced to both Mage-3DP4 (Fig. 3 a and c) and Mage-3.DR13 peptides (Fig. 3 b and d) in most patients (11/13 $DP4^+$ [84.6%] and 10/10 $DR13^+$ or DR 15^+ [100%] patients, respectively) and at all DC doses used (12×10^6: patients 05, 08, 10, and 18; 20×10^6: 15, 16, 27, and 28; 32×10^6: 02, 12, 17, and 19–23). The Th1 responses emerged rapidly, but high and often plateau levels were reached only after a few additional vaccinations (Fig. 4). Simultaneous Elispot analyses of pre and postvaccination PBMC samples confirmed the induction of Th1 responses, and depletion assays showed that these were mediated by $CD4^+$ T cells (data not shown). Interestingly, immunity to Mage-3.DP4 was also induced in 1/2 HLA-DP4 negative, and to Mage-3.DR13 in 7/11 HLA-DR13 negative patients (Fig. 3). Remarkably, most (5/7) HLA-DR13 negative patients who got immunized to the Mage-3.DR13 peptide were HLA-$DR15^+$. Out of those six patients who were not fully evaluable but received at least two vaccinations only patient 09 (DR13-, $DR15^+$, $DP4^+$) developed a positive Elispot (no positivity before and after vaccination no. 1, but 77 Mage-3.DR13-specific and 155 Mage-3.DP4-specific IFN-γ spot-forming cells after vaccination no. 2). Of note was also, that vaccination with Tyrosinase DR4 or gp100 DR4 peptide-loaded DCs did not induce unequivocal immunity in any patient including the four HLA-$DR4^+$ patients 10, 12, 14, and 20 (data not shown except examples in Fig. 4).

Vaccination with Mage-3DP4 or Mage-3.DR13 Tumor Peptide-pulsed DCs Induces Th1 Recall Responses. Recall assays of batched pre and postvaccination PBMC samples provided evidence for the expansion of Mage-3.DP4 and Mage-3.DR13–specific $CD4^+$ T cells, but not Tyrosinase DR4 and gp100 DR4 peptide-specific ones (Fig. 5). Mage-3 peptide-specific proliferative responses (cpm with peptide − cpm without peptide) increased in 12/16 patients, whereby the responses were equivocal in two patients (08 and 28) due to an increased background postvaccination. Recall Elispot analyses were performed in select patients, and confirmed the expansion of Mage-3 peptide-specific $CD4^+$ T cells after vaccination, and showed that they represented Th1 cells (Fig. 6).

The elicited Mage-3 peptide-specific Th1 cells release IFN-γ also in response to DCs loaded with Mage-3 protein and even after direct recognition of Mage-3 protein-expressing HLA-$DP4^+$ tumor cells.

We next established several $CD4^+$ T cell clones from patients 12 and 15 (who exhibited particularly strong Th1 responses) to analyze the Th1 responses in more detail. Such clones readily recognized Mage-3 protein processed by autologous DCs (Fig. 7 a), and released large amounts of IFN-γ. This proved their specificity for Mage-3, and indicated their potential biological relevance. Mage-3.DP4 clones were special in that they were also able to directly recognize autologous EBV-B cells transduced with retro-Ii.Mage-3 (resulting in IFN-γ release by the T cell clone and lysis of the targets, data not shown), and were even capable of direct recognition of Mage-3 expressing, HLA-$DP4^+$ (but not HLA-DP4-) melanoma cells (Fig. 7 b).

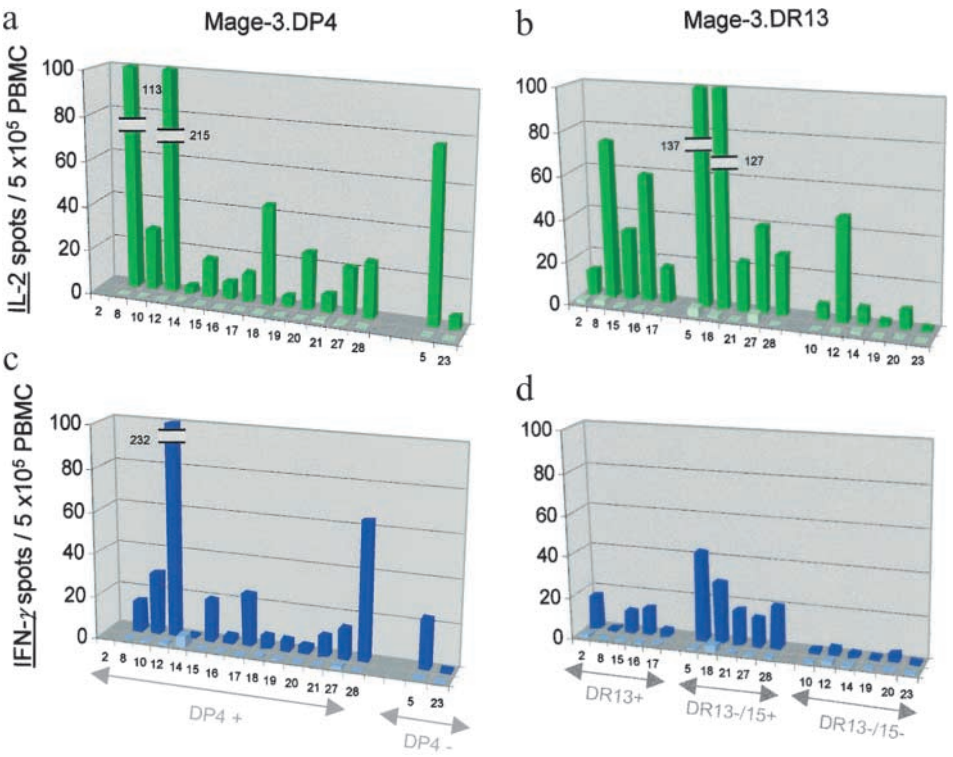


Figure 3. Induction of tumor-specific Th1 effector cells by DC immunization Mage-3.DP4 (a and c) and Mage-3.DR13 (b and d) peptide-specific IL-2 (a and b), IFN-γ (c and d), and IL-4–producing cells were quantitated every 14 d in freshly isolated uncultured PBMCs. Baseline spots (light green and blue) and highest spot numbers (dark green and blue) reached upon DC vaccination are shown. Patient 02 was not vaccinated to Mage-3DP4 peptide so that the respective data are missing. IL-4 spot-forming cells were in general ≤25% of the IL-2 and IFN-γ ones, and are not shown. Background without antigen was subtracted and was less than three spots for all cytokines measured throughout the study (except for patient 28 with nine spots background postvaccination). Patients are grouped on the x-axis according to their expression of HLA.DP4, .DR13, or .DR15. SEM for measurements was <25%.

Clinical Responses. At the evaluation 1 mo after the fifth vaccination (i.e., 4 mo after the onset of the vaccinations) seven of the fully evaluable patients (08, 14, 16, 17, 18, 19, and 28) showed disease progression, eight (05, 10, 12, 15, 20, 21, 23, and 27) exhibited stable disease, and one patient (02) had experienced complete regression of disease (metastases in lung and pancreas).

Discussion

Active specific immunotherapy of cancer has focused so far on the induction of $CD8^+$ CTL responses, primarily by using MHC class I–restricted tumor peptides (most often in the setting of melanoma as the prime model; references 1 and 2). Remarkably, $CD8^+$ effectors were detectable in

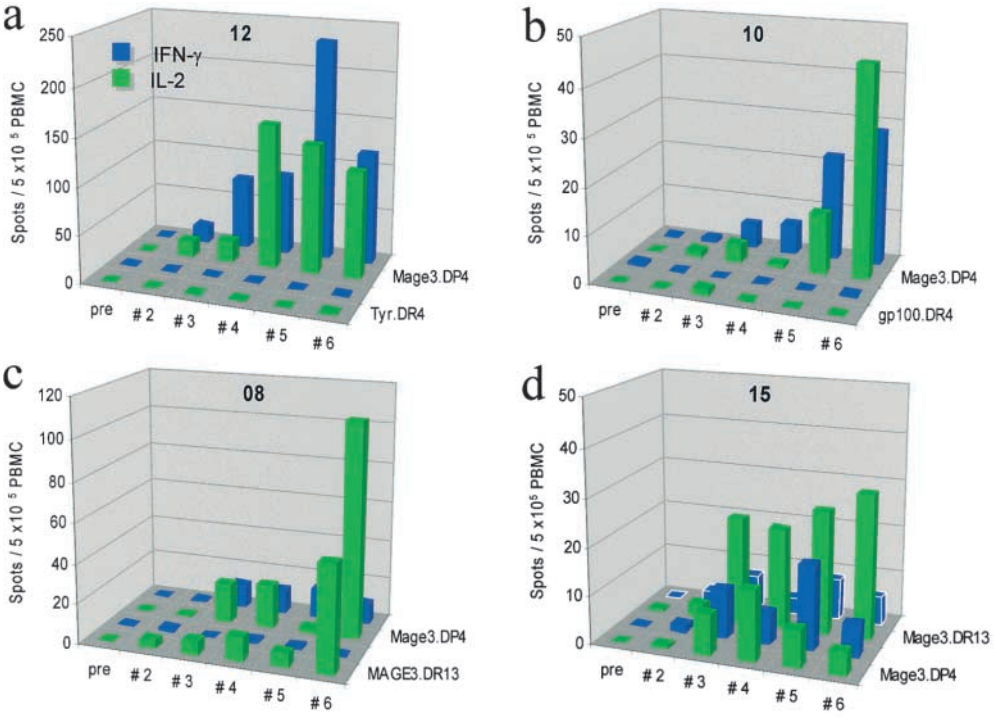
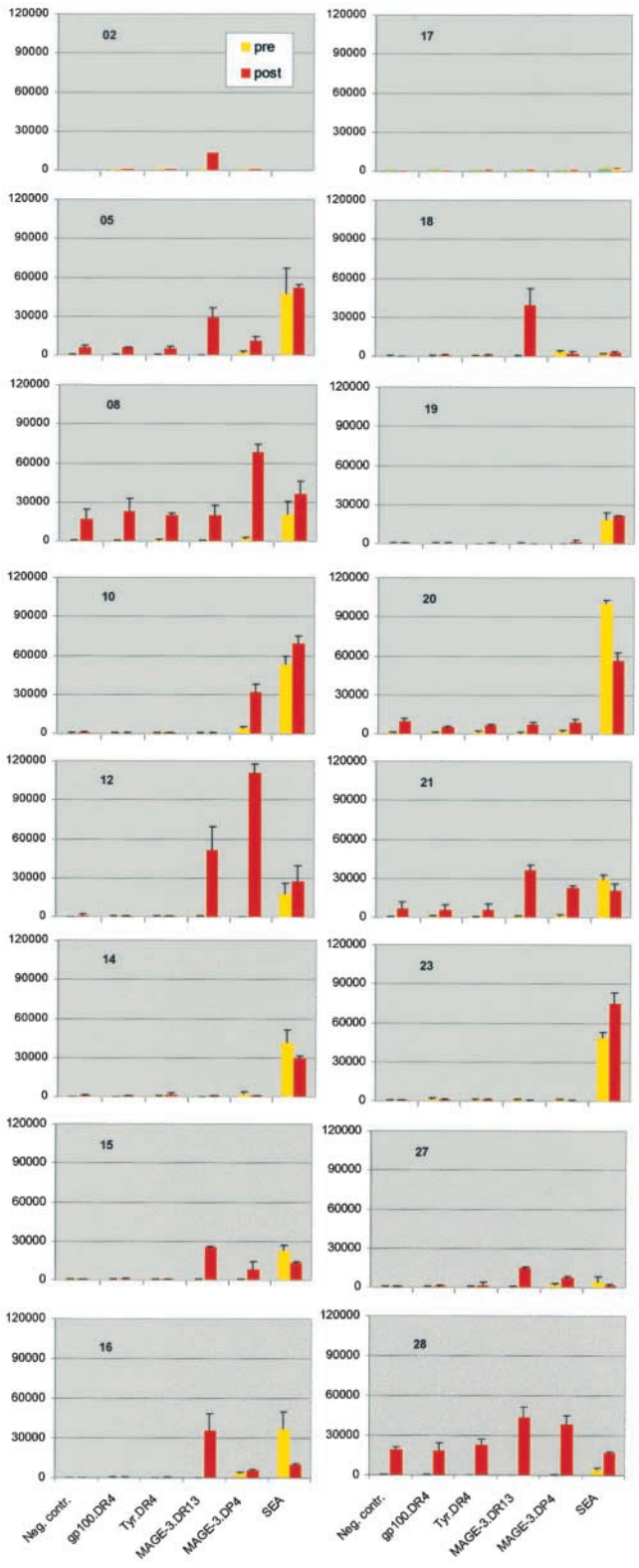


Figure 4. Kinetics of tumor-specific Th1 effector induction PBMCs of patients were serially tested every 14 d by ex vivo Elispot analysis as in Fig. 3 to quantitate the IL-2 and IFN-γ–producing cells specific for Mage-3DP4, Mage-3DR13, gp100DR4, and TyrosinaseDR4 (Tyr.DR4) peptides. Results are shown for four representative subjects (HLA-$DR4^+$/$DP4^+$ patients 10 and 12, HLA-$DR13^+$/$DP4^+$ patients 08 and 15) Background without antigen was subtracted and was less than three spots at all time points. SEM for measurements was <25%.

blood directly ex vivo only after administration of such peptides on DCs, while in vitro restimulations were required for their detection after peptide vaccination (10, 20, 21, for review, see references 2 and 7). The induction of tumor-specific $CD4^+$ Th cells has so far been tried primarily by peptide vaccination albeit with limited success (2–5). Even in the most convincing studies reported to date (22, 23) such Th cell responses were neither shown to be detectable ex vivo nor to be of the desired Th1 type. This ap-



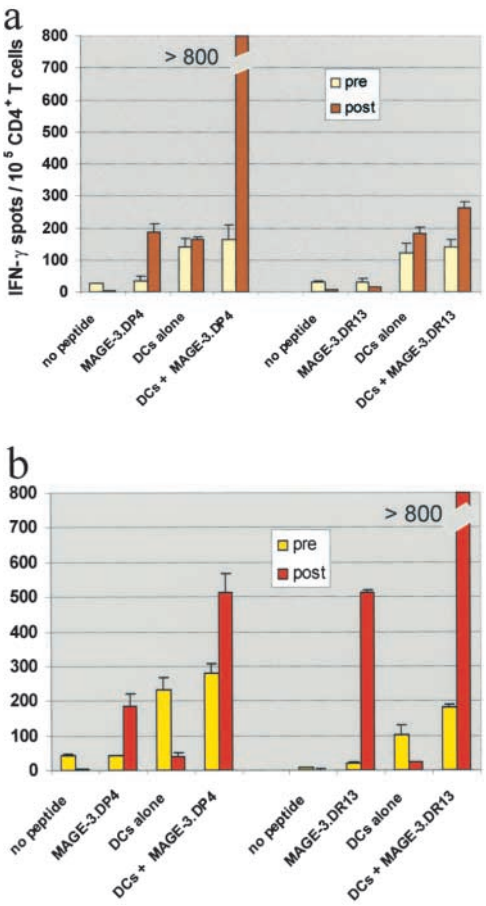


Figure 6. Induction of tumor-specific recall Th1 responses by DC immunization Pre and posttherapy (i.e., 4 wk after vaccination no. 5) $CD4^+$ T cells from patients 12 (a) and 15 (b) were stimulated once in vitro with autologous, peptide-loaded mature DCs (10 μg/ml). On day 8 T cells were stimulated either with peptide alone (Mage-3.DP-4 or Mage-3.DR13 peptide) or with peptide-loaded mature DCs and IFN-γ spot-forming cells were measured by the Elispot technique. The results shown represent the average and SD of triplicate cocultures. Note that Mage-3.DP4-specific and Mage-3.DR13-specific IFN-γ spot-producing $CD4^+$ Th1 cells are clearly expanded after DC vaccination in patients 12 and 15, respectively which fits to the results of the respective proliferative assays (Fig. 5). Note also that (auto-) reactivity to peptide-unloaded DCs (generated in patient 12 from post, and in patient 15 from prevaccination PBMCs) is not increased, but rather decreased upon DC vaccination and induction of specific immunity.

Figure 5. Induction of tumor-specific recall Th responses by DC immunization pre- and post therapy (i.e., 4 wk after vaccination no. 5) PBMCs were thawed, CD8 depleted, pulsed with gp100 DR4, tyrosinase DR4, Mage-3.DP4, and Mage-3.DR13 peptides and proliferation measured on day 5. In parallel cultures SEA (staphylococcal enterotoxin A) was added as a control both for the functioning of $CD4^+$ T cells and an enhanced background reactivity, and proliferation measured on day 3. The results shown represent the average cpm ^{3}[H]thymidine incorporation) and SEM of triplicate cultures. An increased proliferative response to Mage-3 peptide is seen in 12/16 patients but is ambiguous in patients 08 and 28 due to an increased background postvaccination.

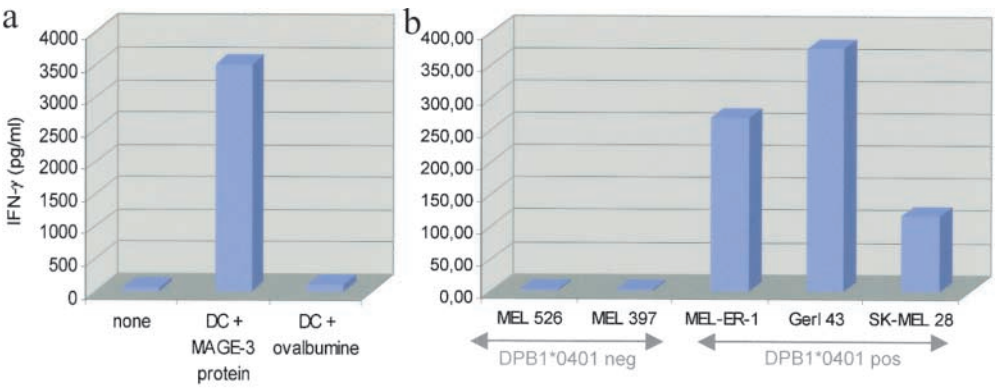


Figure 7. Mage-3.DP4-specific Th clones recognize autologous DCs that have processed Mage-3 protein and even directly recognize Mage-3 expressing, HLA-DP4$^+$ melanoma cells. (a) Autologous, immature monocyte-derived DCs were loaded with Mage-3 protein or OVA (as a negative control) during their maturation, then 15 $\times$ 10^3 mature, protein-loaded DCs were cocultured with CD4$^+$ T cell clone R12–57 (4 $\times$ 10^3 cells per 96 well), and after 16 h IFN-γ was measured in the supernatants. (b) In a parallel set of experiments CD4$^+$ T cell clone R12–57 (4 $\times$ 10^3 cells per 96 well) was added to monolayers of several Mage-3 expressing melanoma cell lines, either HLA-DP4$^+$ or HLA-DP4-, and IFN-γ assayed by ELISA in 16 h supernatants. Data are triplicate values. SEM was <15%, bars. Several clones of patients 12 and 15 were tested with identical results.

plies also to antiidiotype-specific T cell proliferative responses that have been induced in B cell lymphoma by vaccination with idiotype protein and KLH as adjuvant (24). Such antiidiotype responses have also been reported after vaccination with idiotype-pulsed DCs (25 and 26), but in these studies idiotype protein was simultaneously administered also subcutaneously with KLH so that the role of DC vaccination for the induction of the antiidiotype responses is unclear. Therefore, we wanted to learn whether DCs would be valuable adjuvants for the induction of CD4$^+$ helper responses, notably of the desired Th1 type to genuine human tumor antigens. We found that the subcutaneous injection of mature, cryopreserved, peptide-loaded, monocyte-derived DCs rapidly induces tumor-specific Th1 cells that are readily detectable in fresh blood without restimulation in the majority of metastatic melanoma patients including those with a heavy tumor load. Several additional specific observations made in our study appear noteworthy.

The single subcutaneous injection of 4 million KLH-pulsed DC-induced unambiguous Th1 responses (proliferating memory cells as well as ex vivo detectable IFN-γ–producing effectors) in most patients. This rapid induction of a Th1 response by DCs matured by IL-1β, TNF-α, IL-6, and PGE_2 was most noteworthy as it clearly contradicted the results of in vitro experiments predicting that such DCs would release little measurable p70 (27) and, therefore, induce Th2 and not Th1 responses (28, 29). The demonstration that monocyte-derived DCs that are generated under the aegis of GM-CSF and IL-4 from apheresis products, matured by a defined cocktail (IL-1β, TNF-α, IL-6, PGE_2) and cryopreserved according to our recently described method (12) are functional in vivo is important as cryopreserved, antigen-preloaded, mature DC aliquots bear obvious logistical advantages. Of note is also that immunization to KLH occurred even in Multitest Merieux negative patients (anergic: 04, 05, 08, 12, 13, 17, and 27; hypoergic: 02, 03, 07, 09, 14, 18, 19, 20, and 24; normergic: 10, 11, 15, 16, 21, 23, and 28). It will be interesting to test in future studies whether the intensity and/or quality of immune responses following DC-mediated sensitization to KLH and/or another control antigen will be a useful parameter for predicting whether antitumor responses can be expected.

With respect to the induction of ex vivo detectable tumor-specific Th1 responses again several observations were notable besides the mere fact that to the best of our knowledge such induction has not previously been reported. Immune responses to the Mage-3DP4 and DR13 peptides were remarkable in strength and quality, particularly as they occurred also in patients whose metastases expressed Mage-3 (data not shown) and thus could have tolerized to Mage-3. IFN-γ–producing Mage-3 specific Th1 effectors were also absent in all patients at onset, but became readily detectable upon a few vaccinations by ex vivo Elispot analysis. This is noteworthy given the fact that spontaneously occurring tumor-specific CD4$^+$ T cell responses appear to be detectable only after repetitive in vitro stimulations in the presence of cytokines (3–5, 15–18). Recall assays indicated that DC vaccination had also induced proliferating Mage-3 peptide-specific Th cells. The proliferative responses were detectable even though no cytokines were added and cells were stimulated only once. The Mage-3DP4–specific CD4$^+$ T cells were not only able to produce IFN-γ but also lysed autologous EBV-B cells when loaded with Mage-3DP4 peptide or transfected with Ii.Mage-3 to generate Mage-3/HLA-DP4 complexes by natural processing. Other experiments indicated that the Mage-3DP4-specific CD4$^+$ T cells release IFN-γ also after recognition of either Mage-3$^+$/HLA-DP4$^+$ melanoma cells or DCs that had taken up and processed Mage-3 protein. Ongoing experiments also demonstrate the recognition of DCs that have processed necrotic, Mage-3–expressing melanoma cells. This clearly illustrates the potential biological relevance of these Th cells in the microenvironment of the tumor as based on the in vitro properties they should also be able to take up and process Mage-3 protein that is released from tumor cells after their lysis by CTLs. This assumption is supported by the finding that the regressing metastases which we could study so far were infiltrated both by CD8$^+$ and CD4$^+$ T cells.

In contrast to the unambiguous induction of Th1 responses to Mage-3 peptides we found no immunization to the HLA-DR4–restricted gp100 and Tyrosinase (anchor-modified for high-affinity binding to DR4) melanoma peptides, possibly simply because only four patients (no. 10, 12, 14, and 20) were HLA-DR4$^+$. Besides the small sample size the fact that these DR4 peptides were loaded on to half of the DCs (to avoid competition for DR4 molecules) while the others were loaded on to all DCs, competition by T cells specific for the Mage-3 versus tyrosinase/gp100 peptides at the level of the presenting DCs, and major differences in precursor frequencies are other testable explanations. As it is well known that MHC class II–restricted peptides might bind to several different class II molecules (30) we had loaded them onto the DCs irrespective of the actual expression of the restricting MHC class II molecule. Indeed, 1/2 HLA-DP4 negative patients also developed Mage-3DP4–specific Th1 cells. A more striking finding was, that the majority (11/16) of the fully evaluable patients who were immunized to the Mage-3DR13 peptide were actually HLA-DR13 negative, and that 5/11 expressed HLA-DR15. Interestingly, the binding specificity for HLA-DRB1*1501 has been found to be an L, V, or I in the relative position 1, an F, Y, or I in the relative position 4 and an I, L, V, M, or F in the relative position 7 (31). Comparing the amino acid sequence of the MAGE-3DR13 peptide (LLKYRAREPVTHAE) with this binding motif suggests good binding to HLA-DRB1*1501 as there is an L in position 1 and an Y in position 4. Interestingly, an overlapping binding repertoire of DR13 and DR15 has already been described previously (30). The fine specificity of Th1 clones that recognize the Mage-3 peptides will be the subject of further studies.

The current clinical trial was designed to clarify in the first phase (five biweekly vaccinations followed by a clinical evaluation 1 mo later, i.e., 3 mo after vaccination no. 1) whether tumor peptide–specific Th1 responses can be induced by DC vaccination without significant toxicity, and we report here that the answer to this question is yes. After conclusion of the second, currently ongoing period of prolonged vaccinations we will be in a position to address additional important questions, in particular whether the induction of tumor-specific Th1 cells (which appear to be maintained so far) correlates with the induction and longevity of CTL responses and clinical efficacy. It will also be interesting to study whether tumor-specific Th1 cells will direct any emerging humoral antitumor responses (32) to the IgG1 subclass of antibodies which might be of therapeutic value by their ability to fix complement and to bind to macrophage Fc receptors. As half of the MHC class I–restricted peptides have been administered on DCs pretreated with Rankl (14) (a molecule identical to TRANCE; reference 33) it will also be possible to determine whether the respective CTL responses are markedly enhanced. Given the large number of MHC class I peptides used (Materials and Methods) we will perform a simultaneous analysis of blood samples taken at selected time points as soon as patients have been followed for at least 1 y. Interestingly, currently all patients (except patient 05) who were responders at 4 mo (one patient [02] with complete regression, eight with stable disease) continue to respond, and two of these stable patients have now almost cleared their metastases.

The finding that DC vaccination besides inducing tumor-specific CTL (7, 8) can also readily induce ex vivo detectable human tumor peptide-specific Th1 immune responses underscores the potent adjuvant properties of DCs, and encourages further optimization of this novel immunization approach. Th1 induction by DCs should be valuable to explore the factual role of Th1 cells for vaccines targeting cancer, chronic viral and intracellular infections (2–5), and possibly Th2-mediated diseases (34).

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The authors regret that a footnote was missing in Table I. The footnote is for Intestine D under the Others heading. The corrected table appears below.

Table I. *Patient's Characteristics, Status Before and Response to DC Vaccination*

				Metastases at study entry								
				Regional		Distant						
Patient code	Sex age	Center	Previous therapy	Skin	LN	Skin	LN	Lung	Liver	Skeleton	Others	Clinical response
				Fully evaluable patients (five vaccinations)								
02	m-48	E	S, ILP, IT, RT, CIT	–	–	–	–	D/5	–	–	Pancreas 2/35	CR
05	f-77	E	S, ILP, IT, RT, CT	6/34	–	–	–	–	–	–		SD
08	f-64	E	S, IT, CIT, CT	–	–	1/3	1/4	–	2/80	–	Muscle 1/60	OP
10	m-41	E	S, IT	–	2/13	–	–	–	–	–		SD
12	f-65	E	S, CT	–	–	–	1/15	D/10	–	–		SD
14	m-58	E	S, IT, CT	–	–	–	–	D/25	–	–		OP
15	f-35	E	S, CIT	–	2/20	–	–	–	–	–		SD
16	m-63	I	S	–	2/30	–	1/25	–	–	1/20		OP
17	f-60	I	S, CIT	–	–	D/10	3/26	1/3	–		Kidney 2/15, suprarenal 2/60	OP
18	m-77	I	S	–	–	–	–	3/30	1/14	3/60		OP
19	m-70	E	S, IT	–	–	–	–	9/25	–	2/25		OP
20	m-64	E	S, IT, RT	D (>50)/5	–	D/5	–	–	–	–		SD
21	m-59	E	S, IT	–	2/9	–	–	–	–	–		SD
23	f-36	E	S, IT, CT	3/14	–	–	–	–	–	–	Intestine D[a]	SD
27	f-44	I	S, IT	–	–	–	1/51	1/16	–	–		SD
28	m-55	E	S, CIT, ILP	–	2/6	–	3/30	–	–	–		OP
				Not fully evaluable patients (less than five vaccinations)								
01	f-29	E	CIT	–	–	–	D/8	–	1/5	–	Spleen 3/35 peritoneum D muscle 2/30	†
03	m-59	E	S, IT, CT	1/70	D/15	–	–	–	–	–	Sinus cavernosus infiltration; bone	†
04	f-55	E	S, IT, CT	–		–	D/30	–	–	–		OP
07	f-48	I	S, CIT, RT	–	1/20	–	D/50	–	–	–		OP
09	m-57	E	S, CIT	1/11	–	–	D	–	D	–	Pancreas, duodenum, gastric D	OP
11	f-71	E	S	D (>50)/5	–	–		–	–	–		OP
13	f-49	I	S	–	–	–	D/35	–	–	–		OP
24	f-37	I	S, CT, IT, RT	–	–	–	1/45	1/20	–	–		OP

Treatment Centers: E, Erlangen; I, Innsbruck.
Pretreatment therapy: IT, immunotherapy; C(I)T, chemo(immuno)therapy; S, surgery.
RT, radiotherapy; ILP, isolated limb perfusion.
Metastases at study entry: the number and diameter of the largest metastases present at study entry are listed (number/diameter in mm); D, diffuse.
Clinical Response: CR, complete regression; OP, overall progression; SD, stable disease.
†Deceased.
[a]Surgically removed prior to therapy.