Targeting CD4$^+$ T-Helper Cells Improves the Induction of Antitumor Responses in Dendritic Cell–Based Vaccination

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Abstract

To evaluate the relevance of directing antigen-specific CD4$^+$ T helper cells as part of effective anticancer immunotherapy, we investigated the immunologic and clinical responses to vaccination with dendritic cells (DC) pulsed with either MHC class I (MHC-I)–restricted epitopes alone or both MHC class I and II (MHC-I/II)–restricted epitopes. We enrolled 33 stage III and IV HLA-A*02:01–positive patients with melanoma in this study, of whom 29 were evaluable for immunologic response. Patients received intranodal vaccinations with cytokine-matured DCs loaded with keyhole limpet hemocyanin and MHC-I alone or MHC-I/II–restricted tumor-associated antigens (TAA) of tyrosinase and gp100, depending on their HLA-DR4 status. In 4 of 15 patients vaccinated with MHC-I/II–loaded DCs and 1 of 14 patients vaccinated with MHC-I–loaded DCs, we detected TAA-specific CD8$^+$ T cells with maintained IFN-γ production in skin test infiltrating lymphocyte (SKIL) cultures and circulating TAA-specific CD8$^+$ T cells. If TAA-specific CD4$^+$ T-cell responses were detected in SKIL cultures, it coincided with TAA-specific CD8$^+$ T-cell responses. In 3 of 13 patients tested, we detected TAA-specific CD4$^+$CD25$^+$FoxP3$^-$ T cells with high proliferative capacity and IFN-γ production, indicating that these were not regulatory T cells. Vaccination with MHC-I/II–loaded DCs resulted in improved clinical outcome compared with matched control patients treated with dacarbazine (DTIC), median overall survival of 15.0 versus 8.3 months ($P = 0.089$), and median progression-free survival of 5.0 versus 2.8 months ($P = 0.0089$). In conclusion, coactivating TAA-specific CD4$^+$ T-helper cells with DCs pulsed with both MHC class I and II–restricted epitopes augments TAA-specific CD8$^+$ T-cell responses, contributing to improved clinical responses. *Cancer Res; 73(1); 19–29. ©2012 AACR.*

Introduction

Dendritic cells (DC) are considered the most effective antigen-presenting cells to activate naïve T cells (1). Immunotherapy exploiting *ex vivo* generated autologous DCs pulsed with tumor peptides has shown proof of principle (2). We and others have shown that tumor-specific immune responses can be induced in patients with both stage III and IV melanoma (2). Nevertheless, further optimization is warranted before this approach is accepted for clinical practice (3).

The role of CD8$^+$ cytotoxic T cells (CTL) in the eradication of tumor cells that express tumor-associated antigens (TAA) in the context of MHC class I (MHC-I), has clearly been established (4). For melanoma, many HLA-A*01-, A*02-, and A*03–restricted epitopes derived from gp100, tyrosinase, MAGE-3, or MART-1 have been identified (5). The majority of clinical studies have been conducted with MHC-I peptide-pulsed DCs (6–11). A potential disadvantage of this method is that exclusively CD8$^+$ CTLs are targeted, without involving CD4$^+$ T-helper cells in the induction of antitumor responses.

The role of T-helper cells has recently been better characterized. It has been shown that the presentation of tumor peptides in both MHC class I and II (MHC-I/II) induces high-affinity T cells reactive to multiple MHC-I/II epitopes (12). Subsequent direct activation of APC by T-helper cells leads to stimulation of precursor CTLs to become effector CTLs (13). Several studies have shown a critical role for T-helper cells in the maintenance of long-term protective immunity (14, 15). Furthermore, T-helper cells themselves can also have a direct antitumor effect (13). This effect may be particularly relevant to melanoma, as melanoma cells often constitutively express MHC-II molecules (16), which are not downregulated during

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progression (17). Recently, MHC-II epitopes derived from gp100 and tyrosinase have been made available for use in clinical trials (18–20). To determine the additional value of coactivating $CD4^+$ T-helper cells, we investigated the immunologic and clinical responses after vaccination with DCs either pulsed with MHC-I or MHC-I/II–restricted epitopes of g100 and tyrosinase.

Materials and Methods

Patient population

Patients with melanoma with regional lymph node metastases [American Joint Committee on Cancer (AJCC) criteria stage III] scheduled for radical lymph node dissection (RLND) or with distant metastases (AJCC stage IV) were included. Additional eligibility criteria included HLA-A*02:01 genotype, known HLA-DR4 status, expression of melanoma-associated antigens gp100 and tyrosinase, WHO performance status 0 or 1, and lactate dehydrogenase within 2× upper limit of normal (reference value = 450 U/L). Patients with symptomatic brain metastases, serious concomitant disease, or a history of second malignancy were excluded. The trial was registered at ClinicalTrials.gov, identifier NCT00243529, and approved by the local Institutional Review Board. Written informed consent was obtained from all patients.

Study protocol

Patients received the vaccine intranodally (i.n.) injected in a clinically tumor-free lymph node under ultrasound guidance. All administered vaccines consisted of autologous mature monocyte-derived DCs pulsed with gp100 and tyrosinase peptides and keyhole limpet hemocyanin (KLH) protein. Patients were assigned to 2 different groups, depending on their HLA-DR4 genotype. Patients with HLA-DR4 genotype were vaccinated with DCs loaded with MHC-I/II peptides (group A), patients without HLA-DR4 genotype were vaccinated DCs loaded with MHC-I peptides only (group B). Patients were evaluable for immunologic response, as they completed at least 1 cycle of 3 vaccinations with a biweekly interval, followed by delayed-type hypersensitivity (DTH) skin test (8). All 7 patients with RDLN metastases received 1 extra vaccination 2 days before scheduled RLND for additional imaging studies (Aarntzen and colleagues, manuscript submitted) and started thereafter with the standard schedule of 3 biweekly vaccinations. Patients without progression after the first vaccination cycle were eligible for a maximum of 2 maintenance cycles at 6-month intervals. All vaccinations were administered between February 2002 and February 2009. The primary study endpoint was vaccine-specific immune response; secondary endpoints included the clinical response [progression-free survival (PFS) and overall survival (OS) on intention-to-treat basis, calculated from the time of apheresis to event] and toxicity.

DC preparation and characterization

DCs were generated from peripheral blood mononuclear cells (PBMC) prepared from leukapheresis products as described previously (8). Part of the PBMCs was used to generate monocyte-conditioned medium. Plastic-adherent monocytes were cultured in X-VIVO-15 medium (BioWhittaker) supplemented with 2% pooled human serum (HS; Bloodbank Rivierenland), interleukin (IL)-4 (500 U/mL), and granulocyte macrophage colony-stimulating factor (GM-CSF; 800 U/mL; both CellGenix). Immature DCs were pulsed at day 3 with KLH (KLH, 10 μg/mL; Calbiochem). Two days before the harvesting, cells were matured with autologous monocyte-conditioned medium with prostaglandin E_2 (10 μg/mL; Pharmacia & Upjohn) and 10 ng/mL of recombinant TNF-α (provided by Dr. G. Adolf, Bender Wien, Vienna, Austria). This protocol gave rise to mature DCs (21). Patients received at maximum 15×10^6 DCs per injection.

Peptide pulsing of DC

DCs were pulsed with the MHC-I–restricted peptides gp100:154–162 (KTWGQYWQV; ref. 22) and gp100:280–288 (YLEPGPVTA; ref. 23) and tyrosinase:369–377 (YMDGTMSQV); MHC-II–restricted peptides gp100:44–59 (WNRQLYPEWTEAQRLD; ref. 24); and tyrosinase:448–462 (DYSYLQDSDPDSFQD; ref. 25). Peptide pulsing was conducted as described previously (8), and cells were suspended in 0.1 mL for injection.

Flow cytometry

The following fluorescein isothiocyanate (FITC)-conjugated mAbs were used: anti-HLA-Class-I (W6/32), anti-HLA-DR/DP (Q5/13); and phycoerythrin (PE)-conjugated mAbs: anti-CD14, anti-CD83, anti-CD28 (Beckman Coulter, and anti-CD80, anti-CD8, anti-CD86, anti-CD45RA, anti-CD62L (BD Biosciences). Directly labeled mAbs against CD4, CD8, CD25, CD127, CTLA-4 (BD Pharmingen) and FoxP3 (eBioscience), all according to the manufacturer's protocol, were used to characterize T cells. Regulatory T cells were defined as $CD4^+FoxP3^+CD25^{high}CD127^{low}$ cells. The FACSCalibur flow cytometer equipped with CellQuest software (BD Biosciences) was used.

Evaluation of immunologic responses

Peripheral blood sampling was conducted before and after each vaccination and at every follow-up visit thereafter, for the evaluation of KLH-specific responses and tetramer staining. DTH skin test procedures were conducted at the completion of each cycle of 3 biweekly vaccinations, up to a total 3 cycles at a 6-month interval in case of no progression. For evaluation of the immunologic responses, we compared the maximum vaccine-induced responses per individual, as this most accurately reflects the competence of an individual to mount an immune response (Aarntzen and colleagues, manuscript submitted).

KLH-specific proliferation

PBMCs were isolated from heparinized blood by Ficoll-Paque density centrifugation, stimulated with KLH (4 μg/2×10^5 PBMCs) in X-VIVO with 2% HS. After 3 days, cells were incubated with ^{3}H-thymidine for 8 hours, incorporation was measured with a β-counter. Experiments were carried out in triplicate.

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KLH-specific antibodies

Antibodies against KLH were measured in the serum of vaccinated patients using ELISA (8). Microtiter plates were coated with KLH, and different concentrations of patient serum were allowed to bind. After washing, patient antibodies were detected with mouse anti-human IgG, IgA, or IgM antibodies labeled with horseradish peroxidase; 3,3′-5,5-tetramethyl benzidine was used as a substrate. An isotype-specific calibration curve for the KLH response was included in each plate, the detection limit was determined at above 20 mg/L (26).

Skin test infiltrating lymphocyte analyses

Skin tests were conducted as described before (8, 27). Briefly, 2×10^6 to 10×10^6 DCs pulsed with the indicated peptides were injected intradermally at different sites. After 48 hours, punch biopsies (6 mm) were taken, half of the biopsy was manually cut and cultured in RPMI-1640 containing 7% HS and IL-2 (100 U/mL).

No skin test infiltrating lymphocyte cultures were obtained before therapy as we previously showed that although induration might be present, no vaccine-specific T cells were detected before DC-based vaccination (28).

Tetramer staining

SKIL cultures and PBMCs were stained with tetrameric MHC complexes containing the MHC-I epitopes gp100:154–168, gp100:280–288, or tyrosinase:369–377 (Sanquin) or MHC-II epitopes gp100:44–59 and tyrosinase:448–462 (provided by W.W. Kwok, Benaroya Research Institute, Seattle, WA) as described previously (8). In addition, PBMCs were restimulated for 8 days with DR4-binding gp100 or tyrosinase peptides and stained with tetrameric MHC complexes containing MHC-II epitopes gp100:44–59 and tyrosinase:448–462. Tetrameric MHC complexes recognizing HIV were used as correction for background binding. Tetramer positivity was defined as at least 2-fold increase in the double-positive population.

Antigen and tumor recognition

SKIL cultures were challenged with T2 cells pulsed with the indicated peptides or control antigen G250; or an allogenic HLA-A*02:01–positive, HLA-DR4–negative, gp100-positive, and tyrosinase-positive tumor cell line (MEL624), which was stimulated with IFN-γ (400 U/L) for 48 hours before stimulation. Cytokines were measured in supernatants after 16 hours by the cytometric bead array (Th1/Th2 Cytokine CBA 1; BD Pharmingen). Positive and specific cytokine production was defined as a 2-fold increase compared with stimulation with the cell lines pulsed with an irrelevant peptide.

Cytotoxic activity

Cytotoxic activity of SKILs was measured using the chromium release assay as described previously (29). Briefly, T2 or MEL624 cells were incubated with 100 μCi $Na_2[^{51}Cr]O_4$ (Amersham) and, after washing, added to lymphocytes (1×10^5 cells) and unlabeled K562 cells (1×10^4 cells) in triplicate wells of a round bottom microtiter plate (E:T ratio = 10:1). After 4 hours, supernatants were harvested and radioactivity was measured. The specific percentage of cytotoxicity was defined by the following formula:

$$\text{Specific cytotoxicity (\%)} = [\text{experimental release (cpm)} - \text{spontaneouse release (cpm)}]/[\text{maximum release (cpm)} - \text{spontaneous release (cpm)}] \times 100$$

Matched controls

Matched controls were identified from records of patients with metastatic melanoma from the Radboud University Nijmegen Medical Centre (Nijmegen, the Netherlands), The Netherlands Cancer Institute/Antoni van Leeuwenhoek Hospital (Amsterdam, the Netherlands), and University Hospital Essen (Essen, Germany) who had received first-line dacarbazine (DTIC) chemotherapy at 850 to 1,000 mg/m^2 i.v. at 3 weekly intervals, between March 2000 and March 2010. All matched controls were HLA-A*02:01–positive and were required to have received at least 3 infusions, a therapy time frame that is consistent with one cycle of vaccinations.

Control patients were matched to study subjects, in ratio of 1:3, primarily for M substage at baseline according to AJCC criteria, number of distant metastases, number of metastatic sites, localization of distant metastases, and baseline serum lactate dehydrogenase (LDH). These criteria currently represent the most important prognostic factors for survival (30). In case of more than 3 matches for 1 study subject, demographic criteria (age, gender) and systemic salvage treatment after progression on DTIC were used to select the closest match.

Statistical analysis

Differences between the groups were evaluated using an unpaired nonparametric *t* test (Mann–Whitney *U* test). Differences between pre- and post-vaccination were evaluated by a Wilcoxon signed ranks test, *P* values are 2-tailed. Kaplan–Meier probability estimates of PFS and OS were calculated, statistical differences between groups were determined by a log-rank test. Statistical significance was defined as $P < 0.05$. SPSS19.0 was used for all analyses.

Results

Patient and vaccine characteristics

A total of 33 patients were enrolled (Supplementary Fig. S1). Three patients were considered as nonevaluable for immunologic response, as they did not complete 3 vaccinations and DTH skin test because of rapid progressive disease. One patient did not meet eligibility criteria. Thus, a total of 29 patients with stage III ($n = 7$) and IV ($n = 22$) melanoma completed at least 1 cycle of vaccinations. Group A (vaccination with MHC-I/II–loaded DCs) consisted of 15 patients (4 stage III and 11 stage IV) and group B (vaccination with MHC-I loaded DC) of 14 patients (3 stage III and 11 stage IV. Patient and treatment characteristics are summarized in Table 1.

The genotype of the *ex vivo* generated DCs was determined by flow cytometry, and in both groups, the vaccine met the standard release criteria, with respect to expression of MHC-class I and II, costimulatory molecules, CD83 and CCR7 (Supplementary Fig. S2.

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Table 1. Patient characteristics

	Sex	Age (y)	AJCC stage	N stage	M stage	LDH[a] (U/L)	Number of mets	Localization of mets	Prior systemic treatment
IV-A-01	m	69	IV	N1b	M1a	384	>10	LN, skin	Cx
IV-A-03	m	57	IV	Nx	M1b	n.a.	n.a.	LN, lung	No
IV-A-04	m	51	IV	Nx	M1c	417	6	liver	No
IV-A-05	m	37	IV	Nx	M1a	253	3	skin	Ix[b]
IV-A-06	m	66	IV	Nx	M1c	345	6	adrenal gland, intestine	No
IV-A-07	m	38	IV	N1b	M1b	315	6	LN, skin, lung	No
IV-A-08	m	55	IV	N3	M1c	652	3	LN	No
IV-A-09	f	44	IIIa	N1a	M0	319	2	LN	No
IV-A-10	f	54	IV	Nx	M1c	432	2	liver, lung	No
IV-A-11	m	64	IV	Nx	M1b	361	3	lung	Ix[b]
IV-A-12	m	41	IV	N3	M1c	334	4	liver, LN, skin	Cx, Ix[c]
IV-A-13	f	55	IV	Nx	M1c	532	6	liver, LN, lung	No
IV-A-14	m	22	IIIb/c	N1b	M0	267	1	LN	No
IV-A-15	f	70	IIIb/c	N1b	M0	318	1	LN	No
IV-A-16	m	56	IIIc	N3	M0	346	9	LN	No
IV-A-17	m	59	IV	N2c	M1c	459	6	LN, lung, intestine	No
IV-B-01	m	48	IIIb/c	N2b	M0	298	3	LN	No
IV-B-02	f	57	IIIb/c	N1b	M0	403	1	LN	No
IV-B-03	m	54	IV	N2b	M1a	456	6	skin	No
IV-B-04	f	65	IV	Nx	M1b	469	2	lung	No
IV-B-05	m	50	IV	N3	M1c	1834	>10	liver, LN, lung, bone, skin	No
IV-B-06	m	50	IV	N3	M1c	735	6	liver, LN	No
IV-B-07	f	42	IV	Nx	M1c	487	>10	LN, lung, bone, skin	Cx
IV-B-08	m	43	IV	N2b	M1c	324	5	LN, lung, skin, bladder	No
IV-B-09	m	65	IV	N1b	M1c	449	6	liver, bone, skin	No
IV-B-10	f	37	IV	N1b	M1b	334	6	LN, lung, skin	No
IV-B-11	m	65	IV	Nx	M1c	640	>10	liver, LN, lung	Cx
IV-B-12	f	38	IV	Nx	M1b	387	>10	lung, skin	No
IV-B-13	m	30	IV	N3	M1c	464	3	LN, bone	Cx
IV-B-14	f	20	IV	N2a	M1b	293	5	LN, lung, skin	No
IV-B-15	m	69	IV	N2b	M1c	788	6	LN, lung, skin, adrenal gland	No
IV-B-16	f	46	IIIb	N2b	M0	292	3	LN	No

Abbreviations: Cx, chemotherapy; Ix, immunotherapy; LN, lymph nodes; mets, metastases; n.a., not available.

[a]Upper limit of normal (ULN) = 450 U/L.

[b]IFN-α adjuvant.

[c]MAGE-A3 peptide vaccination.

No grade 3 or 4 toxicities were observed (Table 2). In group A, 4 patients experienced grade 1 and 4 patients experienced grade 2 flu-like symptoms; 3 patients had grade 2 injection site reaction consisting of induration and redness. One patient developed vitiligo during the course of vaccination. In group B, 7 patients developed grade 1 flu-like symptoms and 6 patients had grade 1 injection site reactions. No grade 2 toxicities were observed in group B.

Immunologic response to the control antigen KLH

To test the capacity of the patients in this study to generate an immune response, we loaded the DCs with the control antigen KLH. Most patients showed increased T-cell proliferation upon stimulation with KLH, to comparable extent in both groups (Supplementary Fig. S3). Anti-KLH IgG antibodies were detected in 10 of 15 patients tested in group A and 7 of 14 patients in group B, to comparable levels. Anti-KLH IgA and IgM antibodies were detected in a minority of patients (Supplementary Fig. S3).

Tumor-specific $CD8^+$ T-cell responses in SKIL cultures

In group A, 7 of 14 patients tested showed tetramer-positive $CD8^+$ T cells against at least 1 epitope and 3 patients had tetramer-positive $CD8^+$ T cells against multiple epitopes present in SKIL cultures. In group B, this was 6 and 5 of 14 patients, respectively (Fig. 1).

To test the functionality of TAA-specific $CD8^+$ T cells, we measured cytokine production upon *in vitro* challenge

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Table 2. Toxicity and clinical outcome

	Number of vaccinations	Flu-like symptoms (CTC grade)	Injection site reaction (CTC grade)	Other immune related side effects side effects (CTC grade)	Best response	PFS (mo)	Salvage systemic treatment	OS (mo)
IV-A-01	9	2	2	Vitiligo	MR	39	No	119+
IV-A-03	6	0	0	No	SD	12	CIx	16
IV-A-04	3	1	0	No	SD	5	Cx	19
IV-A-05	6	0	0	No	SD	12	Cx	20
IV-A-06	3	1	0	No	PD	2	Cx	14
IV-A-07	3	1	0	No	SD	4	Cx	9
IV-A-08	1	0	0	No	PD	1	Cx	5
IV-A-09	9	2	0	No	NED	36	No	86+
IV-A-10	6	1	0	No	SD	14	Unknown	51
IV-A-11	3	0	0	No	SD	7	No	14
IV-A-12	3	0	0	No	PD	1	No	14
IV-A-13	3	0	0	No	SD	5	Cx	20
IV-A-14	9	2	2	No	NED	83+	n.a.	83+
IV-A-15	3	0	0	No	NED	5	No	6
IV-A-16	9	2	2	No	NED	40	No	41
IV-A-17	3	0	0	No	PD	2	No	7
IV-B-01	9	0	1	No	NED	19	Cx	22
IV-B-02	9	1	1	No	NED	18	No	23
IV-B-03	3	0	0	No	PD	2	CIx	7
IV-B-04	3	1	0	No	PD	2	CIx	16
IV-B-05	3	1	0	No	PD	2	Cx	3
IV-B-06	3	0	0	No	PD	2	Cx	7
IV-B-07	3	0	0	No	PD	1	No	3
IV-B-08	3	1	0	No	PD	4	Cx	7
IV-B-09	3	0	1	No	PD	2	Cx	6
IV-B-10	3	0	0	No	SD	6	Cx	12
IV-B-11	3	0	1	No	SD	5	No	9
IV-B-12	3	0	0	No	PD	2	No	4
IV-B-13	3	1	0	No	PD	3	No	3
IV-B-14	3	0	1	No	SD	8	No	46
IV-B-15	3	1	0	No	PD	2	No	3
IV-B-16	9	1	1	No	NED	69+	n.a.	69+

Abbreviations: CIx, chemoimmunotherapy; Cx, chemotherapy; MR, mixed response; n.a., not available; NED, no evidence of disease; PD, progressive disease; SD, stable disease.

with either peptide pulsed T2 cells or a gp100 and tyrosinase expressing HLA-A*02:01–positive melanoma cell line (Fig. 1). In 4 of 11 patients tested in group A, we observed increased levels of IFN-γ production upon challenge with the melanoma cell line, indicative of development of high-affinity CTLs that maintain tumor reactivity in a suppressive microenvironment. In contrast, in group B, antigen-specific IFN-γ production was measured in all 6 of 6 patients tested, but only 1 patient maintained IFN-γ production upon encounter of the melanoma cell line, suggesting that most T cells developed peptide specificity but cannot recognize naturally processed tumor antigen. In 2 patients, IV-A-05 and IV-A-07, the cytokine production was dominated by IL-5 (Fig. 1).

Tumor-specific CD4+ T-cell responses in SKIL cultures

Similarly, we analyzed SKIL cultures for the presence of TAA-specific CD4+ T cells in group A. In 4 of 10 patients tested with cytokine bead assays, we observed specific IFN-γ production to stimulation; in 3 patients (A-01, A-06, and A-16) to both gp100 and tyrosinase; and in 2 patients (A-09 and A-10) to one TAA. Interestingly, in 4 of 6 patients with a CD4+ T-cell response s in either peripheral blood or in SKIL cultures, concurrent TAA-specific CD8+ T cells were detected.

Tumor-specific responses in peripheral blood

We detected TAA-specific CD8+ T cells in 2 of 12 patients tested in group A and none in group B (Fig. 1). In 3 of 13 patients tested in group A, we detected tetramer-positive CD4+ T cells.

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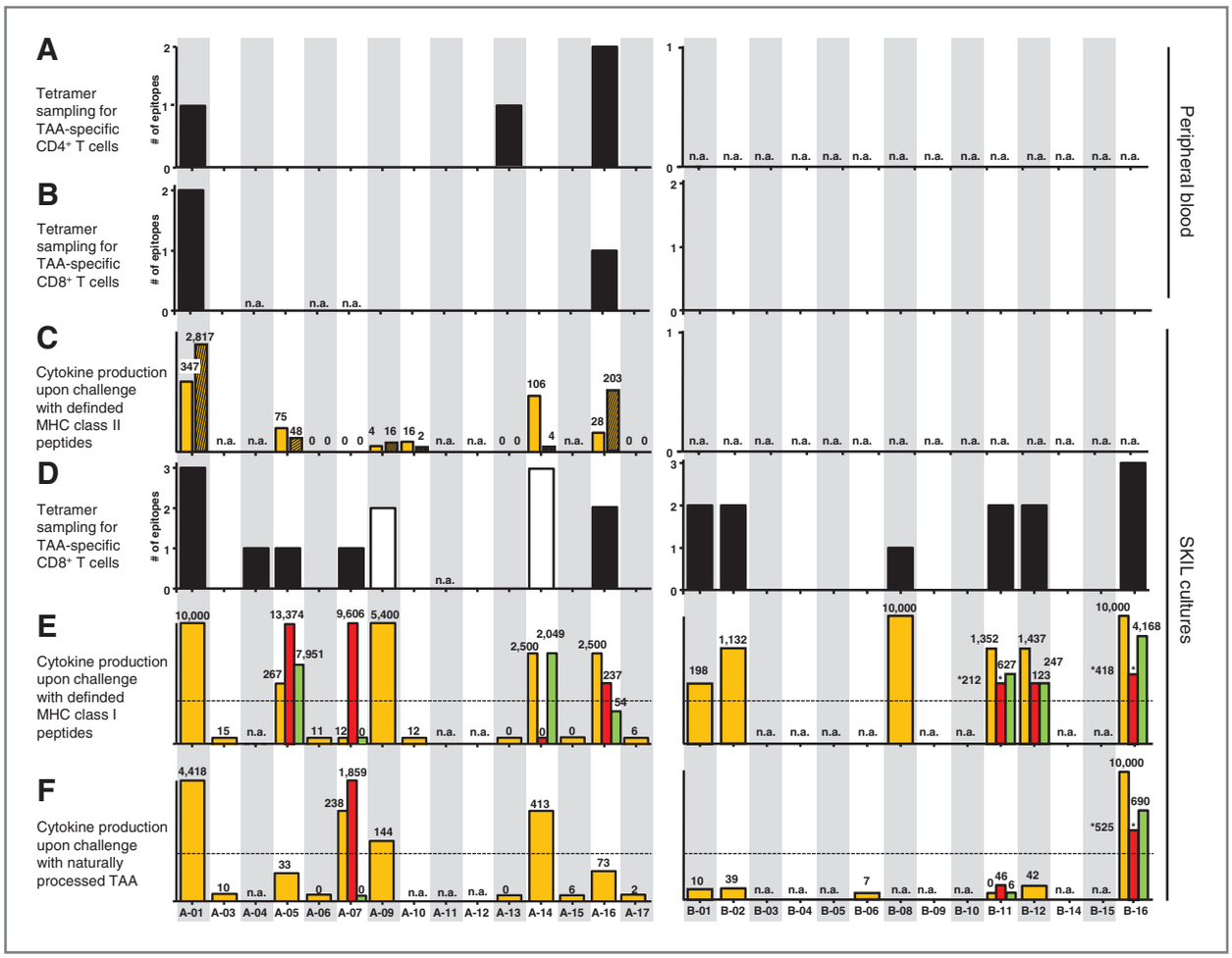

Figure 1. Tumor-specific immune responses upon vaccination with MHC-I or MHC-I/II–loaded DC vaccination. A graphical representation per patient of their immunologic responses after vaccination. A, tetramer screening for vaccine-induced CD4$^+$ T-cell responses in peripheral blood. B, tetramer screening for vaccine-induced CD8$^+$ T-cell responses in peripheral blood. C, the production of IFN-γ in SKIL cultures upon stimulation with gp100 (open yellow bars) or tyrosinase (hatched yellow bars). D, tetramer screening for vaccine-induced CD8$^+$ T-cell responses in SKIL cultures (open bars represent percentages of tetramer-specific T-cell populations that did not meet the predefined criteria for positivity, but positive IFN-γ production was measured upon stimulation with their cognate antigen). To test their functionality, TAA-specific CD8$^+$ T cells were challenged with specific epitopes presented by T2 cell line (E) or with naturally processed gp100 and tyrosinase presented by an allogeneic tumor cell line Mel 624 (F); cytokine production was measured (pg/mL). n.a., not available; dashed line is cutoff value for positivity as defined in Materials and Methods; yellow bars, IFN-γ production upon challenge; red bars, IL-5 production upon challenge; and green bars, IL-2 production upon challenge. The *y*-axis is not scaled. Bar heights are for visualization only; actual cytokine concentrations (pg/mL) are denoted on top of the bars.

In all 3 patients, TAA-specific CD4$^+$ T cells were characterized as CD25$^+$FoxP3$^-$ with maintained capacity to proliferate and produce IFN-γ (Fig. 2).

Clinical responses

Clinical responses are summarized in Table 2. The median PFS of stage III patients was 37 months (range, 5–76); however, their number is too limited to draw meaningful conclusions on the clinical response. For patients with distant metastatic disease, we retrospectively compared survival data with carefully matched controls (Supplementary Table S1). The median PFS in group A was significantly improved compared with controls, with 5.0 versus 2.8 months ($P = 0.0089$); HR, 0.39 [95% confidence interval (CI), 0.19–0.79; Fig. 3A]. There was no significant difference in median PFS between patients vaccinated in group B versus matched controls; 2.0 versus 2.5 months ($P = 0.891$); HR, 1.05; 95% CI, 0.52–2.14.

With regard to OS, patients in group A showed increased 1-year survival rates as compared with matched controls, 80% versus 36%. Median OS was improved, 15.0 compared with 8.3 months for matched historic controls ($P = 0.089$); HR, 0.57; 95% CI, 0.30–1.09. In comparison, patients in group B did not show improved 1-year survival rates, nor median OS rate over DTIC; 27% versus 36% and 7.0 versus 7.9 months ($P = 0.202$); HR, 1.32; 95% CI, 0.64–2.71.

In group A, the presence of vaccine-specific CD4$^+$ T-cell responses in peripheral blood or SKIL cultures was not significantly associated with OS: HR, 0.21; 95% CI, 0.03–1.70; P =

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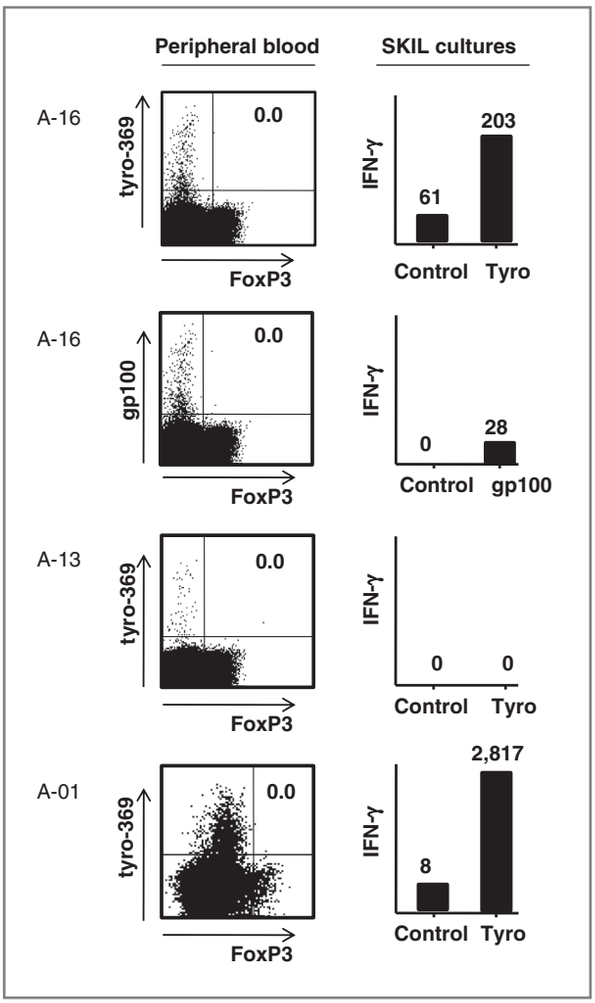

Figure 2. Vaccine-induced CD4+ T cells are not regulatory T cells. In 3 patients, TAA-specific CD4+ T cells were detected in peripheral blood, recognizing one or more MHC class II epitopes (Table 3). A flow cytometric analysis was conducted to further characterize these cells. In all patients, the tetramer-positive cells were CD25+FoxP3−, maintained their proliferative capacity (Table 3), and produced marked levels of IFN-γ upon restimulation.

0.144. In groups A and B, the presence of vaccine-specific CD8+ T-cell responses in peripheral blood or SKIL cultures was not associated with OS: HR, 0.65; 95% CI, 0.19–2.29; $P = 0.507$; and HR, 1.16; 95% CI, 0.30–4.50; $P = 0.834$.

Discussion

The efficacy of DC-based vaccination in patients with cancer has significantly improved over the past decade, as several vaccine parameters have been optimized. We initiated the current study to compare immunologic responses to DCs pulsed with MHC-I–restricted melanoma epitopes alone or MHC-I/II–restricted epitopes.

Upon vaccination with MHC-I/II–loaded DCs, we detected highly functional vaccine-specific CD8+ T cells. These cells maintained their IFN-γ production even in a suppressive milieu of an IL-10–producing melanoma cell line. Interestingly, only in patients vaccinated with MHC-I/II–loaded DCs, we found circulating TAA-specific CD8+ T cells.

The induction of regulatory CD4+ T cells is of particular concern in immunotherapies targeting MHC-II–restricted antigens (31, 32). We showed in all patients that TAA-specific CD4+ T cells were FoxP3-negative, which is in line with the high proliferative capacity and production of significant levels of IFN-γ. Furthermore, in most patients, we detected concomitant TAA-specific CD8+ T-cell responses. These findings strongly support the hypothesis that coactivating TAA-specific CD4+ T helper cells augments the induction and proliferation of TAA-specific CD8+ T cells.

Our findings are in line with previous reports on vaccination with MHC-I/II–loaded DCs (33–36), but direct comparison is hampered by the large variation in study protocols. This is the first article that evaluates the immunologic responses upon vaccination with MHC-I/II–loaded DCs in direct comparison with MHC-I–loaded DCs.

However, our findings are in contrast to previously published trials on peptide vaccination in patients with melanoma (37–39). In a recent randomized multicentre trial, Slingluff and colleagues report on 167 patients with stage IIB to IV melanoma vaccinated with either MHC-I- or MHC-I/II–restricted peptides, with or without cyclophosphamide pretreatment (37). In this trial, the inclusion of melanoma-associated helper peptides paradoxically decreased CD8+ T-cell responses. The discrepancy with our findings might be explained by the intradermal/subcutaneous delivery of peptides, as it might target different DC subsets, compared with direct delivery of antigen-loaded DCs into lymph nodes. To our opinion, controlling antigen presentation is crucial as the induction of CD4+ T cells with improved functionality depends on density and potency of peptide–MHC complexes (40, 41).

Although in a previous vaccination study in 108 patients with melanoma, no benefit was found for HLA-DR4 expression (42), we cannot exclude that this haplotype forms a possible confounding bias in our study when interpreting the clinical data. To obtain more insight in the clinical efficacy of our DC vaccination, we compared the clinical outcome with carefully selected control patients treated with standard DTIC chemotherapy. Comparison with control patients is based on the assumption that the observed historical control response rate is equal to the true control response rate (3). In this respect, survival rates of our matched controls treated with DTIC chemotherapy are comparable with the survival rates reported in recent large randomized trials using DTIC as comparative arm (43, 44). The observed clinical responses are in line with the trend in the differences in the immunologic responses. Only patients who received MHC-I/II–loaded DCs showed increased PFS and 1-year OS as compared with matched control patients. Strikingly, the presence of circulating TAA-specific CD4+ or CD8+ T cells or IFN-γ production upon challenge with naturally processed antigens was clearly linked to a more beneficial clinical course of disease (Fig. 1 and Table 1). It is tempting to speculate that part of the clinical response is related to action of the CD4+ T-helper cell at the tumor site itself. CD4+ T-helper cells have been shown to play an important role in assisting infiltration of the tumor by CTLs and can exert a direct cytotoxic

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Table 3. Immunologic responses in SKIL cultures and peripheral blood

	Tetramer specific CD8$^+$ T cells in SKIL cultures						Tetramer specific CD8$^+$ T cells in blood						Tetramer specific CD4$^+$ T cells in blood				After restimulation			
	gp100–154		gp100–280		Tyrosinase		gp100–154		gp100–280		Tyrosinase		gp100		Tyrosinase		gp100		Tyrosinase	
	% gated	% control	% gated	% control	% gated	% control	% gated	% control	% gated	% control	% gated	% control	% gated	% control	% gated	% control	% gated	% control	% gated	% control
Group A																				
IV-A-01	4.18	0.05	0.70	0.02	10.9	0.20	0.09	0.00	—	—	0.08	0.00	—[a]	—	0.12	0.02	—	—	0.37	0.12
IV-A-03	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
IV-A-04	0.99	0.17	—	—	—	—	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	—	—	—	—	—	—	—	—
IV-A-05	0.20	0.00	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
IV-A-06	—	—	—	—	—	—	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	—	—	—	—	—	—	—	—
IV-A-07	0.04	0.02	—	—	—	—	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	—	—	—	—	—	—	—	—
IV-A-09	—[a]	—	—	—	—[a]	—	—	—	—	—	—	—	—	—	—[a]	—	—	—	—	—
IV-A-10	—	—	—	—	—	—	—	—	—	—	—	—	—[a]	—	—	—	—	—	—	—
IV-A-11	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	—	—	—	—	—	—	—	—	—	—	—	—	—	—
IV-A-12	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
IV-A-13	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.02	0.00	—	—	2.74	0.07
IV-A-14	0.44	0.00	—[a]	—	—[a]	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
IV-A-15	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
IV-A-16	1.80	0.04			0.41	0.06	10.37	1.18	—	—	—	—	0.1	0.02	0.08	0.02	3.03	0.3	0.45	0.01
IV-A-17	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Group B																				
IV-B-01	1.60	0.17	0.40	0.17	—	—	—	—	—	—	—	—	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
IV-B-02	0.25	0.09	0.43	0.09	—	—	—	—	—	—	—	—	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
IV-B-03	—	—	—	—	—	—	—	—	—	—	—	—	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
IV-B-04	—	—	—	—	—	—	—	—	—	—	—	—	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
IV-B-05	—	—	—	—	—	—	—	—	—	—	—	—	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
IV-B-06	—	—	—	—	—	—	—	—	—	—	—	—	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
IV-B-08	—	—	0.09	0.01	—	—	—	—	—	—	—	—	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
IV-B-09	—	—	—	—	—	—	—	—	—	—	—	—	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
IV-B-10	—	—	—	—	—	—	—	—	—	—	—	—	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
IV-B-11	0.35	0.07	0.36	0.07	—	—	—	—	—	—	—	—	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
IV-B-12	0.45	0.01	—	—	0.12	0.02	—	—	—	—	—	—	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
IV-B-13	—	—	—	—	—	—	—	—	—	—	—	—	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
IV-B-14	—	—	—	—	—	—	—	—	—	—	—	—	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
IV-B-15	—	—	—	—	—	—	—	—	—	—	—	—	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
IV-B-16	0.34	0.01	2.52	0.01	0.05	0.01	—	—	—	—	—	—	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Abbreviation: n.a., not available.

[a]The percentages of tetramer-positive cells did not meet the predefined criteria for positivity, nevertheless positive levels of cytokines were measured upon stimulation with their cognate antigen.

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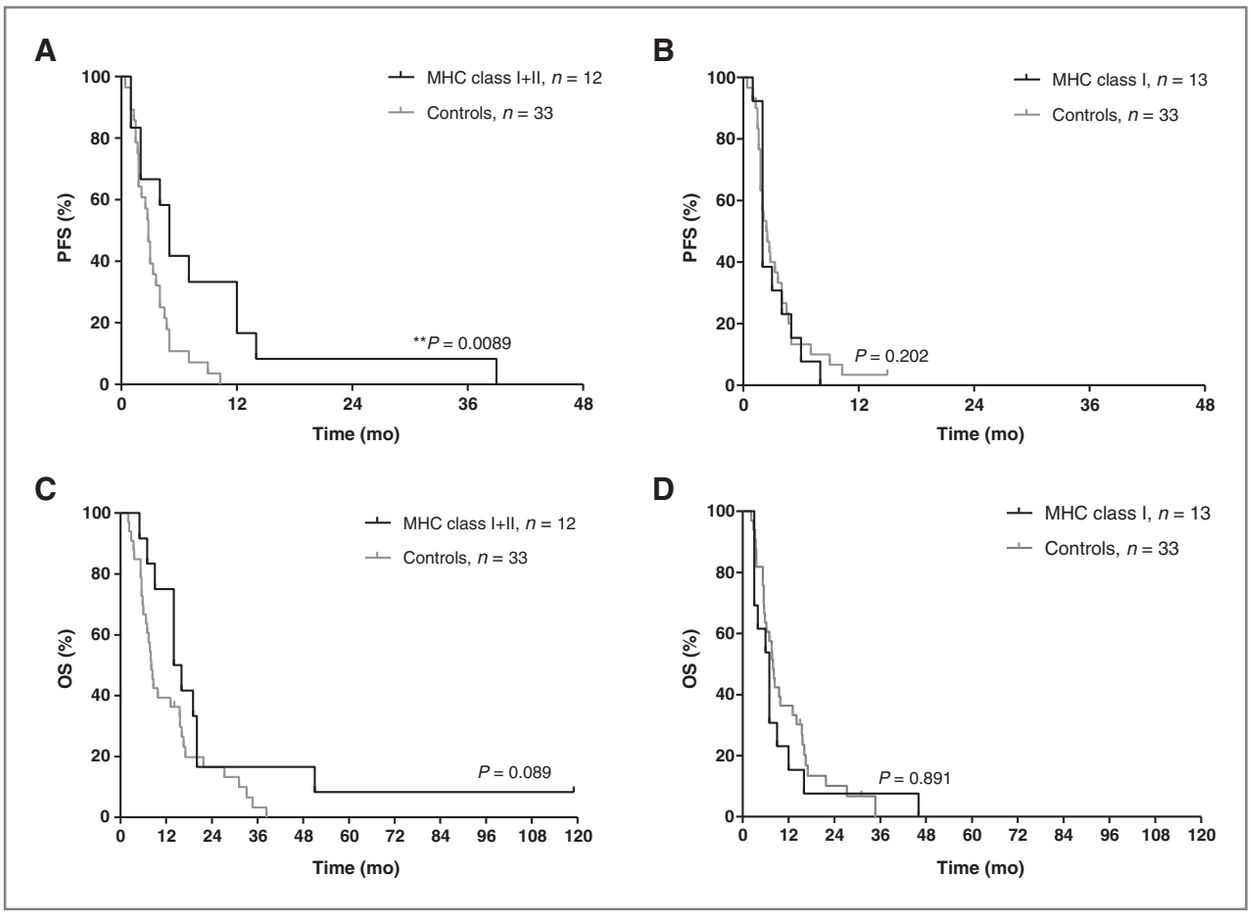

Figure 3. Improved clinical responses upon vaccination with MHC class I and II–loaded DCs. In patients with distant metastatic or irresectable melanoma (stage IV), vaccination with MHC-I/II–loaded DCs resulted in significantly improved PFS compared with controls (A). Vaccination with MHC-I–loaded DCs only did not improve PFS (B). Median OS was improved for vaccination with MHC-I/II–loaded DCs compared with DTIC chemotherapy (C). Accordingly, 1-year survival rates improved upon vaccination with MHC-I/II–loaded DCs. Vaccination with MHC-loaded DCs did not result in improved OS (D).

effect themselves as well (45, 46). In 2006, a randomized trial comparing MHC-I/II–loaded DC vaccination with DTIC chemotherapy was reported (42). This study failed to show improved clinical outcome to DC-based vaccination. As discussed previously (47), the exploited DC-based vaccine in this study was far from optimized. Because of the subcutaneous delivery and low number of injected DC, it is questionable how many antigen-loaded DCs were available for immune induction. Furthermore, injected DCs mostly had a low mature genotype and lacked a nonspecific T-helper antigen, so their immunostimulatory capacity is suboptimal (47). Our study suggests that optimized DC-based vaccination harbors more potential than has been concluded from this study. The importance of antigen-specific CD4$^+$ T-cell stimulus by the same antigen-presenting cell that present tumor-antigen in MHC class I to CD8$^+$ T cells has been shown in previous studies (48, 49). Optimal CD4$^+$ T-cell help might mediated by CD40–CD40L interaction and cytokines such as IL-2. These studies support our findings of superior immune responses in patients vaccinated with DCs loaded with tumor antigen in both MHC class I and II.

Finally, the current vaccination protocol might be further improved by overcoming the disadvantages of peptide pulsing of DCs, for example, the dissociation of peptides from MHC complexes and the lack of posttranslational modification by the use of mRNA electroporation (50, 51). This method has shown to result in mature and migratory DCs expressing the encoded antigens *in situ* (51).

In conclusion, our results show that targeting CD4$^+$ T-helper cells with DCs pulsed with both MHC class I and II–restricted epitopes enhances vaccine-specific immunologic responses.

Disclosure of Potential Conflicts of Interest

The authors declare that the manuscript contains original work only. All authors have directly participated in the planning, execution, and analysis of this study. All have approved the submitted version of the manuscript. The described data have not been published nor submitted elsewhere. No potential conflicts of interest were disclosed.

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