

Results of a phase I clinical study using autologous tumour lysate-pulsed monocyte-derived mature dendritic cell vaccinations for stage IV malignant melanoma patients combined with low dose interleukin-2

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We conducted a pilot study to assess the feasibility and efficacy of immunotherapy for stage IV malignant melanoma patients resistant to conventional therapies involving vaccination with mature dendritic cells (mDCs) combined with administration of low dose interleukin-2. Autologous monocytes were harvested from a single apheresis and cultured for 7 days with granulocyte-macrophage colony-stimulating factor and interleukin-4, yielding immature dendritic cells (iDCs), which were then cryopreserved until use. For 4 days prior to vaccination, iDCs were exposed to autologous tumour lysate combined with tumour necrosis factor- α to induce terminal differentiation into mDCs. Patients were then vaccinated weekly with 10^7 mDCs for 10 weeks and given 350–700 kIU of interleukin-2 three times per week. Of the 10 patients in the study, one showed stable disease, seven showed progressive disease, and two showed mixed responses, including partial tumour regression, and were therefore given 20 additional injections. Only minimal adverse events were noted, including localized skin reactions and mild fever (NIH-CTC grade 0–1). Median survival from the first vaccination was 240 days (range 31–735 days). *In vitro*, melanoma patient-derived dendritic cells (DCs) showed reduced cell surface expression of CD1a antigen on iDCs and reduced CD86 and HLA-DR expression on mDCs. In addition, antigen uptake,

chemotaxis and antigen presentation were all attenuated in DCs from the patients. In summary, although improvement of clinical efficacy will require further research, autologous tumour lysate-pulsed monocyte-derived mDCs could be safely harvested, cryopreserved and administered to patients without obvious complications. *Melanoma Res* 13:521–530 © 2003 Lippincott Williams & Wilkins.

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Introduction

Dendritic cells (DCs) are unique major specialist antigen-presenting cells (APCs) capable of stimulating naive T-cells during primary immune responses more potently than either peripheral blood monocytes/macrophages or B-cells [1]. Previous studies have shown that immature DCs (iDCs) exhibit several characteristic features, including (i) vigorous endocytotic ability; (ii) a high capacity to produce pro-inflammatory cytokines; and (iii) potent chemotactic responses to inflammatory chemokines such as regulated on activation normal T-

cell expressed and secreted (RANTES) and macrophage inflammatory protein (MIP)-1 α via chemokine receptors CCR-1 and CCR-5, respectively [1–3]. The development of iDCs into mature DCs (mDCs) can be induced by a variety of stimuli, including bacterial components such as lipopolysaccharide, pro-inflammatory cytokines such as tumour necrosis factor- α (TNF α) and cognate CD4+ T-cell help via interaction with CD40/CD40 ligand. These events are accompanied by proteolytic cleavage within phagolysosomes, presentation of antigens at the cell surface by major histocom-

patibility complex (MHC) proteins, upregulation of costimulatory molecules, the ability to stimulate T-cells, downregulation of the internalization of exogenous soluble antigen, and production of pro-inflammatory cytokines [1–3]. This maturation process also involves the downregulation of the cell surface expression of CCR-1 and CCR-5, resulting in diminished chemotactic responses to inflammatory chemokines, and enhanced expression of CCR-7, resulting in mDCs homing into secondary lymphoid tissues via the interaction of CCR-7 with MIP-3 β , where they prime naive T-cells and initiate primary immune responses [1–3].

Evidence from both humans and animal models suggests that by enhancing tumour-specific T-cell responses, DCs actively contribute to a protective immunity against cancer [1]. Unfortunately, the use of DC-based tumour vaccines in clinical applications has been limited by the fact that there are relatively few DCs present in peripheral blood and other tissues, making isolation of sufficient cells for vaccination difficult [1–4]. Recently, however, a method was developed to generate DCs from peripheral blood, *in vitro*, by culturing their progenitor cells in cytokine-driven systems, a procedure that has profoundly changed preclinical research as well as the clinical evaluation of these cells [5,6]. Indeed, several groups have reported that vaccination with peripheral blood monocyte-derived iDCs or mDCs pulsed with tumour lysate or a cocktail of peptides derived from tumour-associated antigens (TAAs) appears to be potentially useful as an antitumour immunotherapy, although the clinical effectiveness and the impact on the survival of tumour patients remains unclear [7–9].

In this report, we describe a clinical pilot study in which vaccination with autologous, tumour lysate-pulsed, monocyte-derived mDCs combined with administration of recombinant human (rh) interleukin-2 (IL2) was used as immunotherapy to treat stage IV malignant melanoma patients resistant to conventional therapy. In addition, we also examined the immunological features of these DCs derived from peripheral blood monocytes from cancer patients to assess their suitability for the preparation of a DC-based tumour vaccine.

Materials and methods

Patient selection

This study protocol was approved by the Institutional Review Board of the Institute of Medical Science, University of Tokyo, Japan. All eligible patients were histologically proven to have melanoma with distant metastases. Ten patients (five males, five females; aged 24–75 years, mean 47 years) who had previously tried various other therapies entered the study (Table 1). No alternative therapy was given. Inclusion criteria were an

Table 1 Patient characteristics, status before DC vaccination and response to DC vaccination

Patient no.	Age (years)	Sex	Site of primary tumour	Status before vaccination		No. of DC vaccinations	Clinical response	Response to DTH skin test			Survival (days from first vaccination)
				Previous therapy	Sites of metastases			Prevaccination	5 weeks	10 weeks	
1	24	Male	Skin	Surgery, chemotherapy	Liver, lymph node	6	PD	ND	ND	ND	59
2	27	Female	Skin	Surgery, chemotherapy	Skin, lymph node, lung	7	PD	±	+	ND	50
3	48	Female	Skin	Surgery, chemotherapy	Brain, liver, stomach, lymph node, lung	10	PD	±	+	+	328
4	24	Female	Unknown	None	Skin, lymph node, multiple organs (brain, lung, liver, ovary, etc.)	10	PD	±	ND	+	152
5	75	Male	Skin	Surgery, chemotherapy	Skin, lymph node	10	PD	ND	+	+	629
6	74	Male	Skin	Surgery, chemotherapy	Skin, adrenal, brain, orbit	10	SD	ND	+	+	342
7	35	Male	Skin	Surgery, chemotherapy	Skin, muscle, lung, pancreas, pelvis	8	PD	+	–	ND	52
8	58	Female	Unknown	Surgery, chemotherapy	Lymph node, skin, pericardium, retroperitoneum	30	MIR	–	–	–	561
9	57	Male	Skin	Surgery, chemotherapy	Lung, muscle, adrenal	30	MIR	–	±	ND	735
10	43	Female	Skin	Surgery, chemotherapy	Skin, bone, dura mater	5	PD	–	±	–	31

PD, progressive disease; SD, stable disease; MIR, mixed response; ND, not done.

European Cooperative Oncology Group (ECOG) score of 0–2, adequate hepatic and renal function (total bilirubin < 2 mg/dl, serum creatinine < 2 mg/dl) and a life expectancy of more than 12 weeks. Patients with severe cardiac, pulmonary or psychiatric disease, or with acute uncontrollable infection, were excluded. As a control, peripheral blood samples from 12 normal healthy volunteers (five males, seven females; aged 23–79 years, mean 43 years) were collected and analysed. All of the study participants gave signed informed consent according to the Declaration of Helsinki before enrolling in the study.

Media and reagents

The medium used throughout was RPMI 1640 (Sigma, St Louis, Missouri, USA) supplemented with antibiotic-antimycotic (Gibco BRL, Gaithersburg, Maryland, USA) and 5% heat-inactivated human serum type AB (BioWhittaker, Walkersville, Maryland, USA). Recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) was kindly provided by Kirin Brewery (Tokyo, Japan); rhIL2, rhIL4, rhTNF α , rhRANTES and rhMIP-3 β were purchased from Pepro-Tech (London, UK).

In vitro generation and culture of iDCs from stage IV malignant melanoma patients and normal healthy volunteers

iDCs were generated from leukapheresis products as described elsewhere [10–12]. Briefly, peripheral blood mononuclear cells (PBMCs) were collected from single 15 l leukaphereses from the patients and healthy volunteers using a COBE SPECTRA (Cobe Laboratories, Lakewood, Colorado, USA). The collected cells were then separated by density gradation using Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden), and the light density fraction from the 42.5–50% interface was recovered. The cells were then resuspended in cold phosphate buffered saline (PBS) and allowed to adhere to 10 cm plastic dishes (Primaria, Becton Dickinson, Mountain View, California, USA) for 30 min at 37°C, after which non-adherent cells were removed and the remaining adherent cells were collected for DC preparation. The collected cells (> 10⁸ cells/apheresis) were > 95% pure, as indicated by anti-CD14 monoclonal antibody (MAb) staining (BD Pharmingen, San Diego, California, USA).

To prepare the iDCs, the cells were cultured in 10 cm plastic dishes (> 10⁷ cells/dish) with 6 ml of medium containing rhGM-CSF (final concentration 50 ng/ml) and rhIL4 (250 ng/ml) for 7 days at 37°C under a humidified atmosphere of 5% CO₂ in air, after which the resultant iDCs were harvested. Phenotypical analysis of the iDCs was carried out using a FACS Calibur flow cytometer (Becton Dickinson), CELL Quest Software (Becton Dickinson) and fluorescein isothiocyanate

(FITC)- or phycoerythrin (PE)-conjugated isotype-matched control MAbs (BD Pharmingen). DC family markers (CD1a and CD11c), co-stimulatory molecules (CD40, CD80 and CD86) and HLA-DR were detected in iDCs from both melanoma patients and healthy donors. To assess the endocytotic activity of the iDCs, mannose receptor-mediated endocytosis of FITC-conjugated dextran (FITC-DX) (Molecular Probes, Eugene, Oregon, USA) and macropinocytosis of lucifer yellow (LY) (Molecular Probes) via cytoskeleton-dependent fluid-phase endocytosis were evaluated [10,11]. Chemotaxis of DCs to RANTES or MIP-3 β (100 ng/ml) was assessed as described previously [10,11]. Data were expressed as the number of migrated cells/high power field (HPF).

Freezing and thawing procedures

The washed and counted iDCs were diluted in ice-cold medium consisting of 50% human type AB serum, 10% dimethyl sulphoxide and 40% RPMI 1640. The cells were distributed in 1 ml aliquots (> 10⁷ cells) into pre-cooled plastic vials, after which the vials were placed in an isopropanol-containing biofreezing vessel (BICELL, Nihon Freezer, Japan) and the temperature was reduced to –135°C at a rate of 1°C/min. The cryopreserved samples were then stored until used.

For experimentation, the frozen vials were quickly thawed in a 37°C water bath, after which the cells were washed twice in washing medium consisting of 10% human type AB serum, 5% dextran and 85% RPMI 1640, plated in 10 cm plastic dishes, and cultured in the absence of cytokine for 24 h at 37°C. Preliminary studies showed that frozen and thawed samples contained greater than 95% viable cells as determined by trypan blue exclusion (data not shown).

Tumour lysate extraction

Tumour lysates were used as a tumour antigen since TAA-derived antigenic peptides suitable for our melanoma patients were not available. To prepare the lysates, tumour mass was obtained by exclusion of non-malignant tissues from tumour biopsies with a scalpel. The isolated mass was then homogenized, and tumour cells were obtained by depleting lymphocytes from the total cell suspension using immunomagnetic bead-conjugated MAbs against CD2, CD14 and CD19 (Dyna, Oslo, Norway) according to the manufacturer's instructions. Aliquots of the isolated tumour cells (> 10⁷ = melanoma cells/tube) were then lysed by putting them through three freeze (in liquid nitrogen) and thaw (in a 37°C water bath) cycles. The lysed cells were centrifuged at 800 *g* for 5 min, and the supernatants were passed through a 0.22 μ m filter (Millipore Corporation, Bedford, Massachusetts, USA). The protein contents of the resultant cell-free lysates were determined using DC protein assay kits (Bio-Rad Laboratories, Hercules,

California, USA). Aliquots (500 µg/tube) were then stored at -135°C until use.

Preparation of tumour lysate-pulsed mDCs for vaccination
iDCs (> 10⁷ cells/dish) in 10 cm plastic dishes were cultured for 24 h in 3 ml of medium containing 100 µg/ml of autologous tumour lysates, and then in 6 ml of medium containing 50 ng/ml rhTNFα for an additional 4 days to induce terminal differentiation into mDCs.

Vaccination of patients with tumour lysate-pulsed monocyte-derived mDCs

One week before vaccination, the patients were interviewed and medical histories taken, and the following baseline studies were carried out. A physical examination and complete blood work up consisting of differentiation, blood chemistry and serology, including assays for C-reactive protein and tumour markers lactate dehydrogenase (LDH) and 5-S-cysteinyldopa (5-SCD) were performed. Whole-body computed tomography (CT) and delayed-type hypersensitivity (DTH) skin testing was carried out. Antinuclear antibodies (ANA) titres were determined in all patients upon enrolment in the study and after receiving 10 vaccinations to detect any serological autoimmune reaction. Each week for 10 weeks the eligible patients were injected intradermally with tumour lysate-pulsed monocyte-derived mDCs (10⁷ cells/injection) in close proximity to the cervical and inguinal lymph nodes. The clinical response was evaluated according to World Health Organization (WHO) criteria. Patients were deemed to have had a 'mixed response' if some of their tumours showed regression by > 25% of the pretreatment mass while others showed progression by > 25% of the pretreatment mass or new metastases appeared. Adverse events were evaluated by grading the toxicity according to the National Cancer Institute (NCI) Common Toxicity Criteria (CTC) guidelines version 2. Two patients with mixed responses were enrolled in an additional 20-vaccination protocol after obtaining signed informed consent and the recommendation of the institutional review board.

Concurrent administration of rhIL2 with DC-based tumour vaccine

rhIL2 (Imunace, 350 000 IU/vial, Shionogi Pharmacy, Osaka, Japan) was administered subcutaneously into the forearm three times a week. The dose of rhIL2 was generally 700 000 IU/day, though it had to be reduced in one patient because of side effects (mainly fever lower than 38°C and eosinophilia).

DTH skin testing

DTH skin tests were performed 4 days before the first vaccination and then 5 and 10 weeks after it. Irradiated (150 Gy from a ¹³⁷Cs source) autologous melanoma cells (10⁶ cells) or their lysates were injected intradermally

into the patients' forearm. In addition, irradiated autologous PBMCs (10⁶ cells) and their lysates were used as a negative control. Forty-eight hours after each injection, the diameter of the erythema and the induration were measured. Erythemas < 10 mm in diameter were defined as negative (-), those 10 mm in diameter were defined as neutral (±), and those > 10 mm in diameter were defined as positive (+). Erythemas > 10 mm with induration were defined as strongly positive (++)

Migration assay

In order to investigate the chemotactic migration of iDCs and mDCs toward RANTES and MIP-3β, polycarbonate membrane filters (pore size 8.0 µm) were precoated with 5 µg of gelatin. The lower chambers contained 600 µl of medium with or without 100 ng/ml RANTES and MIP-3β. The upper chamber contained 100 µl of cell suspension (10⁶ cells). The chambers were incubated at 37°C for 4 h. After staining with haematoxylin and eosin, the number of migrated cells per HPF was counted (magnification ×400).

Allogeneic MLR

T-cells were isolated to > 98% purity from PBMCs as described previously [13]. Allogeneic T-cells (10⁵ cells) were co-cultured with irradiated (15 Gy from a ¹³⁷Cs source) DCs (10⁴ cells) in 96-well flat-bottom microplates (Coster, Cambridge, Massachusetts, USA). Thymidine incorporation was measured on day 5 following an 18 h pulse with [³H]thymidine (1 µCi/well, specific activity 5 Ci/mmol, Amersham Life Science, Little Chalfont, Buckinghamshire, UK).

Cytotoxicity assay

PBMCs were obtained from four melanoma patients (patients 5, 6, 8 and 9) 10 weeks after the first vaccination and cultured for 4 days in medium containing 100 U/ml rhIL2 plus 100 µg/ml autologous tumour lysates. CD2+ lymphocytes were then positively selected from IL2-activated tumour lysate-pulsed PBMCs using anti-CD2 MAb conjugated immunomagnetic beads. CD2+ cells (10⁴ to 5 × 10⁵ cells) were co-cultured for 4 h in 96-well round-bottom plates with autologous or allogeneic melanoma cells (10⁴ cells) labelled with Na₂⁵¹CrO₄ (100 µCi/10⁶ cells, NEN Life Science Products, Boston, Massachusetts, USA) at effector cell-to-target cell ratios ranging from 25 to 200 (Coster). The supernatants were subsequently harvested, the radioactivity counted, and the percentage of specific lysis calculated [13]. The counts per minute (c.p.m.) for spontaneous release were < 20% of the total release c.p.m.

Statistical analysis

The statistical significance of differences between the two groups was evaluated using the non-parametric Mann-Whitney *U*-test.

Results

Patient characteristics and clinical outcome

Ten stage IV malignant melanoma patients whose clinical status varied widely were enrolled into our study; their previous therapies and clinical responses are summarized in Table 1. Following the 10 week therapeutic protocol, analysis of total body CT images revealed a reduction in tumour metastasis in two patients (patients 8 and 9). Figure 1a–c shows the disappearance of multiple lung metastases in patient 9, while Figure 1d–f shows necrosis and regression of a metastatic lesion in patient 8. Necrosis of numerous metastatic lesions was observed during treatment in both patients 8 and 9. Figure 2 shows a pathological specimen from a metastatic skin lesion after treatment in patient 8; note the prominent central necrosis. Necrosis of metastatic lesions was accompanied by local pain, elevated C-reactive protein and sometimes fever; necrosis of skin lesions was also accompanied by local erythema. However, because other metastatic lesions in patients 8 and 9 showed increased tumour mass, the final response of these patients was deemed to be mixed. The clinical response of patient 6 was stable disease, while that of the remaining seven patients was

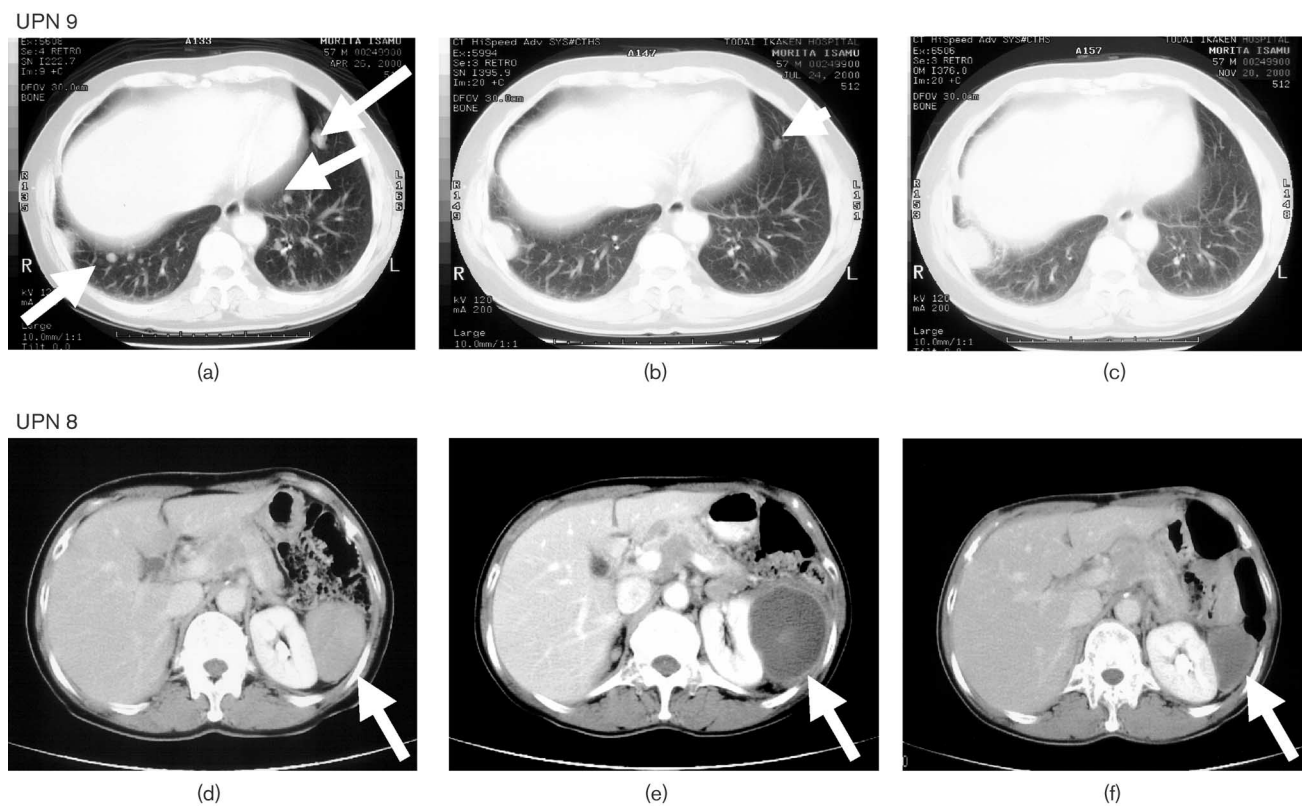
progressive disease, although the rate of increase in total tumour mass declined slightly following treatment in patient 5 (data not shown).

Adverse events are summarized in Table 2. Mild fever (38°C) was occasionally seen for 1–2 days, or a transient erythema and induration occurred around the vaccination sites. We observed no clinical signs of autoimmune disease, and the antinuclear antibody titres were not elevated after therapy. There was no sign of NCI-CTC grade 3–4 toxicity following DC-based vaccination. We therefore conclude that our phase I immunotherapy protocol using autologous, tumour lysate-pulsed, monocyte-derived mDCs plus rhIL2 can be applied repeatedly without substantial side effects.

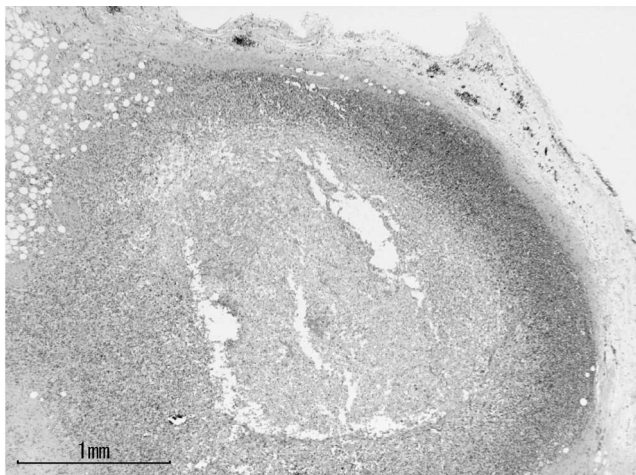
DTH reaction of melanoma patients

To evaluate tumour-specific immunity, we examined the DTH responses toward irradiated autologous melanoma cells and their lysates (Table 1). Before treatment, there was little or no DTH response against these samples, except in patient 7. By contrast, 5 and 10 weeks after the first vaccination, DTH responses against irradiated autologous melanoma cells and their

Fig. 1



CT of the lungs at about the same level in patients 9 (a–c) and 8 (d–f) obtained before (a,d), during (b,e) and after (c,f) treatment. Arrows indicate metastases. The disappearance of multiple lung metastases in patient 9 (a–c) and the regression of retroperitoneal metastasis in patient 8 (d–f) can be seen.

Fig. 2

Resected specimen of a metastatic skin lesion from patient 8; note the central necrosis. Scale bar: 1 mm.

lysates were detected in five of the patients (patients 2, 3, 4, 5 and 6). No responses to irradiated autologous PBMCs or their lysates were detected (data not shown).

Quality control of administered patient-derived DCs and comparison with those from healthy volunteers

To compare the biological properties of monocyte-derived DCs from melanoma patients and healthy volunteers, we first examined the cell surface expression of DC-family markers (CD1a and CD11c), co-stimulatory molecules (CD40, CD80 and CD86) and HLA-DR (Fig. 3). Flow cytometric analysis revealed levels of CD1a expression on iDCs from melanoma patients that were significantly lower than on those from healthy donors (Fig. 3a, $P < 0.001$). Expression of

the other molecules tested was similar in the two groups of iDCs (Fig. 3b–g). TNF α -stimulated mDCs from melanoma patients and healthy volunteers expressed similar levels of CD83, which is known to be a maturation marker for a family of DCs [6] (Fig. 3e). On the other hand, expression of CD1a, CD40, CD80, CD86 and HLA-DR was upregulated on mDCs from healthy volunteers, but not on those from melanoma patients (Fig. 3a, c, d, f and g); indeed, cell surface expression of CD86 and HLA-DR was significantly downregulated ($P < 0.001$) on melanoma patient-derived mDCs (Fig. 3f,g).

To assess induction of DC endocytotic activity by TAA/tumour lysates from melanoma patients and healthy volunteers, mannose-receptor-mediated endocytosis of FITC-DX and macropinocytosis of LY via cytoskeleton-dependent fluid-phase endocytosis were evaluated (Fig. 4). The amounts of FITC-DX (Fig. 4a) and LY (Fig. 4b) internalized by iDCs from melanoma patients tended to be lower than the amounts internalized by iDCs from healthy volunteers, though the effect was only significant for LY. The capacity to internalize FITC-DX and LY was downregulated in both types of mDC.

iDCs are known to migrate toward sources of inflammatory chemokines, while mDCs migrate toward sources of homeostatic chemokines [2,3,11]. To compare the responsiveness to chemokines of DCs from melanoma patients and healthy volunteers, we examined the chemotactic migration induced by RANTES and MIP-3 β . We found that iDCs from melanoma patients responded less to RANTES than those from healthy volunteers (Fig. 5a, $P < 0.05$), and that RANTES-induced chemotaxis was diminished in both types of mDC (Fig. 5b). mDCs from both melanoma patients and healthy volunteers responded to MIP-3 β , though

Table 2 Adverse events accompanying DC vaccination (NCI-CTC grading)

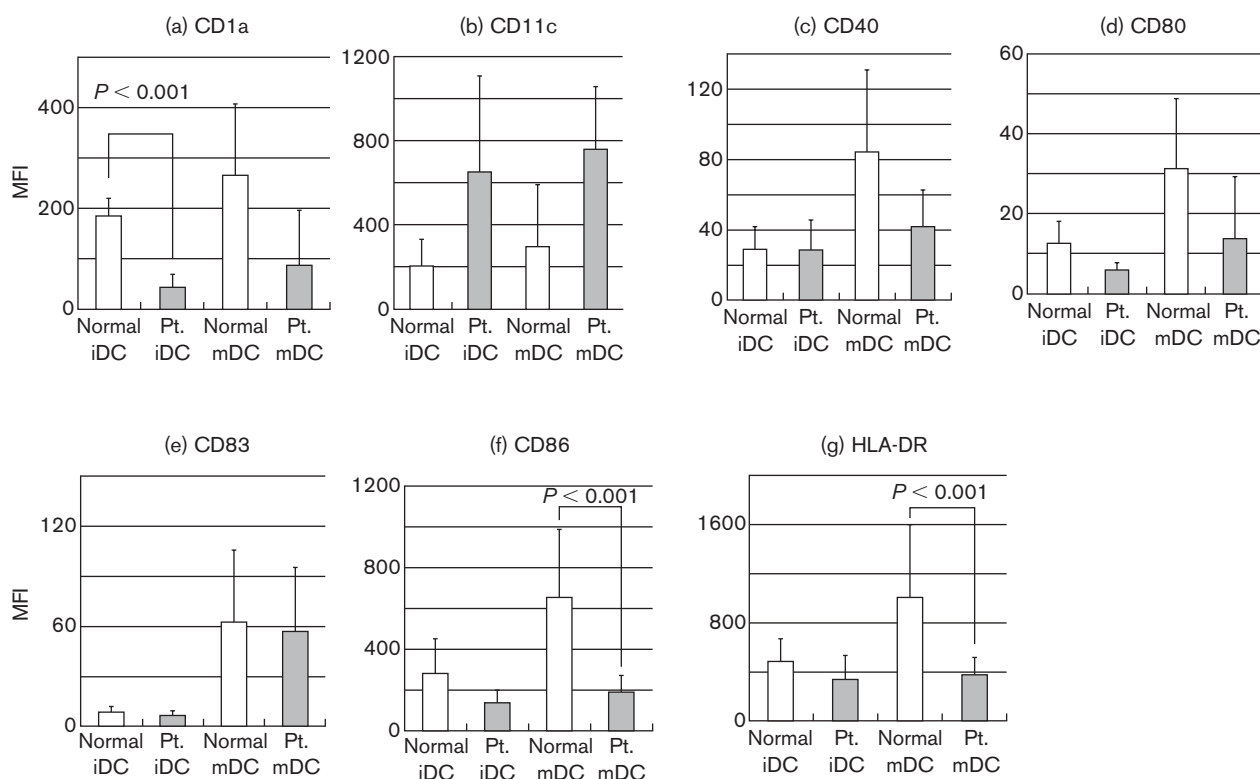
	Patient no.									
	1	2	3	4	5	6	7	8	9	10
Blood/bone marrow										
Platelets	0 ^a	0	0	0	0	0	0	0	0	0
Constitutional symptoms										
Fever	0	0	0	0	0	0	0	0	0	0
Weight gain	0	0	0	0	0	0	0	0	0	0
Weight loss	0	0	0	0	0	0	0	0	0	0
Dermatology/skin										
Injection site reaction	1	0	0	0	0	0	1	0	0	0
Tumour site reaction concurrent with tumour regression	0	0	0	0	0	0	0	1	1	0
Autoimmune reaction										
ANA before treatment ^b	< 40 \times	< 40 \times	< 40 \times	< 40 \times	< 40 \times	< 40 \times	< 40 \times	< 40 \times	< 40 \times	< 40 \times
ANA after completion of treatment ^b	< 40 \times	< 40 \times	< 40 \times	< 40 \times	< 40 \times	< 40 \times	< 40 \times	< 40 \times	< 40 \times	< 40 \times

ANA, antinuclear antibody titre.

^aThis case exhibited lower platelet counts (grade 2–3) due to complicated disseminated intravascular coagulation; consequently this adverse event was considered to be unrelated to the DC vaccination.

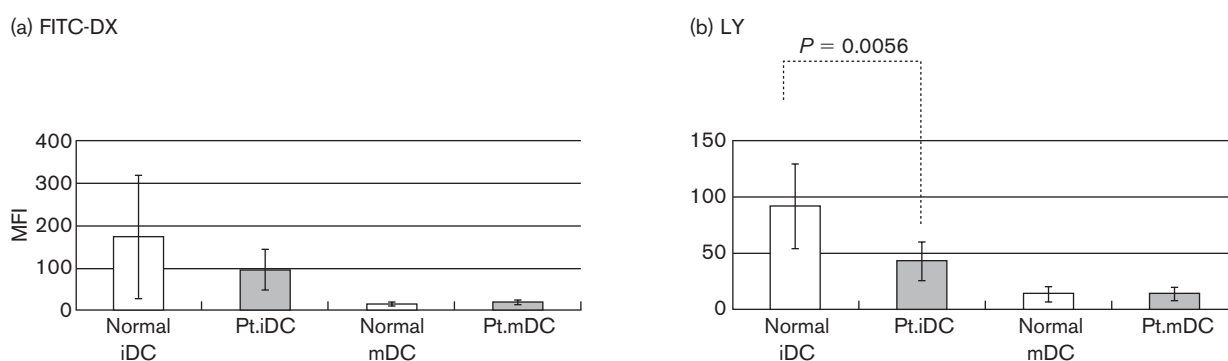
^bFold of titre.

Fig. 3



Immunophenotyping of iDCs and normal mDCs derived from patients and healthy volunteers. Cell surface expression of CD1a (a), CD11c (b), CD40 (c), CD80 (d), CD83 (e), CD86 (f) and HLA-DR (g) was analysed by flow cytometry. The bars depict the mean \pm SD for iDCs and normal mDCs from healthy volunteer donors (Normal; $n = 12$; white bars) and melanoma patients (Pt.; $n = 10$; grey bars). Values were compared using the Mann-Whitney's *U*-test. MFI, mean fluorescence intensity.

Fig. 4



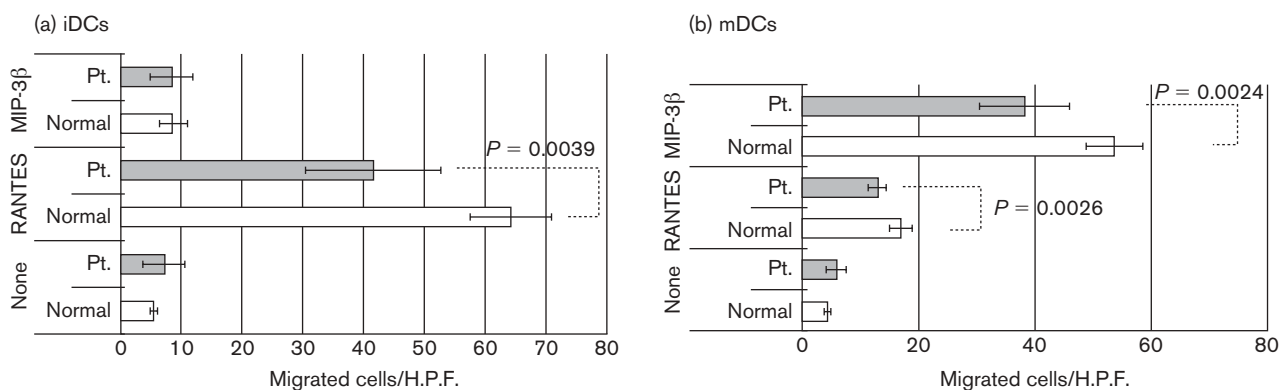
Flow cytometric analysis of the antigen uptake capacities of iDCs and mDCs. The bars depict the mean \pm SD amount of FITC-DX (a) and LY (b) internalized at 37°C over a period of 1 h by iDCs and mDCs from healthy volunteers (Normal, white bars) and melanoma patients (Pt., grey bars). MFI, mean fluorescence intensity.

patient-derived mDCs were less responsive than those derived from healthy volunteers (Fig. 5b, $P < 0.05$ for both RANTES and MIP-3 β).

We also found that iDCs from the patients and healthy

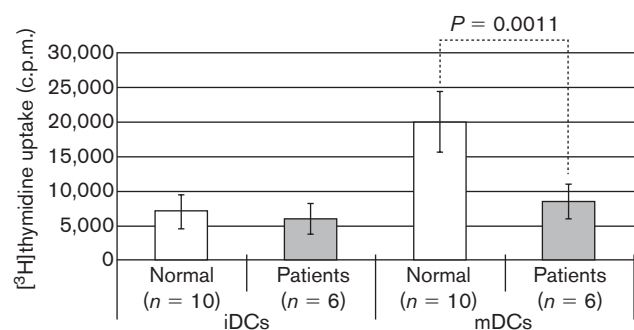
volunteers exhibited similar capacities to stimulate allogeneic T-cells (Fig. 6). This capacity was not enhanced in mDCs from melanoma patients, but was enhanced in mDCs from healthy volunteers ($P < 0.05$). It thus appears that changes in the expression of MHC pro-

Fig. 5



Chemotactic migration of iDCs (a) and mDCs (b) from melanoma patients (grey bars) and healthy volunteer donors (white bars) toward RANTES and MIP-3 β . Polycarbonate membrane filters (pore size 8.0 μ m) were precoated with 5 μ g of gelatin. The lower chambers contained 600 μ l of medium with or without 100 ng/ml RANTES and MIP-3 β . The upper chamber contained 100 μ l of cell suspension (10^6 cells), which were incubated at 37°C for 4 h. After staining with haematoxylin and eosin, the numbers of migrated cells per HPF were counted at magnification $\times 400$.

Fig. 6



Stimulation of allogeneic T-cells by DCs from melanoma patients and normal healthy volunteers. T-cells isolated from peripheral blood were cultured for 5 days with irradiated DCs (10^4 cells), after which [3 H]thymidine incorporation was determined.

duct/co-stimulatory molecules paralleled the ability of these cells to stimulate allogeneic T-cells.

In vitro cytolytic activities of IL2-activated CD2+ lymphocytes

We examined the cytotoxic activity of PBMCs against autologous and allogeneic melanoma cells derived from four melanoma patients (patients 5, 6, 8 and 9). When PBMCs were stimulated with tumour lysate plus rhIL2 *in vitro*, we detected cytolytic activity by CD2+ lymphocytes against autologous melanoma cells, but not against HLA-mismatched allogeneic melanoma cells (Fig. 7). In contrast, no such activity was detected with CD2+ lymphocytes obtained from melanoma patients before treatment (data not shown).

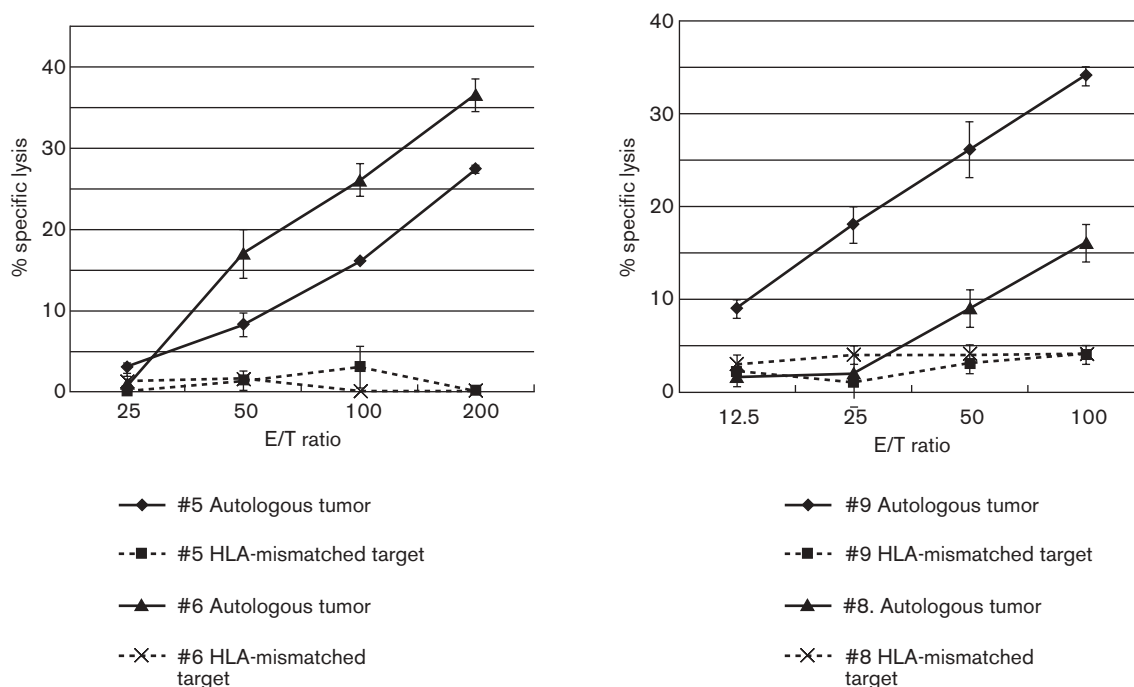
Discussion

We have described a pilot study in which an immunotherapy protocol involving administration of a DC-

based vaccine plus low dose rhIL2 was used to treat stage IV malignant melanoma patients unresponsive to other therapies. Our protocol entailed several modifications of a previously described immunotherapy with a DC-based vaccine [7]. First, we carried out a single 15 l leukapheresis on each melanoma patient to obtain $> 10^8$ monocyte-derived DCs, which enabled us to vaccinate each patient 10 times (10^7 tumour lysate-pulsed mDCs per injection). Thus, leukapheresis provided us with a means to obtain large numbers of DC-based cells without requiring patients to undergo frequent blood collection. Second, we used mDCs, rather than iDCs, as a source of DC-based vaccine because (i) mDCs show superior ability to induce Th1 and cytotoxic T-lymphocyte responses [1]; (ii) mDCs are more resistant to the immunosuppressive effect of IL10 than iDCs [14]; and (iii) mDCs more efficiently home into and accumulate within T-cell-dependent areas of secondary lymphoid tissues than iDCs [1]. Third, we combined DC-based vaccination with low dose administration of rhIL2. Shimizu *et al.* [15] reported that administration of IL2 enhances the therapeutic efficacy of DC-based vaccines in murine experimental models [15]; it is also well known that IL2 acts not only as a growth factor for lymphocytes but also enhances their cytolytic activities. Moreover, DCs directly activate natural killer (NK) [16] and natural killer T (NKT) [17] cells, and rhIL2 may potentiate this effect.

Our data show that the side effects of this approach were negligible, though most of the patients remained unresponsive to therapy. However, two patients did show a mixed response, and others showed enhancement of *in vivo* DTH responses against irradiated autologous melanoma cells and their lysates (Table 1) as well as cytolytic responses of *in vitro*-activated CD2+ lymphocytes against autologous melanoma cells (Fig.

Fig. 7



Cytolytic activity of *in vitro*-activated, patient-derived CD2+ lymphocytes against autologous or allogeneic melanoma cells. Lysis was measured in terms of the percentage ^{51}Cr release as described in Materials and methods.

7). Moreover, our finding that patient-derived melanoma cells are a heterogeneous population in terms of their expression of MHC products and the CD86 co-stimulatory molecule suggests that tumour lysate-pulsed DCs and rhIL2 synergistically activated lymphocytes to kill melanoma cells through both MHC-dependent and MHC-independent mechanisms. It therefore appears that immunotherapy with a DC-based vaccine and rhIL2 has the potential to give protective immunity against melanoma cells in stage IV with no NCI-CTC grade 3–4 side effects. Taken together, these findings provide a clinical safety rationale for the use of a DC-based vaccine in patients with advanced melanoma and indicate that further research into improving the efficacy of this approach is clearly warranted.

We were also interested in whether our therapeutic protocol could induce tumour-specific immunity in melanoma patients. Our data showed enhanced DTH responses against melanoma cells and enhanced cytolytic responses by *in vitro*-activated CD2+ lymphocytes against autologous melanoma cells in some patients. The diminished lung metastasis observed in patient 9 and the central necrosis observed in both patients 8 and 9 appear to reflect the antitumour immunity induced by DC therapy, though the precise cause of these

effects is not yet certain. Nestle *et al.* [7] previously reported an association between the clinical response and the DTH response in advanced melanoma patients receiving DC-based vaccine. However, our findings and those of Thurner *et al.* [8] suggest that, although we were able to induce a degree of protective immunity against melanoma cells (e.g. DTH responses), there was not necessarily an association with clinical outcome. This discrepancy probably reflects differences in the clinical status of the patients and the experimental designs, which suggest that a balance between the degree of protective immunity induced and the patient status (e.g. the tumour burden) may determine the clinical outcome in malignant melanoma patients given this type of immunotherapy.

Central necrosis of metastatic tumours was a prominent feature of the antitumour response observed in our study. It is now well established that tumour angiogenesis is regulated by the vascular endothelial growth factor (VEGF) family of cytokines [18–20], and that DCs produce large amounts of interferon- γ (IFN γ) in response to IL12 stimulation [12]. The observed necrosis may therefore reflect inhibition of the VEGF cascade, and thus tumour angiogenesis, by IFN γ released from administered DCs or from activated Th1/NK cells.

Why the treatments were ineffective in most patients remains unclear. It may be that melanoma cells escape host protective immunity during the course of treatment. For example, melanoma cells reportedly produce large amounts of IL10 and transforming growth factor- β (TGF β), which suppress host anticancer immunity [21–23]. Yue *et al.* [21] described the transcription and translation of IL10 and IL10 receptor (IL10R) in patient-derived melanoma cells, as well as decreased cell surface expression of HLA class I, class II and intercellular adhesion molecule-1 (ICAM-1), which reciprocally regulates IL10R expression [21]. In addition, we have previously shown that IL10 interferes with TNF α -induced maturation of iDCs [10], and expression of HLA-DR was diminished on mDCs from melanoma patients in the present study. Taken together, these findings strongly suggest an IL10-mediated escape of melanoma cells from host immune surveillance. Steinbrink *et al.* [22] described a melanoma antigen-specific anergy caused by treating DCs with IL10, and Enk *et al.* [23] suggested that DCs themselves mediate tumour-induced tolerance in metastatic melanoma. This means that IL10 and TGF β 1 may induce the dysfunctional immune properties seen in the patient-derived DCs examined in the present study. The use of HLA-matched unrelated donor-derived monocytes as sources of DCs, or gene transduction of appropriate immunostimulatory molecules into autologous DCs and tumour cells, may enable us to overcome the impaired host antitumour immunity.

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