

Intravenous and intradermal TriMix-dendritic cell therapy results in a broad T-cell response and durable tumor response in a chemorefractory stage IV-M1c melanoma patient

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Abstract Dendritic cells (DCs) electroporated with mRNA encoding CD70, CD40L and a constitutively active toll-like receptor 4 (TriMix-DC) have an increased T-cell stimulatory capacity. In a prospective phase IB clinical trial, we treated melanoma patients with intradermal and intravenous injections of autologous TriMix-DC co-electroporated with mRNA encoding full-length MAGE-A3, MAGE-C2, tyrosinase and gp100. We report here the immunological and clinical results obtained in one patient with a particularly favorable outcome. This patient had stage IV-M1c melanoma with documented progression during dacarbazine chemotherapy and received 5 TriMix-DC injections. Following DC therapy, a broad CD8⁺ T-cell response against multiple epitopes derived from all four treatment antigens was found in the blood and among T cells derived from DTH biopsy. In addition, CD4⁺ T cells recognizing different MAGE-A3-derived epitopes were detected in DTH-derived

cells. A spontaneous anti-MAGE-C2 CD8⁺ T-cell response was present prior to TriMix-DC therapy and increased during treatment. The tumor response was assessed with 18-fluorodeoxyglucose-positron emission/computed tomography. We documented a partial tumor response according to RECIST criteria with a marked reduction in ¹⁸F-FDG-uptake by lung, lymph node and bone metastases. The patient remains free from progression after 12 months of follow-up. This case report indicates that administration of autologous TriMix-DC by the combined intradermal and intravenous route can mediate a durable objective tumor response accompanied by a broad T-cell response in a chemorefractory stage IV-M1c melanoma patient.

Keywords Dendritic cell · TriMix · Immunotherapy · Melanoma · Administration route

Introduction

Melanoma is the most aggressive form of skin cancer, and its incidence is increasing worldwide [1]. Early stages of melanoma can be cured by surgery, but the prognosis for patients with metastatic melanoma is grim, with an expected 2-year survival rate of 10–20% [2]. Melanoma is known to be an immunogenic cancer, and different melanoma-associated antigens (MAAs) have been described [3]. Recently, ipilimumab, an antibody against cytotoxic-T-lymphocyte-associated antigen 4 (CTLA-4) and the combination of gp100 peptide vaccine with interleukin (IL)-2 showed an improved overall survival in three randomized phase III trials, underlining the potential of immunotherapy in metastatic melanoma patients [4–6].

Dendritic cells (DCs) are known for their unique capacity to induce the activation of naïve tumor-specific

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T lymphocytes [7]. For this reason, a growing number of clinical trials are being performed using tumor antigen-loaded DCs as cellular immunotherapy in cancer patients [8, 9]. DC-based immunotherapy has shown to induce anti-tumor immune responses, but so far with limited clinical efficacy. Both the maturation of DC and the route of administration might play a role in determining the quantity and quality of the immune response [10–15]. We previously described a single-step approach for effective antigen loading and maturation of DCs by mRNA electroporation offering multiple advantages [16]. First, electroporation with full-length MAA-encoding mRNA facilitates cellular processing and presentation of the full range of antigenic peptides. Consequently, a broader MAA-specific T-cell response can be induced, irrespective of the patient's HLA type. Furthermore, enhanced MAA presentation in both HLA class I and II can be achieved by fusion of the MAA-encoding sequence with an HLA class II-targeting signal [17]. Also, the T-cell stimulatory capacity of DCs can be greatly enhanced by co-electroporation with CD40L, CD70 and a constitutively active toll-like receptor 4 (caTLR4)-encoding mRNA (TriMix-DC) [18]. The combination of CD40L and caTLR4 electroporation mimics CD40 ligation and TLR4 signaling of the DCs and generates phenotypically mature, cytokine-secreting DCs. Additionally, CD70 electroporation provides a costimulatory signal to CD27⁺ naïve T cells by inhibiting activated T-cell apoptosis and by supporting T-cell proliferation [18]. A phase I pilot clinical trial demonstrated that intradermal administration of autologous TriMix-DC is feasible, is safe and effectively stimulates CD8⁺ T-cell responses [19].

New insights into the organ-specific trafficking of vaccine-induced T-cell populations indicated that combination of different routes of administration may be beneficial to target different tumor locations [20]. Accumulating evidence from immunization studies in animals has shown that vaccination route impacts on the migratory capacity of the induced effector T cells [14, 21, 22]. In a mouse melanoma model, the intravenous injection of DCs was shown to be essential for responses against visceral metastases, whereas subcutaneous vaccination resulted in a response against non-visceral metastases [12]. Following intravenous injection, the DCs were only found in the spleen, whereas subcutaneously injected DCs were mainly found in the skin-draining lymph node and to a minor extent in the spleen [12, 23]. The same compartmentalization was found for the stimulated CD8⁺ T cells (both primed and memory T cells), and consequently intravenous DC administration was more protective for lung metastasis, whereas subcutaneous injection was protective for subcutaneous tumors [12]. Similarly, injection route-dependent distribution of the DCs has been found in human subjects, with intradermal DCs migrating to the skin-draining lymph nodes and

intravenously injected DCs migrating to the lungs, with subsequent redistribution to the liver, spleen and bone marrow [24]. Moreover, compartmentalization of the immune response has also been described in melanoma patients: intralymphatic DC injection led mainly to skin-homing CD8⁺ T cells [25]. Therefore, the administration route might play a role in the outcome of DC therapy.

To further optimize the immunogenicity and clinical efficacy of autologous TriMix-DC in melanoma patients, we are conducting a prospective phase I clinical trial on combined intravenous and intradermal administration. We here describe a patient with chemotherapy-refractory metastatic melanoma who participated in this clinical trial and responded favorably. The patient experienced a sustained partial tumor response according to RECIST criteria accompanied by a broad CD8⁺ and CD4⁺ T-cell response to the MAA presented by the TriMix-DC.

Patient and methods

Patient

In August 2002, this 54-year-old Caucasian male patient had a pigmented skin lesion excised from his back. Anatomopathologic examination revealed a nodular melanoma with ulceration, Clark level IV and Breslow thickness of 4 mm. There was no lymphatic or venous invasion and a high mitotic rate (10/mm³). A left axillar lymphadenectomy was performed, and metastatic melanoma cells were detected in two out of ten nodes. The final pathologic staging was pT3bN2aMx. In October 2002, he initiated adjuvant high-dose interferon- α -2b that had to be stopped after 1 month because of grade 3 toxicity. In June 2010, a computed tomography (CT) revealed newly developed lung, lymph node, bone and liver metastases (American Joint Committee on Cancer (AJCC) stage IV-M1c melanoma). After 2 cycles of dacarbazine chemotherapy, a complete response of the liver metastasis and stabilization of other metastases were documented. The patient received 2 additional cycles of dacarbazine, but progressive disease was confirmed with an increase in size of the lung, lymph node and bone metastases. In October 2010, the patient consented to enroll in a phase I clinical trial (EudraCT2009-015748-40) with autologous TriMix-DC therapy. His baseline Karnofsky score was 70%, and laboratory evaluation was unremarkable with normal lactate dehydrogenase (LDH) and C-reactive protein serum measurements. The patient's HLA type is HLA-A2, HLA-A3, HLA-B35, HLA-B44, HLA-Cw4 and HLA-Cw5 for HLA class I and HLA-DR1, HLA-DR13, HLA-DP04, HLA-DQ5 and HLA-DQ6 for HLA class II.

Table 1 TriMix-DC quality control assessment

Yield	871.5 × 10 ⁶ cells
CD70 expression	86.2%
CD40 expression	43.9%
CD80 expression	52.6%
CD83 expression	66.8%
CCR7 expression	29.6%
CD14 expression	15.1%
Viability	88%
Purity	73%
IL-12p70 secretion, 0–24 h	109.2 pg/mL
IL-12p70 secretion, 24–48 h	6.1 pg/mL
<i>IL</i> interleukin	

Treatment DC production and administration

Following leukapheresis, monocytes were enriched by plastic adherence and cultured in the presence of 1% autologous plasma, 1,000 U/mL GM-CSF and 500 U/mL IL-4 [11, 16]. On day 6, immature DCs were harvested and co-electroporated with TriMix-mRNA (CD40L, CD70 and caTLR4 encoding mRNA) and 1 of 4 mRNA encoding a MAA (either MAGE-A3, MAGE-C2, tyrosinase or gp100) linked to an HLA class II-targeting signal [19]. Equal ratios of TriMix-DC (expressing one of the four treatment antigens) were mixed and cryopreserved after a rest period of 2 h post-electroporation. DCs were thawed 2–3 h before injection. An in-process quality control (QC) as well as a QC of the final cellular product was performed before administration (Table 1). Results were as expected based on our previous experience [19]. TriMix-DC were administered 5 times: at weeks 0, 2, 4, 6 and 14 (Fig. 1). The patient received per session 11 × 10⁶ DCs intradermally (right axillary and right inguinal region, 2 injections per site) and 20 × 10⁶ DCs intravenously.

Clinical monitoring

Adverse events were recorded and graded according to the National Cancer Institute Toxicity Criteria, version 3.0. Tumor evaluations by whole-body 18-fluorodeoxyglucose-positron emission/computed tomography (18F-FDG-PET/CT) were performed at baseline and every 8 weeks thereafter. Tumor response was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECISTv1.1).

Monitoring specific T-cell responses in peripheral blood

Immunomonitoring of the peripheral blood of the patient was performed as described elsewhere [16]. Briefly, CD8⁺

T cells were isolated from the patient's PBMC before and after treatment using anti-CD8 MACS beads (Miltenyi), obtaining a >90% purity.

Ten million CD8⁺ T cells were co-cultured with autologous TriMix-DC co-electroporated with each of the 4 different tumor antigens at a 10:1 ratio in stimulation medium [IMDM, 1% human AB serum, 1 mM sodium pyruvate, non-essential amino acids, 0.24 mM L-asparagine and 0.55 mM L-arginine (all from Lonza, Verviers, Belgium)] without any further addition of exogenous cytokines. A separate co-culture was performed per treatment antigen. CD8⁺ T cells were restimulated weekly. The culture medium was changed every 2–3 days, and after 2 and 3 rounds of stimulation, CD8⁺ T cells were harvested and their antigen specificity and function were determined. To this end, they were stimulated overnight with autologous EBV-transformed B cells (aEBV-B) that were electroporated with the treatment MAAs. Upregulation of CD137 and cytokine secretion were investigated in response to antigen-specific stimulation. To identify the epitopes recognized by the CD8⁺ T cells, the cells were restimulated with aEBV-B cells pulsed with pools of 10 peptides (10 µg/mL in stimulation medium) consisting of 15-mers [covering the entire MAA sequence, each with 11 amino acid (aa) overlap] or with the individual peptides composing the recognized pools. The gp100 protein was covered by 163 synthetic peptides (16 pools), tyrosinase by 130 peptides (13 pools), MAGE-C2 by 91 peptides (9 pools) and MAGE-A3 by 76 peptides (8 pools) (all purchased from EMC Microcultures, Tübingen, Germany). Recognized peptides are indicated by the number of their first and last aa. The corresponding aa sequence can be found in Table 2. Full-length MAA responses were considered positive when the CD137 upregulation and IFN-γ or TNF-α secretion showed a twofold increase upon stimulation compared to control mRNA. Peptides recognized by the CD8⁺ T cells were considered positive when the IFN-γ secretion was increased 2.5-fold compared to a control peptide.

Monitoring specific T-cell responses in DTH skin biopsies

Delayed type IV hypersensitivity (DTH) immunomonitoring was performed as described elsewhere (An M.T. Van Nuffel, in preparation) [19]. Briefly, at week 6, the patient was injected intradermally with 2 × 10⁶ treatment DCs (i.e., 5 × 10⁵ per antigen) at three different sites on his lower back, and DTH biopsies were taken 72 h later. After 2.5 weeks of culture in IL-2 (100 IU/mL)-supplemented medium, skin-infiltrating lymphocytes (SKILs) (CD8⁺ and CD4⁺) were harvested and their antigen specificity was determined as for the blood-derived CD8⁺ T cells. In addition, CD107a and CD40L upregulation was investigated on CD8⁺ and CD4⁺ SKILs, respectively. Where indicated,

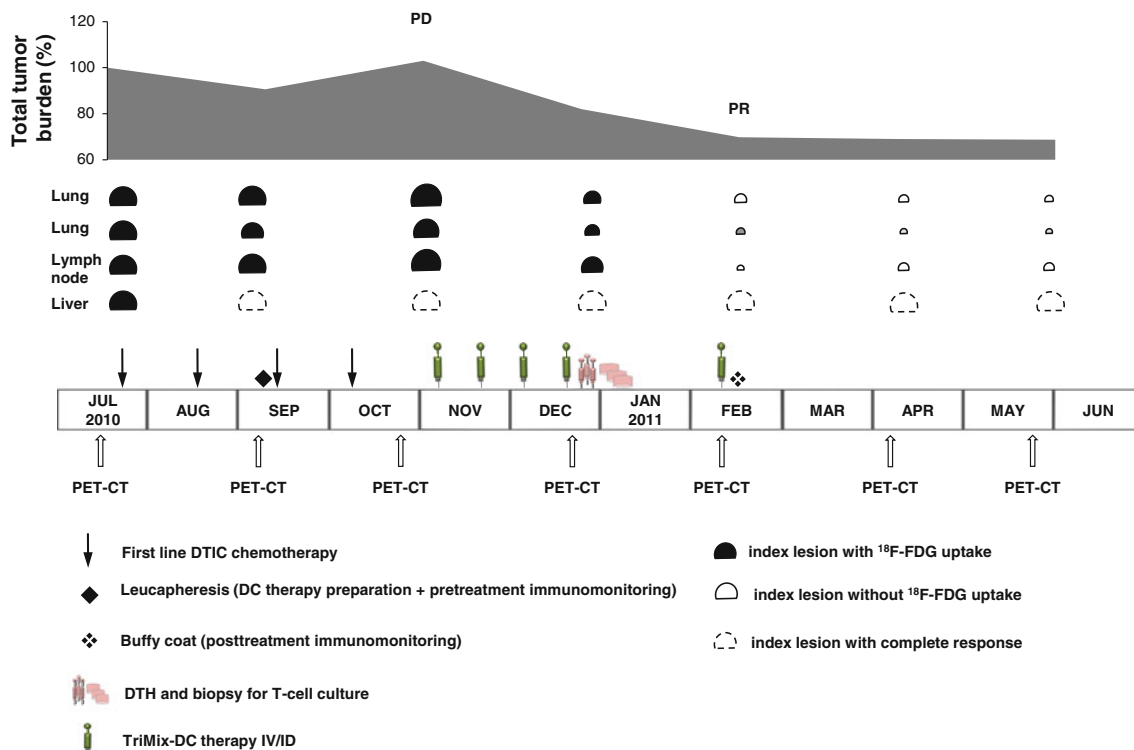


Fig. 1 Clinical evolution, treatment schedule and follow-up

antigen-specific CD4⁺ SKILs were enriched by MACS following the manufacturer's instructions (Miltenyi, Bergisch Gladbach, Germany). A peptide was considered recognized by the CD8⁺ T cells when CD137 upregulation or IFN- γ or TNF- α secretion showed a twofold increase compared to control. CD4⁺ T cells were considered to be antigen-specific when secretion of one of the quantified cytokines showed a fivefold increase compared to control.

Results and discussion

Toxicity and clinical outcome

A stage IV melanoma patient with documented progression during dacarbazine chemotherapy was treated with TriMix-DCs co-electroporated with MAGE-A3, MAGE-C2, tyrosinase or gp100. He received 5 TriMix-DC injections (at weeks 0, 2, 4, 6 and 14): 11×10^6 DCs were injected intradermally and 20×10^6 DCs intravenously.

TriMix-DC administrations were well tolerated. Grade 2 local skin reactions (swelling and erythema) resolving after 24–72 h were observed at the intradermal axillary and inguinal injection sites after each TriMix-DC administration. These skin reactions were as expected based on our previous study [19].

Remarkably, the patient experienced grade 2 chills (moderate tremor of the entire body) starting 20 min after

TriMix-DC administration and resolving spontaneously within 30 min. These chills were most pronounced after the first and second administration, attenuated after the third and fourth administration and remained absent after the fifth administration. They are specifically related to the intravenous route of administration since we did not observe any chills in our previous study with intradermal TriMix-DC administration only [19]. The cause of this adverse event is not clear but likely related to a cytokine release syndrome following the intravenous administration. While chills have not been reported in other clinical trials with autologous DCs, chills, fever and constitutional symptoms have been observed in prostate cancer patients treated with intravenous administration of the immunotherapeutic cell therapy sipuleucel-T [26]. Prospective investigation of cytokine serum levels in TriMix-DC-treated patients is currently ongoing in our phase I trial.

A tumor assessment by whole-body ¹⁸F-FDG-PET/CT at week 8 showed a stable disease (SD) according to RECIST criteria with a 20% regression of the lung metastasis and stabilization of all other metastases. At week 16, the patient achieved a partial response (PR) (Fig. 2), which is ongoing 12+ months after initiating TriMix-DC therapy. Uptake of ¹⁸F-FDG has normalized in the lung and lymph node metastases and has strongly diminished in the skeletal metastasis.

Objective tumor responses according to RECISTv1.1 in patients with metastatic melanoma treated with antigen-specific immunotherapy (including peptide and

Table 2 Overview of peptides and their aa sequence recognized before and after DC therapy by peripheral blood CD8⁺ T cells or CD8⁺ and CD4⁺ SKILs

peptide	aa ^a sequence	CD8			HLA restriction	CD4	HLA restriction
		blood pre	blood post	SKILs post	of the underlined/italic known epitopes	SKILs post	of the indicated known epitopes
gp100 ₃₃₋₄₇	LGVSRQLRTKAWNRQ	-	+	-		-	
gp100 ₈₁₋₉₅	NASFSJALNFPGSQK ^b	-	-	+	<u>A3^c</u> , <u>A11</u>	-	
gp100 ₈₅₋₉₉	SJALNFPGSQKVLDP	-	-	+	<u>A3</u> , <u>A11</u>	-	
gp100 ₂₉₃₋₃₀₇	QAAIPLTSCGSSPVP	-	+	-		-	
tyrosinase ₂₀₁₋₂₁₅	AHEAPAFLPWHRLF	-	+	+	<u>A24</u>	-	
tyrosinase ₂₀₅₋₂₁₉	PAFLPWHRLFLLRWE	-	+	+	<u>A24</u>	-	
tyrosinase ₂₀₉₋₂₂₃	PWHRLFLLRWEQEIQ	-	-	+		-	
tyrosinase ₂₈₅₋₂₉₉	HQSLCNGTPEGPLRR	-	-	+		-	
tyrosinase ₃₀₉₋₃₂₃	TPRLPSSADVEFCLS	-	-	+	B35	-	
tyrosinase ₃₆₉₋₃₇₇	<u>YMDGTMSQV</u>	-	-	+	A2	-	
tyrosinase ₄₁₃₋₄₂₇	EANAPIGHNRESYMV	-	+	-		-	
tyrosinase ₄₃₃₋₄₄₇	YRNGDFFISSKDLGY	-	-	+		-	
MAGE-C2 ₁₆₅₋₁₇₉	AEMLMIVIKYDYFP	-	+	-		ND ^d	
MAGE-C2 ₁₈₉₋₂₀₃	MELLFGLALIEVGPD	-	+	-	<u>A2</u>	ND	
MAGE-C2 ₂₄₁₋₂₉₁	pool 7	+	+	-		ND	
MAGE-C2 ₂₅₇₋₂₇₁	GVYAGREHFVYGEPR	ND	+	-		ND	
MAGE-C2 ₂₈₅₋₂₉₉	EYREVPHSSPPYYEF	-	+	+		ND	
MAGE-C2 ₂₈₉₋₃₀₃	VPHSSPPYYEFLWGP	-	+	+		ND	
MAGE-A3 ₆₅₋₇₉	PQGASSLPTTMNYPL	-	-	+		-	
MAGE-A3 ₇₇₋₉₁	YPLWSQSYEDSSNQE	-	+	-		-	
MAGE-A3 ₁₀₅₋₁₁₉	FQAALSRKVAELVHF	-	-	+		-	
MAGE-A3 ₁₀₉₋₁₂₃	LSRK <u>VAELVHFLLLK</u>	-	-	-	<u>A2</u> , <u>A24</u>	+	} DR4 (RKVAELVHFLLLKYR) ^e , DR13 (RKVAELVHFLLLKYRA) DR13 (FLLLYRAREPVTKAE)
MAGE-A3 ₁₁₃₋₁₂₇	<u>VAELVHFLLLKYR</u> AR	-	-	+	<u>A24</u> , <u>B40</u>	+	
MAGE-A3 ₁₁₇₋₁₃₁	VHFLLLKYRAREPVT	-	-	-		+	
MAGE-A3 ₁₂₁₋₁₃₅	LLKYRAREPVTKAEM	-	-	-		+	} DR4, DR7 (VIFSKASSLQL)
MAGE-A3 ₁₄₁₋₁₅₅	GNWQYFFPVIFSKAS	-	-	-	B52	+	
MAGE-A3 ₁₄₅₋₁₅₉	YFFPVIFSKASSLQ	-	-	-		+	} DR7 (VFGIELMEVDPIGHL)
MAGE-A3 ₁₆₁₋₁₇₅	VFGIELMEVDPIGHL	-	-	+		+	
MAGE-A3 ₁₆₅₋₁₇₉	<u>ELMEVDPIGHLYIFA</u>	-	+	+	<u>A1</u> , <u>B18</u> , <u>B35</u> , <u>B44</u>	+	
MAGE-A3 ₁₆₉₋₁₈₃	VDPIGHLYIFATCLG	-	+	-		-	
MAGE-A3 ₁₇₇₋₁₉₁	IFATCLGLSYDGLLG	-	-	-		+	
MAGE-A3 ₁₈₉₋₂₀₃	LLGDNQIMPKAGLLI	-	-	+		+	} DR11(GDNQIMPKAGLLIIV)
MAGE-A3 ₁₉₃₋₂₀₇	NQIMPKAGLLIIVLA	-	-	+		+	
MAGE-A3 ₂₄₁₋₂₅₅	DPKLLTQHFVQENY	-	-	-		+	DP4 , DQ6 (KKLLTQHFVQENYLEY)
MAGE-A3 ₂₉₇₋₃₁₁	PHISYPPLHEWVLR	-	+	-		-	DQ5 (YPPLHEWVLRREG)

^a aa: Amino acid sequence of the recognized 15-mer peptides

^b Known HLA class I epitopes are underlined or italicized based on [33]

^c Bold: HLA molecules expressed by the patient

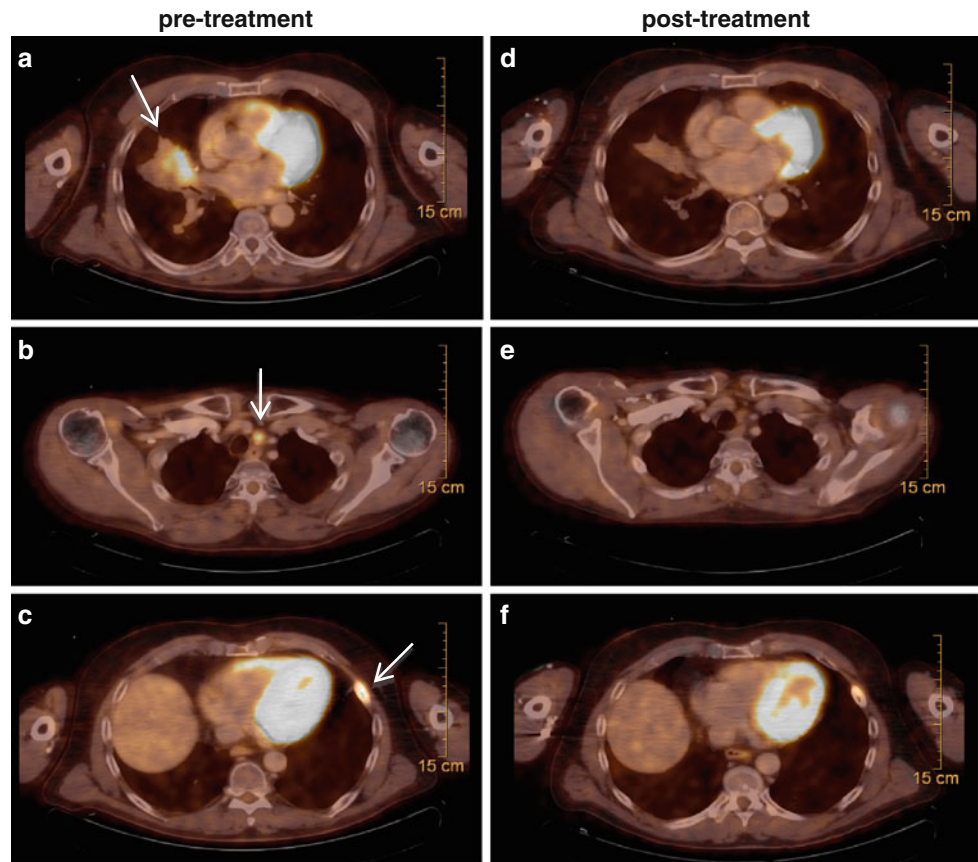
^d ND: not determined

^e HLA restriction and amino acid sequence of the known HLA class II epitopes based on [33] and Van Nuffel AMT et al., submitted. Epitopes can be longer than the peptides used, or their overlap. Curly brackets encompass all peptides containing part of the known epitopes

protein vaccines and DC therapy) have mostly been limited to patients with the most favorable prognosis, having stage IV-M1a/b disease. RECIST responses in chemorefractory stage IV-M1c melanoma patients have

only been very rarely reported in the literature [27]. Our case observation therefore is indicative of the potential of an optimized cell product as well as the route of administration.

Fig. 2 18-Fluorodeoxyglucose-positron emission tomography/computed tomography (^{18}F -FDG-PET/CT) at baseline (**a, b, c**) and 16 weeks after the initiation of autologous TriMix-DC therapy (**d, e, f**). The *arrows* on the pre-treatment scans show the sites of tumor lesions. The patient achieved a partial response (PR) according to RECIST criteria with regression and decreased metabolic activity of a right lung metastasis (**a, d**), paratracheal lymph node (**b, e**) and rib metastasis (**c, f**)



Immune response

Tumor antigen-specific T-cell responses in peripheral blood

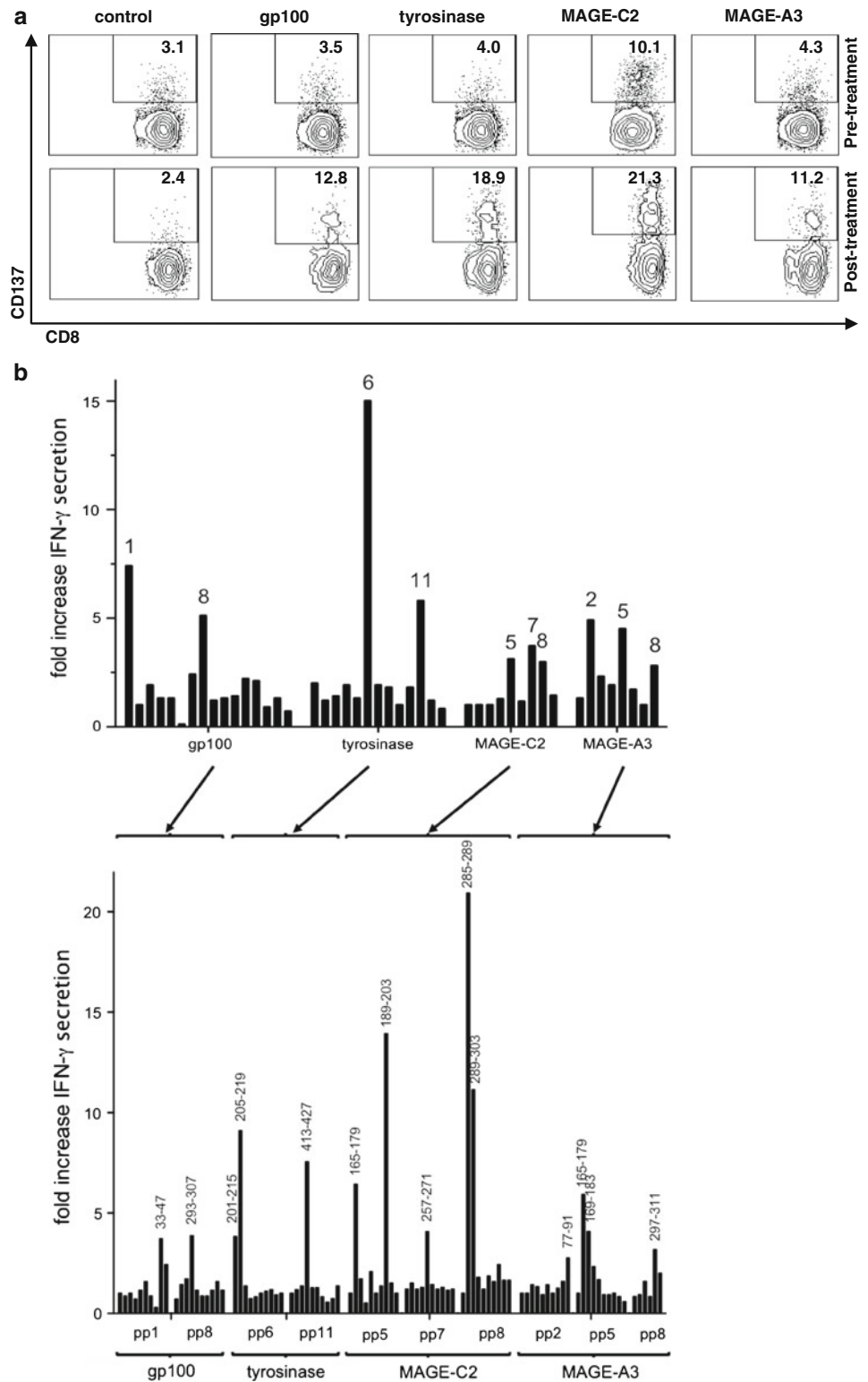
To find out whether the TriMix-DC treatment stimulated the patient's immune response, 10 million purified CD8^+ T cells were stimulated *in vitro* with the treatment DCs in a separate co-culture per antigen. After 3 stimulations, MAGE-C2 was recognized by the CD8^+ T cells collected prior to DC therapy (Fig. 3a). This is not an unexpected finding, because spontaneous tumor-specific T-cell responses in patients are a common observation in different types of cancer including melanoma [28–32]. To localize the recognized region of MAGE-C2, aEBV-B cells were loaded with pools of overlapping 15-mer peptides covering the entire antigen and used to stimulate the CD8^+ T cells overnight. CD137 upregulation and enhanced cytokine secretion indicated that the recognized epitope(s) is located in MAGE-C2 peptide pool no 7 (covering aa 241 up to 291; data not shown). The other MAA were not recognized by pre-treatment CD8^+ T cells after repeated stimulations (Fig. 3a).

In contrast, post-treatment CD8^+ T cells restimulated 3 times *in vitro* with autologous DCs demonstrated an immune response against gp100, tyrosinase, MAGE-C2

and MAGE-A3 (Fig. 3a). Thus, treatment with TriMix-DC further expanded the pre-existing MAGE-C2 response and additionally induced *de novo* responses restricted to other MAA that are included in the DC therapy. Alternatively, the reactivity found in the post-therapy samples might have been present before treatment but below the detection limit of our immunomonitoring method, indicating that DC therapy expanded weak pre-existing responses rather than inducing *de novo* responses.

Here also, we investigated which peptide pools were recognized by these *in vitro*-restimulated T cells. All positive pools were retested with the individual peptides contained in the pool (Fig. 3b). We observed that 2 peptides from gp100, 3 tyrosinase-derived peptides, 5 MAGE-C2-derived peptides and 4 peptides from the MAGE-A3 antigen stimulated the post-treatment CD8^+ T cells (Table 2). Some of the recognized peptides contained previously identified epitopes (Table 2, underlined peptides). One of them was reported to be restricted to an HLA type not presented by the patient [33], indicating that this might be a promiscuous epitope. In addition, several as yet unidentified epitopes were recognized by the T cells, illustrating the advantage of full-length MAA-encoding mRNA-electroporated DCs presenting the complete array of epitopes contained within the antigens.

Fig. 3 Treatment antigen-specific T-cell responses in pre- and post-treatment peripheral blood cells. **a** CD137 expression by in vitro-stimulated peripheral pre- and post-treatment CD8⁺ T cells upon overnight re-exposure to aEBV-B cells electroporated with the full-length MAA mRNAs. mRNA encoding the HIV protein Nef flanked by the same HLA class II-targeting signal served as control antigen. Indicated *numbers* represent the percentage of positive cells within the CD8⁺ population. **b** Fold increase in IFN- γ secretion by stimulated T cells upon overnight re-exposure to aEBV-B cells loaded with the pools of 10 peptides (*upper panel* indicated *numbers* represent the number of the positive peptide pools) and the individual peptides of the positive-tested peptide pools (*lower panel* indicated *numbers* represent the first and last aa of the recognized peptide). *pp* peptide pool



Unfortunately, because we did not have a tumor biopsy at our disposition, we do not know which antigens are expressed by this patient’s tumor lesions. However, due to the prevalence of the antigens used in the DC treatment, it is very likely that at least one of these four antigens is

expressed [34]. The specific CTLs might then form the spark, necessary to induce antigen-spreading, which may contribute to the tumor clearing [35, 36]. An analysis of antigen-spreading was performed for 5 other MAAs, including WT1, MAGE-A1, MAGE-A4, MAGE-A10 and

MAGE-C1. None of these antigens were recognized after TriMix-DC treatment by peripheral blood CD8⁺ T cells after *in vitro* stimulation (data not shown). Unfortunately, we were not able to include more MAAs due to limited patient material.

Tumor antigen-specific T-cell responses in post-treatment DTH

After ID injection of DCs, a correlation has been described between the presence of treatment antigen-specific CD8⁺ SKILs derived from DTH biopsies induced by treatment DCs and a positive clinical outcome [37]. Therefore, we also investigated the presence of specific T cells in a DTH biopsy taken 1 week after the fourth DC administration (Fig. 1). Because we use full-length tumor antigens to load the DCs, we used mRNA-loaded aEBV-B cells as targets and measured antigen-specific surface markers on the SKILs as described elsewhere (Van Nuffel, A. M. T. in preparation). Prior to restimulation with aEBV-B cells, the T cells from the biopsy were cultivated in the presence of IL-2 and without any antigen-specific stimulation.

An elevated CD137 and CD107a expression and cytokine secretion in response to gp100, tyrosinase, MAGE-C2 and MAGE-A3 compared to control antigen were found (Fig. 4a and data not shown). In our experience, this T-cell response is exceptionally broad. So far, out of 33 patients monitored after TriMix-DC treatment (administered intradermally only (ID) or combined intradermally and intravenously (ID/IV)), only 3 patients had specific CD8⁺ SKILs for all four treatment antigens ([19] and unpublished data). Of note, two of them received TriMix-DC ID/IV, suggesting that this injection route might contribute to a broader CD8⁺ T-cell response.

Using the same approach as for the blood-derived CD8⁺ T cells, we could document that this patient's post-treatment CD8⁺ SKILs recognized multiple peptides within all 4 antigens (Table 2). About the same number of peptides was recognized by blood and DTH CD8⁺ T cells. Surprisingly, only five peptides were recognized by both populations (Table 2). All other peptides were recognized by either the SKILs or the peripheral blood CD8⁺ T cells (Table 2). The reason for this is unclear. On the one hand, it might merely reflect the limitations of our *in vitro* immunomonitoring method. Indeed, screening was performed in one culture well per antigen. Therefore, we cannot exclude overgrowth of some CD8⁺ T-cell responses by others targeting the same antigen. This will lead to a bias between the responses found after restimulation of the blood-derived CD8⁺ T cells and the SKILs. On the other hand, it is more and more understood that DCs can inform the T cells about which location they come from [38]. One could hypothesize that the T cells stimulated by the ID-injected DCs might upregulate homing

receptors targeting them toward the skin, whereas those T cells stimulated by the IV-injected DCs might upregulate other homing receptors. However, we observed similar compartment restrictions of antigen-specific T cells in patients receiving their TriMix-DC solely intradermally (Benteyn et al. in preparation). In addition, it has been reported that T cells found in the skin are found in the blood as well [39]. Therefore, the role of combined ID/IV administration of the TriMix-DCs in this observation is unclear.

In addition to CD8⁺ T-cell responses, we also investigated CD4⁺ T-cell responses in the DTH biopsies. CD4⁺ SKILs upregulated CD137 and CD40L upon re-exposure to two antigens: MAGE-C2 and MAGE-A3 (Fig. 4b). We looked further into detail to the recognized peptides of MAGE-A3 on purified CD4⁺ SKILs. Here also a broad response was documented: 12 peptides were recognized (Fig. 4 and Table 2). Five of them overlapped with peptides recognized by CD8⁺ T cells (Table 2). This feature of nested epitopes where HLA class I- and class II-restricted peptides are present within the same antigenic region is not uncommon and has been described before [40–42].

Besides the recognition of several regions of the antigen, measurement of IFN- γ , TNF- α , IL-2, IL-10, IL-5 and IL-13 showed that the CD4 responses had diverse functionalities [43]. A Th1 cytokine profile characterized by a predominant secretion of IFN- γ , TNF- α and IL-2 was displayed by the T cells recognizing MAGE-A3_{141–155} and MAGE-A3_{145–159}; a mixed Th1/Th2 cytokine profile was displayed by the T cells directed against peptide MAGE-A3_{109–123}, MAGE-A3_{113–127}, MAGE-A3_{117–131}, MAGE-A3_{121–135} and MAGE-A3_{241–255}; a Th2 cytokine profile for which IL-13 and IL-5 are common was displayed by the T cells recognizing MAGE-A3_{161–175}, MAGE-A3_{165–179}, MAGE-A3_{177–191}, MAGE-A3_{189–203} and MAGE-A3_{193–207}. The TNF- α and IL-5 secretion of the CD4⁺ SKILs against the individual peptides from the pools that tested positive is shown in Fig. 4c. Although the biological implications of this diverse Th response are unclear, such cytokine patterns including the unconventional mixed Th1/Th2 are regularly observed after immune therapy in cancer [41].

Unfortunately, in the previous study where TriMix-DC were injected ID only, the CD4⁺ Th response has not been investigated into sufficient detail. Therefore, we cannot estimate the contribution of the injection route to this broad functionality.

Conclusion

We previously reported that the T-cell stimulatory capacity of DCs can be enhanced by co-electroporation of mRNA encoding CD40L, CD70 and caTLR4 (TriMix-DC) and that intradermal administration of these TriMix-DC is safe,

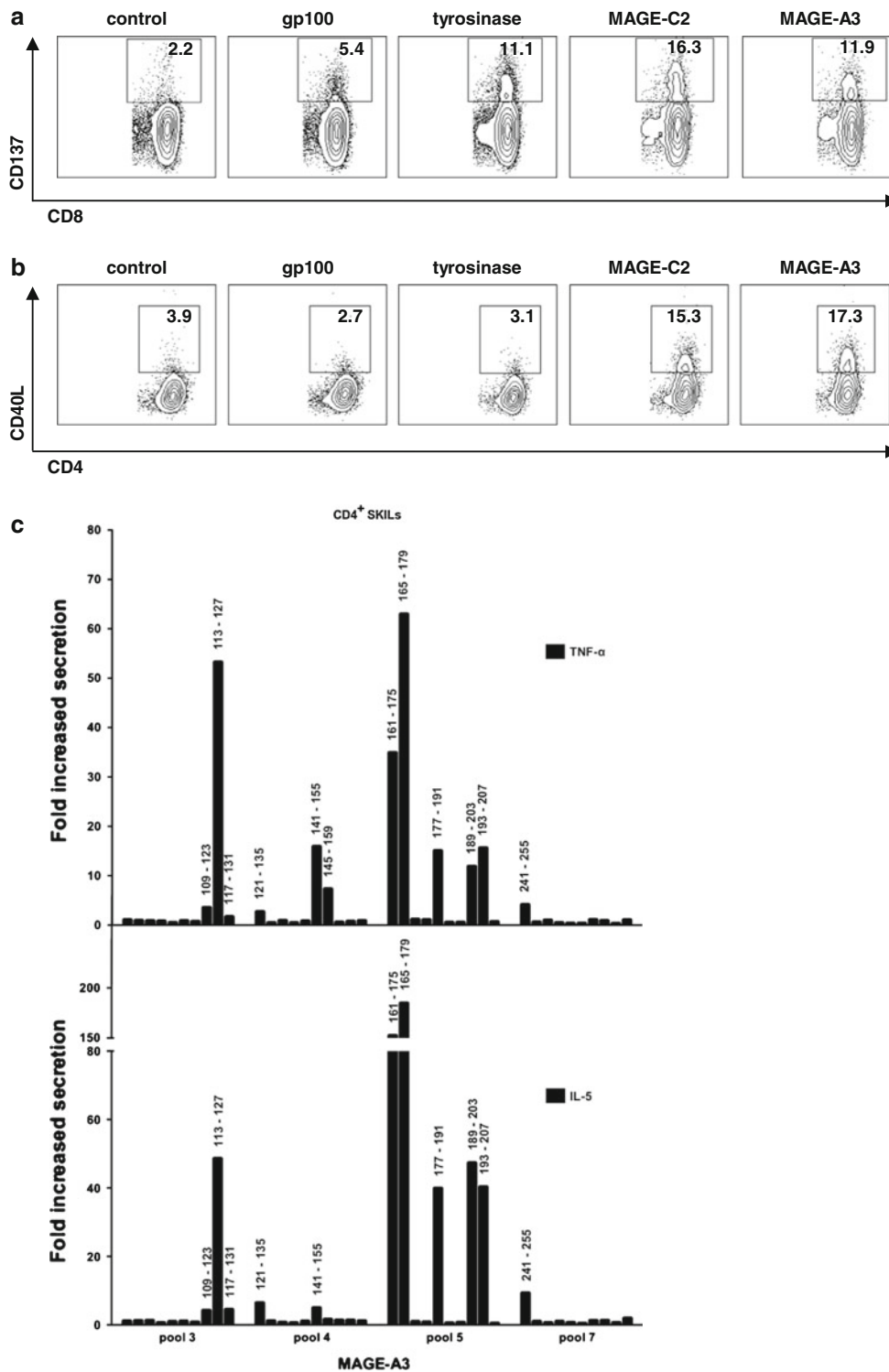


Fig. 4 Treatment antigen-specific T-cell responses in post-treatment DTH. **a** CD137 expression by the CD8⁺ SKILs after stimulation with MAA-presenting aEBV-B cells. mRNA encoding the HIV protein Nef flanked by the same HLA class II-targeting signal as the MAA served as control antigen. Indicated numbers represent the percentage of positive cells within the CD8⁺ population. **b** CD40L expression by the CD4⁺ SKILs after stimulation with MAA-presenting aEBV-B cells.

Indicated numbers represent the percentage of positive cells within the CD4⁺ population. **c** Fold increase in TNF- α and IL-5 secretion by the CD4⁺ SKILs 24 h after stimulation with aEBV-B cells pulsed with MAGE-A3-derived 15-mer peptides. The aEBV-B cells were pulsed with the individual peptides contained within the peptide pools recognized by the CD4⁺ SKILs (data not shown). Indicated numbers represent the first and last aa of the recognized peptide

feasible and immunogenic [16, 18, 19]. We here report that a chemorefractory melanoma stage IV-M1c patient achieved a durable clinical response together with a very broad and diverse T-cell response after combined intradermal and intravenous administration of autologous TriMix-DC. This suggests that besides optimization of the DC formula itself, optimization of the administration of the cell product by the combined ID/IV route may result in further enhancement of the immunogenicity and antitumor activity of this approach while maintaining its low toxicity.

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Conflict of interest TriMix-DCs are the topic of a current patent application (WO2009/034172). AB and KT are mentioned as inventors of this application. None of the authors involved in this study receives any form of support or remuneration related to this platform.

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