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SHORT COMMUNICATION

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A new dendritic cell vaccine generated with interleukin-3 and interferon-β induces CD8$^+$ T cell responses against NA17-A2 tumor peptide in melanoma patients

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Abstract Dendritic cells derived from monocytes cultured in the presence of type I interferon were found to induce efficient T cell responses against tumor antigens in vitro. We vaccinated eight stage III or IV melanoma patients with dendritic cells generated with interferon-β and interleukin-3, activated by poly I: C, and pulsed with the tumor-specific antigen NA17.A2. This dendritic cell vaccine was well-tolerated with only minor and transient flu-like symptoms and inflammatory reactions at the injection sites. In most patients, isotopic imaging documented dendritic cells (DC) migration from the intradermal injection site to the draining lymph nodes. Finally, mixed lymphocyte-peptide culture under limiting dilution conditions followed by tetramer labeling indicated that three out of eight patients mounted a CD8 T cell response against the NA17.A2 antigenic peptide. We conclude that DC generated in type I-IFN represent an interesting alternative to DC generated in IL-4 and GM-CSF for cancer immunotherapy.

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Introduction

Most clinical trials of dendritic cell-based vaccines in cancer patients use dendritic cells (DC) derived either from blood monocytes cultured in the presence of GM-CSF and interleukin (IL)-4 [11, 27] or from CD34$^+$ precursors cultured with GM-CSF, Flt3-L and TNF [1, 20]. Encouraging results have been obtained, and a lot of effort is now devoted to improve this type of immunotherapy and develop it on a larger scale [2, 6, 9]. Belardelli et al. [22, 26] described a population of DC derived from monocytes cultured in the presence of GM-CSF and IFNα (GM/IFN DC). These cells are strongly immunogenic, express high levels of HLA molecules, costimulatory molecules, and CCR7, a chemokine receptor favoring migration in secondary lymphoid organs. They spontaneously produce IL-15, which promotes Th-1 type responses and survival of T-lymphocytes. We observed that all these features were shared by DC generated in the presence of IL-3 and IFN-β (IL3/IFNβ DC) [4]. In addition, we established that both the GM/IFN and IL3/IFNβ DC secreted high amounts of IFN-α in response to Poly I:C; whereas, only low levels were secreted under the same condition by DC differentiated in IL-4 and GM-CSF [4]. Recently, GM/IFN and IL3/IFNβ DC were found capable of stimulating in vitro naïve CD8$^+$ T cells recognizing tumor antigens such as the Melan-A/MART-1 peptide [18, 24, 28]. We report here the first results of a pilot clinical trial

in which melanoma patients received IL3/IFNβ DC presenting the NA17.A2 antigen, a peptide encoded by an intron sequence of the gene coding for N-acetylglucosaminyltransferase V, and presented by HLA-A2 [12].

Materials and methods

Preparation of dendritic cell vaccine

Dendritic cells were generated according to GMP guidelines in a closed culture system using double-tray Cell Factory (Nunc, Roskilde, Denmark). Briefly, blood mononuclear cells isolated from cytapheresis products were washed in PBS supplemented with 5% human serum albumin (300 ml PBS + 75 ml HSA 20%), resuspended at 5×10^6 cells/ml in 320 ml of X-VIVO 20 medium (Cambrex Europe, Verviers, Belgium) supplemented with 1% autologous serum, and transferred into a double-tray cell-factory. Cells were then allowed to adhere for 2 h at 37°C in a 5% CO_2 atmosphere. Non-adherent cells were discarded and adherent cells were washed and cultured in 320 ml of RPMI medium (Cambrex Europe) containing 100 U/ml of IFN-β (Avonex, Biogen, Cambridge, UK) and 50 U/ml of IL-3 (Cell Genix, Freiburg, Germany). After 48 h, IFN-β and IL-3 were added to the cultures, at the same concentrations. At day 5, cells were matured by overnight (18 h) incubation with 10 μg/ml of poly I:C (Sigma, Bornem, Belgium). Non-adherent cells were harvested at day 6 and pulsed for 2 h with NA17.A2 peptide (VLPDVFIRC, Clinalfa, Switzerland) (10 μg/ml). Since 60–65% of Belgian/French population are DPB1*0401 [10], DC were also pulsed with the MAGE-3.DP4 peptide (KKLLTQHFVQENYLEY, Clinalfa, Switzerland) (10 μg/ml) in order to potentially solicit help from $CD4^+$ T cells [13].

Cytokine measurements

Commercially available Elisa Kits were used to determine the concentrations of IFN-α, (BioSource International, Fleurus, Belgium) and IL-12 p70 (Endogen, Woburn, MA, USA) in culture supernatants.

Patients and vaccination protocol

Eight HLA-A2-positive patients with Stage IIIb or IV melanoma expressing the NA17 tumor-associated antigen as determined by RT-PCR were vaccinated with peptide-pulsed IL3/IFNβ DC and tested for blood frequency of anti-NA17.A2 CD8 T cells. Patients were 18 to 75-year old, had a Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1 and a life expectancy of more than 4 months. Three vaccinations were given at 3-week intervals. Each vaccination consisted of 20×10^6 cells injected half subcutaneously and half intradermally, and 2.5×10^6 cells injected in an inguinal lymph node under ultrasound guidance. The Ethics Committee of the Erasme Hospital approved the study and all patients signed an informed consent. Adverse events were graded using the common terminology criteria for adverse events (CTCAE), Version 3.0 (Publish Date December 12, 2003).Clinical responses were measured according to RECIST criteria as a secondary endpoint of this study.

Imaging procedure for dendritic cell migration

Dendritic cells, prepared and matured as described above, then resuspended at a concentration of 10^7 DC/100 μl of RPMI medium [3], were incubated for 30 min at 20°C with 1.11 MBq/10^7 DC of Indium-111 oxine (Mallinckrodt Medical), then washed thrice with the medium. Patients received subcutaneous (SC) or intradermal (ID) injections of 5×10^6 labeled DC (in 0.3 ml of PBS) in the proximal inguinal region of each leg. A 60-min dynamic acquisition centered on the inguinal region was acquired immediately on a Sopha DSX gamma camera equipped with a parallel-hole medium-energy collimator. Ten-minute images were also acquired repeatedly during the first 45 h post-injection. Migration of DC-related tracer labeling was defined as the occurrence of at least one visually detectable focal uptake in the inguinal region. In one patient, a 3.7 MBq injection of Tc-99m nanocolloids (Nanocis^R, CIS bio International)—a radiopharmaceutical used for the sentinel node detection—was performed 19 h after the administration of DC, precisely in the area of labeled DC injection. Dynamic acquisition and 2-min static images centered on the inguinal region were acquired on the same gamma camera.

Detection of anti-NA17 T cells

Blood frequencies of anti-NA17.A2 $CD8^+$ T cells were estimated using mixed lymphocyte-peptide cultures under limiting dilution conditions, followed by labeling with tetramers, as described [5, 14]. Briefly, PBMC collected and frozen 3 weeks after the third vaccination were thawed, resuspended at 10^7 cells/ml in Iscove's medium supplemented with 1% human serum (HS) and 2 μM of peptide NA17.A2 (VLPDVFIRC), incubated over 1 h at room temperature, and washed. These peptide-pulsed cells were distributed at 2×10^5 cells/well in round-bottom microwells in Iscove's medium with 10% HS, L-arginine (116 mg/L), L-asparagine (36 mg/L), L-glutamine (216 mg/L), IL-2 (20 U/ml) and IL-7 (10 ng/ml). On days 5 and 7, 50% of the medium was replaced by fresh medium containing IL-2 and IL-7. Peptide NA17.A2 was added on day 7 at a concentration of 2 μM. During the second week of stimulation, the microcultures were divided according to proliferation, in a medium containing IL-2 alone. At day 14, aliquots of

the microcultures were labeled with anti-CD8 antibodies coupled to fluorescein, an HLA-A2 tetramer containing the NA17.A2 peptide and coupled to phycoerythrin, and a control HLA-A2 tetramer containing an EBV peptide (GLCTLVAML encoded by gene *BMLF1*) and coupled to allophycocyanin. The proportion of microcultures containing $CD8^+$ cells specifically stained with the A2/NA17 tetramer was used to estimate a blood frequency of precursors of cells that can be stained by the tetramer (anti-NA17.A2 TETp frequency), taking into account the proportion of $CD8^+$ cells in the PBMC.

$$\text{TETp frequency among blood CD8 cells} = \frac{-\ln\left(1-(\text{number of positive wells}/\text{total number of wells})\right)\times 100}{\text{number of PBMC per well}\ \times\ \%\ \text{of CD8}}$$

Results and discussion

Characteristics of the IL3/IFNβ dendritic cell vaccine

The resulting DC populations were more than 80% pure as determined by morphology. Contaminating cells were mostly B cells. Although far less potent than DC as antigen-presenting cells, these cells might contribute also to antigen presentation without interfering with the nature of the immune response. The major characteristics of the IL3/IFNβ DC vaccine preparations are given in Table 1. Flow cytometry analysis disclosed a mature phenotype including the expression of high levels of CD80, CD83 and CD86. These cells also produced high amounts of IL-12p70 and IFN-α. These IL3/IFNβ Dc were still able to produce these cytokines upon rechallenge with CD40L (data not shown).This is consistent with previous studies that report that poly(I:C) used for DC maturation preserves their ability to secrete bioactive IL-12 upon rechallenge notably with CD40L [17, 25].

Safety of the IL3/IFNβ dendritic cell vaccine and clinical outcomes

The IL3/IFNβ DC vaccine was well-tolerated with neither serious adverse event, nor grade 3 or 4 adverse reaction, in any of the eight patients. Seven patients developed a grade 1–2 local inflammatory reaction at the injection site (erythema with various degrees of skin infiltration) which faded away after 48 h. Transient grade 1–2 flu-like symptoms and fatigue developed in all patients with fever up to 40°C in one patient within 6 h after vaccine administration; in all cases, symptoms disappeared within 48 h. Neither vitiligo nor other signs of autoimmunity were observed.

Out of the four patients who had measurable disease at the onset of vaccination, three displayed tumor progression after vaccination. One patient remained stable for 9 months with the exception of one small brain metastasis 5 months after inclusion, treated with gamma knife surgery. Out of the four patients with no detectable tumor, one showed tumor progression while the others remained free of disease for >13, >16 and >18 months (table 2).

In vivo migration of IL3/IFNβ dendritic cells

Evidence of IL3/IFNβ DC migration was found after ID, but not after SC injection (Fig. 1a, b). These results are consistent with those published by Morse et al. [19]. Their data indicated that DC trafficking is markedly dependent on their mode of delivery: SC administration seems to be ineffective in causing DC migration to regional lymphatics, IV administration results in DC migration to the spleen, whereas intradermal administration leads to regional transit in some patients.

Migration after ID injection was documented in six out of the eight patients studied. In patient 1, only a faint uptake in the inguinal region draining the vaccination site could be observed. In the other patients, tracer migration was more apparent as illustrated in Fig. 1. Focal uptake in the inguinal sites draining the sites of labeled DC injection represented $0.56 \pm 0.16\%$ (mean ± SD) for loaded (LL) DC, and $0.78 \pm 0.23\%$ (mean ± SD) for non-loaded (NLL) DC of the initially

Table 1 Characteristics of IL3/IFNβ DC injected to patients

	DC enumeration		Immunophenotyping of DC (%)					Cytokine production in DC supernatant (pg/ml)	
	Absolute number×10^6	Purity of DC (%)	CD14	CD1a	CD80	CD83	CD86	IL12 p70	IFNα
Mean	67	86	91	3	88	56	98	422	432
SD	37	8	6	1	14	22	8	484	311

Results are from 24 vaccine preparations: 3 vaccines per patient × 8 patients
SD standard deviation

Table 2 Characteristics of patients vaccinated

Patient number	Age/sex	Stage	Previous treatments	Presence of metastases at inclusion	Clinical outcome
1	36/F	IIIb	S, IFNα	No detectable	NED (>13 months)
2	41/M	IIIb	S, RxTh	No detectable	PD
3	60/M	IVM1c	S	No detectable	NED (>18 months)
4	37/F	IVM1c	S, γK	No detectable	NED (>16 months)
5	37/M	IVM1c	S, RxTh	Liver, subcutaneous	PD
6	58/M	IVM1c	S	Lung, liver, subcutaneous	PD
7	47/M	IVM1c	γK	Lung, subcutaneous	SD[a] (9 months)
8	65/M	IVM1c	S, I, C	Liver	PD

Abbreviations: *NED* no evidence of disease, *ED* evidence of disease = presence of metastases, *F* female, *M* male, *S* surgery; *IFNα*, interferon-α, *RxTh* radiotherapy, *γK* gamma-knife, *C* chemotherapy, *I* immunotherapy, *PD* progressive disease, *SD* stable disease

[a]Except for one new 4 mm brain metastasis 5 months after inclusion, treated by gamma-knife

administered radioactivity. This is in accordance with previous results that observed that a significant percentage of both immature and mature IL-4/GM-CSF generated DC remained at the site of the injection [7]. However in contrast to them, the maximum intensity of migration estimated by visual analysis of radioactivity was around 24 h post-injection, but that could be explained by the use of a different type of DC.

Blood frequencies of anti-NA17.A2 T cells

Frequencies of precursors of CD8 T cells stained by a tetramer containing the NA17.A2 peptide were evaluated in the blood of the eight patients (Table 3). Pre-vaccination frequencies were evaluated for five patients. In four patients, they were below or about 10^{-6} of the blood CD8 T cells. We obtained similar frequencies in several other melanoma patients, not included in this study. In two individuals without cancer, we estimated frequencies at 0.1 and 0.5×10^{-6} of the CD8 cells. Therefore, we believe that these values correspond to the frequency of naïve anti-NA17.A2 T cells. This frequency is similar to that of the naïve CD8 cells against another tumor-specific antigen, peptide MAGE-$3_{168\text{-}176}$presented by HLA-A1 molecules [16]. In patient 4, the pre-vaccination frequency was 4.5×10^{-6}, and a frequency of 7×10^{-6} was observed in blood

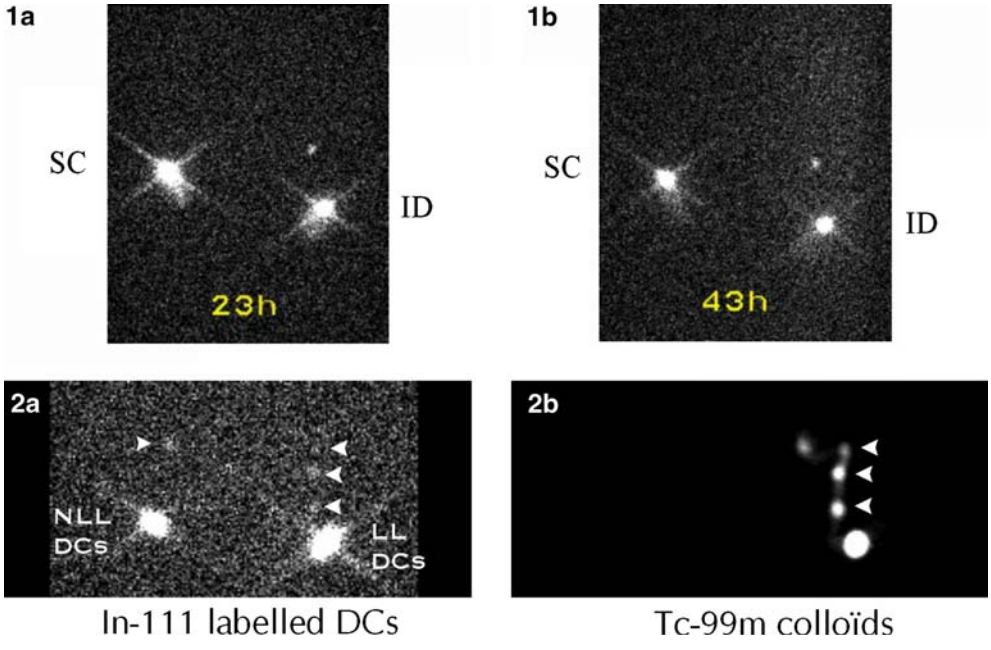

Fig. 1 Migratory capacities of IL3/IFNβ DC. **1a, b** In-111 labeled autologous IL3/IFNβ DC were injected in the proximal inguinal region of each leg of a patient, either subcutaneously (*SC*) on one side, or intradermally (*ID*) on the opposite side. Using a Sopha DSX gamma camera, **1a** and **1b** images, acquired, 23 h and 43 h post-injection, respectively, showed migration only with ID injection. **2a** An image obtained 15.5 h after ID administration of labeled DCs either loaded (*LL*) with NA17.A2 antigen on one side, or non loaded (*NLL*) on the opposite side, demonstrated tracer migration towards nodular inguinal sites (*arrow heads*). **2b** The patient was secondarily injected with Tc-99m nanocolloïds on the site of LL DCs injection. The acquisition obtained 30 min later demonstrated that the migration detected on In-111 **2a** image corresponded to three draining lymph nodes in the inguinal region

Table 3 Migratory and immunostimulatory properties of DC IL3/IFNβ injected to patients

Patient number	Status of disease at inclusion	DC migration	Anti-NA17.A2TETp frequency ($\times 10^{-6}$ of the blood CD8 cells)		T cell response
			Before	After	
1	No detectable tumor	+	< 0.8	9.4	+
2		+	< 0.7	5.7	+
3		–	NT	< 0.5	–
4		+	4.5	21	–
5	Presence of metastases	–	NT	< 0.7	–
6		+	NT	< 0.2	–
7		+	1.1	6.1	+
8		+	< 0.4	< 0.3	–

Abbreviations: *NT* not tested, *TETp* tetramer positive

collected 9 months earlier. We conclude that this patient had a spontaneous anti-NA17.A2 T cell response prior to vaccination.

After vaccination, 4 out of the 8 patients had anti-NA17.A2 TETp at frequencies below 10^{-6} of the CD8 cells, not higher than what we consider to be naïve frequency. Three patients had frequencies that were ≥5-fold times higher than before vaccination, indicating that they responded to the vaccine. In patient 4, the post-vaccination frequency was also 5-fold higher than before vaccination, but this may correspond to fluctuations of a pre-existing response. We conclude that three out of the eight vaccinated patients mounted an anti-NA17.A2 T cells response that can be detected with our methods. As illustrated in Table 3, all patients with a high anti-NA17 CTL frequency displayed migration of Indium-labeled DC, and had small or no tumor burden at the time of inclusion: three had no detectable metastasis, and one had small lung and subcutaneous metastasis.

Concluding remarks

The results of this pilot trial establish that vaccination of cancer patients with IL3/IFNβ DC represents a safe and valid tool to induce $CD8^+$ T cell responses against tumor-antigens in melanoma patients. Together with previous reports demonstrating the efficiency of similar DC in the induction of anti-HIV responses in SCID mice reconstituted with human lymphocytes [15, 26], our data suggest that DC generated in type I IFN might represent an interesting alternative to DC generated in IL-4 and GM-CSF for the development of more efficient cellular vaccines. Indeed, we found that IL3/IFNβ DC produce higher levels of IFN-α [4] and IL-6 [8], the latter cytokine being able to circumvent suppressive signals elicited by regulatory T cells which jeopardize anti-tumor responses in cancer patients [21, 23]. Since simultaneous immune responses to multiple tumor antigens might be required for efficient therapeutic vaccination in cancer patients, we will soon initiate a randomized trial comparing multipeptide vaccines based on IL3/IFNβ or IL4/GM-CSF DC.

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