

IV DISCUSSION

IV. 1 COMPARISON OF THE THERAPEUTIC EFFICACY OF VARIOUS MODALITIES OF IMMUNOTHERAPY INVOLVING TUMOR-SPECIFIC T CELLS IN PATIENTS WITH DETECTABLE MELANOMA METASTASES

Immunotherapy may be active, using antigens to stimulate an immune response *in vivo*, or passive, generating the appropriate effector cells *in vitro* and transferring them back into the patient. Both forms of immunotherapy can use antigens that are undefined at the molecular level, such as those that are present on whole tumor cells, or defined antigens, which can be administered under a wide variety of modalities.

Because we aim at comparing the clinical efficacy of various modalities of immunotherapy, only the tumor evolution of patients with detected melanoma metastases at study entry will be presented here. Studies that were designed in the adjuvant setting, i.e. those which only included patients with no evidence of disease after resection of all visible metastatic lesions, will not be discussed here. Likewise, because the results reported in Section III are focused on vaccination with peptides or with a recombinant MAGE-3 protein, the immunotherapy modalities using viruses will not be further discussed here (221). All the patients who received at least one treatment injection according to the relevant study protocol modalities will be considered for the discussion that follows.

IV. 1.1 Vaccination with undefined antigens : melanoma cell-based vaccines

Prior to the molecular characterization of tumor antigens, tumor cells were the only source of antigens for cancer vaccination. Undefined antigens may be present on autologous or allogeneic tumor cells or in tumor cell lysates. Vaccines can be prepared from freshly isolated melanoma cells or from melanoma cell lines. The former procedure requires large tumors in order to obtain enough material for the vaccine, whereas the latter involves a longer delay. Short-term cultures allow a limited expansion of tumor cells for a period of 4 to 8 weeks, whereas the establishment of a permanent melanoma cell culture usually lasts for 3 to 6 months and has a success rate of about 50% for metastatic melanoma lesions. The first melanoma vaccines involved inoculations of patients with lethally irradiated (100 - 150 Gray) autologous or allogeneic melanoma whole cells, or with cell extracts, with or without immunological adjuvants. More recently, approaches involving gene therapy or antigen-presenting cells were also used. Twenty one selected clinical trials illustrating these various approaches are presented in *Table 23* (234-254). Therapeutic effectiveness of melanoma cell-based vaccines will be discussed in *Section IV. 1.1.4*.

Table 23 Cell-based vaccines in patients with detectable melanoma metastases

Type of vaccine	Cytokine or adjuvant	Injection route ¹	Patients enrolled ² (n)	Tumor response ³ according to maximal extent of disease ⁴		Trials (n) (References)
				non-visceral	visceral	
Autologous melanoma cell-based vaccines						
Whole cells obtained from short-term cultures	BCG ⁵ , GM-CSF ⁶ or IFN ⁷	s.c.	33	1CR	2PR 11SD	(234)
DNP ⁸ -modified whole melanoma cells (M-Vax TM)	BCG	i.d.	97	2PR, 4MxR / 42	2CR, 2PR, 1MxR, 2SD/ 55	(235)
Melanoma / autologous DC ⁹ Hybrids	none	s.c.	17	1MxR / 3	1PR, 1SD / 14	(236)
	low-dose IL-2	s.c.	10	1SD / 4	1PR, 1SD / 6	(237)
Melanoma / allogeneic DC Hybrids	none	i.d.	20	1CR, 1MxR, 1SD / 3	5SD / 17	(238)
Heat shock protein gp 96-peptide complexes	none	i.d. or s.c.	28	1CR / 11	1CR / 17 3SD	(239)
SUBTOTAL			205	14OR, 7MxR or MR, 25SD		6
Allogeneic melanoma cell-based vaccines						
Whole cells obtained from: 1 cell line	BCG	i.d.	15	0 / 6	0 / 9	(240)
3 cell lines	none	i.l.	47	1CR, 6PR, 2SD / 22	1CR, 1PR / 25	(241)
3 cell lines (CancerVax TM or Canvaxin TM)	BCG alone or with: Cy., Indom., or Cimet. ¹⁰	i.d.	40	2CR	1PR 1CR, 5PR, 4SD	(242)
	BCG alone or combined with:	i.d.	54	3CR, 1MxR, 4SD / 18		(243)
	Cimetidine			2CR, 2PR, 4Mxr, 1SD / 21		
	Cyclophosphamide			1CR / 3		
	Indomethacin			1CR, 1SD / 3		
	Ranitidine			0 / 2		
	DNCB ¹¹			0 / 1		
	Indom. plus Cimet.			1MxR, 1SD / 6		
Mechanical lysate of 2 cell lines (Theraccine or Melacine TM)	Detox ¹² alone or combined with:	s.c.	33	1CR, 2PR, 2MR, 2MxR	1CR, 2PR, 1SD,	(244, 245)
	Cyclophosphamide		9	1CR, 1PR, 2MR	1PR	
	Cyclophosphamide plus IFN- α		47	2CR, 2PR, 4MR, 25SD / 9		3SD / 38
Preparation from cell membranes Large multivalent immunogen (LMI)	none	i.d. / s.c.	17	1MxR, 3SD / 7	1PR, 1SD / 10	(247)
SUBTOTAL			262	41OR, 17MxR or MR, 43SD		8
Genetically modified melanoma cell vaccines						
Transfected gene						
Autologous short-term cell cultures	INF- γ	s.c.	20	2CR, 2MR / 20		(248)
Allogeneic cells (HLA-A2 matched)	IL-2	s.c.	12	1SD / 1	1MxR, 3SD / 11	(249)
Autologous melanoma cells mixed with allogeneic transfected fibroblasts	IL-2	i.d. / s.c.	6		2SD / 6	(250)
Allogeneic cells (HLA-A2 matched)	IL-4	s.c.	12	1MxR, 1SD / 5	1MxR / 7	(251)
Autologous cells	IL-7	s.c.	10	0 / 1	2MxR, 4SD / 9	(252)
	IL-12	s.c.	6		3SD, 1MR / 6	(253)
	GM-CSF	i.d. / s.c.	26	1PR, 1MxR, 3MR		(254)
SUBTOTAL			92	3OR, 12MxR or MR, 14SD		7
TOTAL			559	58 OR, 36 MxR or MR, 82 SD		21

Legend to Table 23

- 1) *Cell Injection route*: i.d. = intradermal, i.l. = intralymphatic, s.c. = subcutaneous.
- 2) Patients received at least 1 vaccination.
- 3) *Tumor response*: CR = Complete Response, PR = Partial Response,
OR = Objective Response, MR = Minor Response, MxR = Mixed response,
SD = Stable Disease. The other patients had progressive disease.
- 4) *Melanoma metastases*: non-visceral : American Joint Committee on Cancer 2002
stage III or IV M1a; visceral : stage IV M1b or M1c.
- 5) Bacille Calmette-Guérin.
- 6) Granulocyte-Macrophage Colony Stimulating Factor.
- 7) Interferon- α or γ .
- 8) Dinitrophenyl.
- 9) Dendritic cells.
- 10) Cy.: Cyclophosphamide; Indom.: Indomethacin, Cimet.: Cimetidin.
- 11) Dinitrochlorobenzene.
- 12) Contains "detoxified endotoxin" (monophosphoryl lipid A or MPL) from *Salmonella minnesota*,
cell wall skeletons of *Mycobacterium phlei*, squalane oil, and Tween-80 emulsifier.

IV. 1.1.1 Autologous melanoma cell-based vaccines

The advantage of using the patient's own tumor cells is that they may present unique or rare, highly immunogenic antigens, such as those encoded by individual genetic mutations present in the tumor. However, few patients have sufficient accessible tumor available for most autologous vaccines strategies. Autologous vaccines are limited to single patients, their preparation is costly and time-consuming and the interval of several weeks or months between initial tumor harvest and ultimate availability of the clinical vaccine may result in concurrent tumor progression and associated immunosuppression, which can potentially impair vaccine efficacy, or lead to death before the vaccination was initiated.

IV. 1.1.1.1 Whole melanoma cells

Thirty-three patients with metastatic melanoma were immunized by subcutaneous route with irradiated autologous melanoma cells from short-term cell lines, administered with various immunological adjuvants, including Bacille Calmette-Guérin (BCG), granulocyte colony-stimulating factor (GM-CSF), IFN- α , or IFN- γ (234). Based on intent-to-treat analysis, tumor response 2 months after starting treatment was 1 CR, 2 PR, 11 SD and 19 PD. All 33 patients progressed within 7 months except for the 3 patients who had an objective tumor response. The overall survival of the latter was 36, 46+ and 78+ months, respectively (*Table 23*).

The most studied autologous melanoma vaccine is a whole-cell vaccine treated with the hapten dinitrophenyl (DNP) that binds to molecules on the surface of the cells. M-VaxTM (AVAX Technologies), first developed at the Thomas Jefferson University, Pennsylvania, USA, consists of low-dose cyclophosphamide (300 mg/m² as i.v. bolus) followed by multiple i.d. injections of DNP-modified, irradiated melanoma cells mixed with BCG at 4-week intervals (*Table 23*).

Ninety-seven patients with surgically incurable metastases were included in a phase II non-randomized trial, to determine whether DNP vaccine could induce regression of metastases (235). Six patients had stage III and all the others had stage IV disease at study entry. Fifty-five patients had visceral metastases, including 30 patients with lung as single visceral localization. There were 2 CR, 4 PR, 5 MxR and 2 SD. All the patients who experienced tumor regression had stage IV disease. Tumor regression involved skin, lymph node, or lung metastases. Both CR and 2 of 4 PR occurred in patients with small lung metastases. No tumor regression was observed in the 15 patients with other visceral localizations. Tumor regression required at least 4 months to become evident and in 2 cases maximum regression was not observed until 1 year after the beginning of treatment. The median objective response duration was 20.5 months (range: 5 to 60+ months). Following vaccination treatment, 87% of the patients developed delayed-type hypersensitivity (DTH) to autologous, DNP-modified melanoma cells and about 42% of them also exhibited DTH to autologous, unmodified tumor cells. The induction of DTH to unmodified autologous tumor cells was associated with significantly

longer survival. The assessment of the clinical effectiveness of the autologous, DNP-modified melanoma vaccine would require a randomized trial (*Table 23*).

IV. 1.1.1.2 Autologous melanoma / dendritic cell hybrids

Dendritic cells are antigen-presenting cells that appear to play a central role in stimulating immune responses (255). They have the unique ability to stimulate naive T and B lymphocytes as well as natural killer cells (NK). Somatic hybrids between tumor cells and DC should express the antigens of the parent tumor cells and the co-stimulatory molecules of the antigen-presenting cells. In the absence of appropriate selection markers, the purification of fused hybrids or “dendritomas” from the fusion mixture remains difficult. Wei and colleagues recently described a method, using a double fluorescent dye procedure, that allows easy purification by fluorescence-activated cell sorting of dendritomas (dual colors) from the fusion mixture containing unfused autologous dendritic cells (red color marker) and autologous melanoma cells (green color marker) (*Table 23*) (237).

Seventeen patients with disseminated melanoma refractory to standard therapy were enrolled in a pilot study of vaccination with autologous hybrids of melanoma and dendritic cells, to evaluate the evolution of tumor metastases and the safety of the vaccine (236). Three patients had soft tissue, skin or lymph node metastases and 14 had visceral metastases, including 1 patient with lung as single visceral localization. The treatment schedule included 3 s.c. vaccinations at monthly intervals. In patients with stable disease or tumor regression, vaccinations were repeated until progression or as long as tumor cells for vaccine preparation were available. No serious side effects were observed. Fourteen patients had PD, 1 had a PR and 1 had a MxR and 1 achieved disease stabilization for 6 months. No DTH response was observed in any patient, either before, or after vaccination (*Table 23*).

In another pilot study, 20 patients with non-resectable melanoma that had not responded to previous treatments were vaccinated with hybrids of autologous melanoma cells and mature allogeneic DC (238). In addition to the costimulatory molecules required for T-cell activation, mature allogeneic DC are expected to provide a potent immunostimulatory capacity to the hybrid cells, through the allogeneic MHC molecules that will generate a strong helper T-cell response to support the induction of tumor-specific CTL. Three patients had stage III and 17 had stage IV disease with visceral metastases at study entry. The treatment schedule included 3 i.d. vaccinations at 4-week intervals. Patients who did not require other antitumor treatment received up to 22 additional vaccinations. No serious adverse events were reported. Twelve patients had PD. In addition, 1 CR, 1 MxR and 6 SD were observed (*Table 23*).

These preliminary results indicate that hybrid vaccine of DC and melanoma cells can be safely applied and can induce tumor regression. However, the clinical efficacy of this approach in its present form is still insufficient. A phase II randomized study comparing immunization with autologous DC pulsed with gp100 antigen versus autologous DC fused with autologous tumor cells in patients with stage III or IV melanoma is ongoing at the Dana Farber Cancer Institute, Boston, Massachusetts, USA.

IV. 1.1.1.3 Heat shock protein preparations from autologous melanoma cells

Heat shock proteins (HSP) are molecular chaperones that bind intracellular proteins and peptides. The HSP glycoprotein gp96 was shown to bind peptides of about 10 amino acids, which can then be transferred to MHC class I molecules and presented to T lymphocytes. Mice immunized with optimal doses of purified HSP gp96 derived from autologous tumor develop a protective tumor-specific T cell response (256). However, immunization with high doses of gp96 appeared to down-regulate the anti-tumor immune response (257). Based on these results, careful dose-escalation schedules should be followed in the design of clinical trials.

HSP-peptide complexes (HSPPC) vaccines production requires a resectable tumor sample from each patient. They have been studied in early phase clinical trials, mostly using gp96, for various types of advanced malignancies including melanoma (258). HSPPC-96 (OncophageTM) developed by Antigenics, Inc., Woburn, MA, USA, showed minimal toxicity as well as potential efficacy. Forty-two patients with stage IV melanoma received 4 weekly injections of the vaccine by i.d. or s.c. route (239). No severe treatment-related toxicity was reported. Of 28 patients with measurable disease, 2 had a CR and 3 had SD (*Table 23*). Duration of CR was 18+ and 23+ months, respectively. The SD lasted 5, 6 and 9 months, respectively. However, therapeutic efficacy was not confirmed by a phase III study for patients with stage IV melanoma in which overall survival was the primary endpoint (Trial n° C-100-21) (259). Three hundred and twenty two patients were randomized 2:1 to receive vaccine or various types of treatment, respectively. No significant difference in survival was observed between the 2 groups of patients (260).

IV. 1.1.2 Allogeneic melanoma cell-based vaccines

Allogeneic vaccines have significant advantages over autologous tumor vaccines in terms of availability. Although they may lack unique or rare antigens that could be important antigenic targets, the immunogenicity of HLA alloantigens can increase the immunogenicity of other cell surface antigens, some of them being shared with the patient's tumor. As opposed to autologous vaccines, allogeneic vaccines can be used to immunize several patients and can be manufactured in sufficiently large quantities to allow large-scale randomized trials in multiple institutions. Whole cell- or cell-extracts vaccines can be prepared from one, or from a few different established melanoma cell lines. The lack of defined antigens makes the monitoring of the immune response to such vaccinations quite difficult.

IV. 1.1.2.1 Whole allogeneic melanoma cells

Fifteen patients were immunized with a whole-cell vaccine prepared from irradiated cells of a single melanoma cell line. They received 3 to 12 injections by i.d. route at 3-week intervals. The 15 patients had PD (*Table 23*). There was also no evidence of accelerated tumor growth (240).

Forty-seven patients with evaluable metastatic disease received active specific intralymphatic immunotherapy (ASILI) consisting of irradiated whole cells obtained from 3 different melanoma cell lines. Patients received 2 to 21 immunizations at 3 to 4-week intervals. Nine objective tumor responses were observed, of whom 7 occurred in patients without visceral metastases at study entry (*Table 23*) (241).

Since 1984, DL Morton at the John Wayne Cancer Institute, Santa Monica, California (USA) has developed a polyvalent vaccine consisting of three allogeneic melanoma cell lines. This vaccine, called CancerVaxTM or CanvaxinTM, is developed by the CancerVax Corporation, Carlsbad, CA. It consists of viable but irradiated cells (100 Gy), administered i.d., mixed with BCG alone, or combined with various immunological adjuvants. It was reported to induce objective tumor responses in 9/40 stage IV melanoma patients and in 9/54 patients with stage III in-transit metastases (*Table 23*) (242, 243). Canvaxin trials were licensed to Serono in 2004. In 2005, this pharmaceutical company decided to discontinue the Phase III clinical trials of Canvaxin in melanoma, based upon the recommendation of an independent data and safety monitoring board. The board found that the data were unlikely to provide significant evidence of an overall survival benefit for patients with Stage III or IV melanoma who were treated with Canvaxin vs. those who received placebo.

IV. 1.1.2.2 Cell lysates from allogeneic melanoma cell lines

M. S. Mitchell, at the University of Southern California, Los Angeles (USA) developed a vaccine consisting of two mechanically disrupted allogeneic melanoma cell lines mixed with the immunological adjuvant DetoxTM. The latter includes monophosphoryl lipid A (MPL) and a mycobacterial cell wall skeleton extract. This vaccine, called Terracine or MelacineTM - Corixa Corporation - has been reported to produce occasional responses in stage IV melanoma patients, either alone, or combined with IFN- α and/or with prior low-dose cyclophosphamide (244-246). A preparation of tumor cell membranes affixed to amorphous silica microbeads (Large multivalent immunogen or LMI) had similar effects when administered without immunological adjuvant (247) (*Table 23*).

IV. 1.1.3 Genetically modified melanoma cell vaccines

The goal of somatic gene therapy targeted to melanoma cells is to introduce tissue-specific “suicide” genes (HSVtk) (261), to transfer tumor suppressor genes such as p53 (262), to inactivate aberrant oncogene expression such as *ras*, *c-myc* or *Stat3* (263), or to introduce genes encoding immunologically relevant molecules such as cytokines (248, 252-254), co-stimulatory molecules

(B7.1), or allogeneic MHC class I molecules (HLA-B7). This approach was mainly conducted on autologous melanoma cell lines, but allogeneic melanoma cell lines were also used by some authors (249, 251). To overcome the limitation of generating tumor cell lines for gene transduction, the use of gene-transduced fibroblasts admixed with irradiated melanoma cells, has also been proposed (250). During the last decade, several Phase I/II clinical trials with genetically modified cells were initiated in patients with advanced metastatic melanoma. These vaccines are well tolerated. Occasional tumor responses were reported (*Table 23*). Further studies are needed to determine the real impact of genetically modified vaccines on melanoma treatment (264, 265).

IV. 1.1.4 Therapeutic efficacy of melanoma cell-based vaccines

Vaccines derived from autologous or allogeneic whole cells, cell extracts, or from genetically modified melanoma cells were tested in patients with stage III or IV detectable cutaneous melanoma. Five hundred and fifty nine patients were enrolled in the 21 selected trials presented in *Table 23* and they received at least one vaccination according to the various protocols modalities. Three hundred and eighty eight patients had tumor progression (69%). Objective tumor responses (CR or PR) were observed in 58 patients (10%) and MxR or MR were observed in 31 additional patients (6%). Disease stabilization, usually lasting less than 6 months, was observed in 82 patients (15%). In 15 of these trials (71%), the presentation of clinical data allows to conclude that objective tumor responses were more frequent in patients with non-visceral metastases only ($25/179 = 14\%$) than in patients who had detected visceral metastases at study entry ($10/192 = 5\%$) (*Table 28*).

IV. 1.2 Vaccination with defined antigens recognized by T lymphocytes

Immunization with T-cell defined antigens is currently being explored in human clinical trials by several groups. Vaccines include peptides presented by HLA class I or class II molecules, proteins given alone or mixed with immunological adjuvants or cytokines, recombinant viral or bacterial vectors, dendritic cells, or DNA encoding the antigen. In this Section, we will focus on vaccinations with peptides, proteins and dendritic cells (*Tables 24 to 26*).

IV. 1.2.1 Vaccination with peptides

Vaccines with peptides encoded by cancer-germ line genes or differentiation genes of the melanocytic lineage, most of them presented by HLA class I molecules, have been tested in patients with detectable stage III or IV cutaneous melanoma. Five hundred and nine patients were enrolled in the 25 trials presented in *Table 24* (190, 197, 232, 266-280), including 8 trials performed by our group. Patients received at least one vaccination according to the relevant protocol modalities. Except for 2 trials where 13/31 (42%) and 6/16 (38%) patients who received a gp100.A2 peptide mixed with Montanide ISA 51 and combined with i.v. perfusions of high-dose IL-2 had an objective tumor response, respectively, the therapeutic efficacy was low (*Table 24*) (275, 276).

Table 24 Vaccination with peptides in patients with detectable melanoma metastases

Antigens			Cytokine, adjuvant or other peptide	Injection route ¹	Patients enrolled ² (n)	Tumor response ³ according to maximal extent of disease ⁴		Trials (n) (References)
encoded by <i>gene</i>	amino acids	presented by HLA-				non-visceral	visceral	
Antigens encoded by cancer-germ line genes								
MAGE-3	168–176	A1	none	i.d. / s.c.	39	2CR, 1PR, 3MxR / 23	1CR / 16	(197)
MAGE-3	168–176	A1	MPL ⁵ / QS21 ⁶ (SB-AS1)	i.m.	7	0 / 3	1SD / 4	Marchand, LUD 95–002*
MAGE-3 & MAGE-1	168–176 161–169	A1	none	i.d. / s.c.	17	1MxR, 1SD / 6	1MxR / 11	van Baren, LUD 96–007*
MAGE-3	168–176	A1	none	i.d. / s.c.	45	1MxR, 1SD / 14	2MxR / 18	van Baren, LUD 97–004*
	243–258	DP4				0 / 2	0 / 3	
		both				1SD / 8		
MAGE-1	161–169	A1	none	s.c.	4	0 / 3	0 / 1	Marchand, LB 93–001*
MAGE-1	230–238	Cw*1601	none	s.c.	2	0 / 1	0 / 1	Marchand, LUD 94–002*
MAGE-3	271–279 FLWGPRLV	A2	none	i.d. / s.c.	27	1MxR, 2SD / 14	1MxR / 13	Weynants, LUD 95–004*
MAGE-4 & MAGE-10	230–239 254–262	A2	none	i.d. / s.c.	5	0 / 1	1SD / 2	van Baren, LUD 99–006*
	IL-12		0 / 2					
MAGE-12	170–178	Cw*0702	IFA ⁷	s.c.	9	1PR / 4	0 / 3 0 / 2	(266)
NY-ESO-1 / LAGE-2	157–167, 157–165 155–163	A2	GM-CSF ⁸	i.d.	9	7SD / 7	1SD / 2	(267)
NY-ESO-1	157–165 ana. C165V	A2	IFA	s.c.	37	0 / 19		(268)
	161–180	DP4				1 PR (visceral) / 7		
		both				0 / 11		
GnT- V ⁹	38–46 38–47	A2	none	i.d. / s.c.	10	1CR, 2PR / 10		(232)
SUBTOTAL					211	9OR, 10MxR or MR, 15SD		12
Antigens encoded by differentiation genes of the melanocytic lineage								
Melan-A / MART-1	27–35	A2	IFA	s.c.	23	3MR / 23		(269)
Melan-A / MART-1	26–35 EAAGIGILTV	A2	IL-12 + Flu.A2 (58–66)	i.d.	28	1CR, 1MxR, 2SD / 6	1PR, 2MxR, 4SD / 22	(270)
Melan-A / MART-1	26–35 or 26–35 analogue (A27L) ELAGIGILTV	A2	none + Flu.A2 (58–66)	s.c.	24	1SD / 3		(271)
			MPL/QS21 (AS02B) + Flu.A2			2SD / 6	1MxR / 4	
			IFA + Flu.A2			2SD / 4	0 / 1	
			AS02B + low-dose IL-2			0 / 4		
			IFA + low-dose IL-2			0 / 1	0 / 1	
Melan-A / MART-1	26–35 analogue (A27L)	A2	IFA + CpG 7909	s.c.	5	1SD / 5		(190)
tyrosinase	243–251, 369–377, 206–214, 192–200	A1, A2 A24, B44	GM-CSF	i.d.	15	0 / 7	1MxR, 1SD / 8	(272)
tyrosinase	369–377	A2	QS21	i.d.	7		0 / 7	(273)
gp100	154–162, 209–217, 280–288	A2	IFA	s.c.	28	1OR / 28		(274)
gp100	209–217 native	A2	IFA	s.c.	51	1OR / 9		(275)
	209–217 analogue IMDQVPFSY		3MxR / 11					
	(T210M) "g209–2M"		13OR, 4MxR, 3SD / 31 including 4PR 1CR, 8PR					
gp100	209–217 analogue (T210M)	A2	IFA	s.c.	54	0 / 11		(276)
			IFA + high-dose IL-2			6OR / 16		
			IFA + GM-CSF			0 / 13		
			IFA + IL-12			0 / 14		
Melan-A / MART-1 gp100	26–35 (A27L) 209–217 (T210M)	A2	IFA	s.c.	41	0 / 22		(277)
tyrosinase gp100	240–251, 369–377 280–288, 17–25	A1, A2 A2, A3	IFA + gp100.DR4 (44–59)	i.d. / s.c.	13	1CR (visceral) / 19		(278)
			IFA + GM-CSF + low IL-2 + tetanus helper peptide			2PR, 2SD / 13 3 10		
Melan-A / MART-1 tyrosinase gp100	27–35, 26–35 1–9, 369–377 280–288, 457–466	A2	none	i.d.	6	2SD / 3	0 / 3	(279)
Melan-A / MART-1 tyrosinase gp100	26–35 1–9, 369–377 280–288, 457–466	A2	GM-CSF	i.d.	3	1CR, 1PR / 2	1PR / 1	(280)
SUBTOTAL					298	29OR, 15MxR or MR, 20SD		13
TOTAL					509	38 OR, 25 MxR or MR, 35 SD		25

Legend to Table 24

* Unpublished observation

1) *Antigen Injection route*: i.d. = intradermal, s.c. = subcutaneous, i.m. = intramuscular.

2) Patients having received at least 1 vaccination.

3) *Tumor response*: CR = Complete Response, PR = Partial Response, OR = Objective Response = CR and / or PR
MR = Minor Response, MxR = Mixed response, SD = Stable Disease.
The other patients had PD = Progressive disease.

4) *Melanoma metastases*: non-visceral : American Joint Committee on Cancer 2002 stages III or IV M1a;
visceral : AJCC IV M1b or M1c.

5) *MPL* = Monophosphoryl lipid A.

6) *QS21* = a *Quillaja* saponin.

7) *IFA* = Incomplete Freund's Adjuvant.

8) *GM-CSF* = Granulocyte-Macrophage Colony Stimulating Factor.

9) *GnT-V* = N-acetylglucosaminyltransferase V gene codes for "NA17" peptides.

Four hundred and eleven patients had tumor progression (81%). Objective tumor responses (CR or PR) were observed in 38 patients (7%) and some tumor regression (MxR or MR) was observed in 25 additional patients (5%). Disease stabilization, was observed in 35 patients (7%). Appropriate clinical data obtained from 17 of these trials (68%) indicate that objective tumor response rates were higher in patients with non-visceral metastases only (10/132 = 8%) than in patients who had detectable visceral metastases at study entry (3/123 = 2%) (Table 28).

IV. 1.2.2 Vaccination with proteins

So far, only vaccines with a recombinant MAGE-3 protein with or without immunological adjuvant were performed in patients with detectable stage III or IV cutaneous melanoma [(198, 210, 281) and *Section III 2.3*]. Ninety one patients were enrolled in the 3 trials presented in Table 25 (198, 210, 281), 2 of which were performed by our group. Patients received at least one vaccination according to the relevant protocol modalities. Seventy eight patients had tumor progression (86%). Objective tumor responses (PR only) were observed in 3 patients (3%) and MxR were observed in 7 patients (8%). Disease stabilization was observed in 3 patients (3%). Appropriate clinical data obtained from 2 trials out of 3 indicate that objective tumor response rates were higher in patients with non-visceral metastases only (3/41 = 7%) than in patients who had visceral metastases detected at study entry (0/38 = 0%) (Table 28).

IV. 1.2.3 Dendritic cell-based vaccines

Dendritic cells (DC) are the most potent antigen-presenting cells known. They are specialized for the initiation of T-cell immunity, resulting in appropriate host rejection of virus-infected as well as malignant tumor cells. They are capable to capture, process and present antigens to naive and primed T-cells. Importantly, they are the only cells able of inducing primary responses of both helper and cytotoxic T lymphocytes, because they express the appropriate costimulatory signals, secrete cytokines, including IL-12 and have the unique ability to present exogenous antigens in the context of HLA class I, a process known as cross-presentation. Immunization with antigen-loaded or -transfected DCs efficiently primes CD4⁺ and CD8⁺ T cells in animal models, resulting in protective immunity against infectious agents and tumors. Antigen presentation by DC not only initiates adaptive immune responses, but also stimulates natural killer cells, the effectors of innate immunity. They thus act as “nature's adjuvant”, both *in vitro* and *in vivo*. These features have prompted their use for the development of several therapeutic applications, including cancer vaccines.

In 1996, Hsu and colleagues used autologous DCs from the peripheral blood of patients with B-cell lymphoma (282). DCs were pulsed with idiotypic proteins expressed on their tumor's cell surface. This treatment induced complete responses in 2/4 patients and all patients experienced a tumor regression without evidence of toxicity. Importantly, the capacity of DC to initiate CD8⁺ T

Table 25 **Vaccination with a recombinant MAGE-3 protein in patients with detectable melanoma metastases**

Type of vaccine dose of protein / vaccination	Adjuvant ¹ or peptides	Injection route ²	Patients enrolled ³ (n)	Tumor response ⁴ according to maximal extent of disease ⁵		Trials (n) (References)
				non-visceral	visceral	
MAGE-3 protein alone						
300 µg	none (saline)	i.m.	2	1PR / 1	0 / 1	(198)
		i.d. / s.c.	30	1PR, 3MxR / 29	1MxR / 1	(210)
MAGE-3 protein mixed with an immunological adjuvant						
30, 100 or 300 µg	AS02B	i.m.	47	1PR, 2MxR, 1SD / 11	0 / 36	(198)
300 µg	CpG 7909 (0.5 or 1 mg)	i.m.	12	1MxR, 2SD		(281)
TOTAL			91	3 OR, 7 MxR, 3 SD		3

1) *Adjuvant*: AS02B contains MPL and QS21 in an oil-in-water emulsion

CpG 7909 = synthetic, single stranded oligodeoxyribonucleotide optimized for human use;

2) *Antigen Injection route*: i.d. = intradermal, s.c. = subcutaneous, i.m. = intramuscular.

3) *Patients* having received at least 1 vaccination.

4) *Tumor response*: PR = Partial Response, OR = Objective Response = CR and / or PR
MxR = Mixed response, SD = Stable Disease. The other patients had progressive disease.

5) *Melanoma metastases*: non-visceral: American Joint Committee on Cancer 2002 stages III or IV M1a;
visceral: IV M1b or M1c.

cell responses greatly depends on their maturation status. Immature DC are weak immunogens and can be tolerogenic. In healthy subjects, vaccination with DC loaded with an Influenza peptide was shown to induce specific CTL response, provided the DC were appropriately matured and activated (283, 284). Likewise, in patients with metastatic melanoma, intranodal injection of immature DCs (iDC) loaded with 1 tumor peptide and 1 recall antigen, did not lead to significant immune responses, contrary to the intranodal injection of mature DC loaded with another tumor peptide and a different recall antigen, in the same patient (*Table 26*) (40, 278, 285-303). The protocols used to generate DC for vaccination vary considerably, depending on how the DC are collected and grown *in vitro* and on the source of tumor antigens used for loading, transfection, or transduction. DC can be obtained either from GM-CSF-amplified bone-marrow CD34⁺ progenitors, or from non-proliferating peripheral blood monocytes, the latter being the most commonly used. They are collected by leukapheresis and amplified *in vitro* in the presence of GM-CSF and IL-4 to obtain immature DC. In some protocols, DC are then matured using various pro-inflammatory cytokines or pathogen-associated molecules. Finally, they are loaded with tumor cell-lysates or peptides before being reinfused to patients (*Table 26*). In some studies, DC were also loaded with helper-proteins or -peptides of various origin, that provide a stimulation of the CD4⁺ T cell response in the absence of known, tumor-specific epitopes presented by HLA class II molecules and also serve as 'positive control' for the monitoring of immune responses. Tumor cell-lysates loaded on DC are presented by both MHC class I and class II pathways and can elicit both CD8⁺ and CD4⁺ T cells responses. Because many tumor antigens are mutated, patient-specific antigens, the use of DC loaded with autologous tumor cell lysates should allow the presentation of a broad spectrum of tumor antigens relevant to each individual patient (292, 293, 299-301). On the other hand, loading DC with peptides makes the monitoring of the anti vaccine T lymphocyte response much easier (*Table 26*). DC can also be genetically engineered to provide constitutive, high level of tumor antigen expression, or to introduce genes that further enhance their immune stimulatory ability (304). Gene transfer to DC can be achieved using various viral and non-viral systems. Transfection of RNA or DNA alone (naked) or coated with liposomes, using electroporation or gene guns, can lead to T cell activation (302, 305, 306). DC transduction of recombinant viral vectors derived from various viruses including adeno-, retro-, pox-, or lentiviruses can also lead to foreign transgene expression and to immune cell activation (141, 150, 305, 307, 308). Recently, membrane vesicles derived from DC, such as exosomes, have also been used for the development of cancer vaccines. Exosomes are small membrane vesicles of endosomal origin, measuring 50 to 100 nm in diameter, secreted by many cell types (309). DC-derived exosomes, known as dexosomes, contain all the antigen presenting molecules required for peptide presentation, i.e. MHC class I and class II molecules, costimulatory- and adhesion molecules. They can activate both adaptive and innate immune responses. In mice, they are capable of

Table 26 Dendritic Cell-based vaccines in patients with detectable melanoma metastases

Type of vaccine Source of tumor antigen(s)	Cytokines ¹ or helper antigens ²	Injection route ³	Patients enrolled ⁴ (n)	Tumor response ⁵ according to maximal extent of disease ⁶		Trials (n) (References)
non-visceral visceral						
Dendritic cells loaded with peptides						
MAGE-3 HLA-A1 (1)	TNF- α ¹ Tetanus Toxoid	i.d. / s.c., then i.v.	13	0 / 1	6MxR / 12	(285)
MAGE-3, MAGE-1 HLA-A1 (2), -A2 (1), -B44 (1)	iDC $\pm$ KLH	i.v. / s.c.	9	1CR, 1SD / 3	1MxR, 2SD / 6	(286)
gp100, Tyrosinase HLA-A2 (2)	iDC	i.v.	16	0 / 6	1CR, 2MxR, 2MR / 10	(287)
gp100 (154–167, 280–288) or gp100 (154–167 Q -> A, 280–288 A -> V) and Tyrosinase (369–376) HLA-A2 (5)	iDC + KLH	i.v. / i.d.	9	0 / 2	0 / 7	(288)
	TNF- α , PGE2 ¹ KLH		10	1SD / 1	1PR, 1MxR, 2SD / 9	
gp100, Tyrosinase HLA-A1 (1), -A2 (2), -A3 (1)	iDC Tetanus helper peptide; IL-2	i.d. / s.c.	13	1PR, 1SD / 3 / 10		(210)
MAGE-1 or Melan-A or Tyrosinase HLA-A1 (2), -A2 (2)	iDC $\pm$ IL-1 β , IL-6, TNF- α , PGE2 ¹ TT or Tuberculin ²	i.l.	11	1MxR, 1SD	2MxR, 1SD	(289)
MAGE-3, Melan-A, gp100, Tyrosinase HLA-A2 (4)	TNF- α KLH, FLU MP.A2 ²	s.c.	17	3CR, 1PR, / 6	1MR, 5SD, / 11	(290)
	TNF- α , IFN- α ¹ $\pm$ KLH, + FLU MP.A2	s.c.	19	1SD / 10 / 9		(291)
MAGE-3, Melan-A, gp100, Tyrosinase HLA-A2 (4)	iDC + KLH FLU MP.A2	i.l.	14	0 / 1	5SD / 13	(292)
MAGE-3, -1, Melan-A, gp100, Tyrosinase HLA-A1 (2), -A2 (4)	iDC + KLH	i.l.	12	1CR / 3	2PR, 1MR / 9	(293)
MAGE-3, -1, Melan-A, gp100, Tyrosinase HLA-A1 (2), -A2 (4)	TNF- α	i.v.	13	1MR, 2SD / 4	4SD / 9	(294)
MAGE-3, -1, -2, Melan-A, gp100, Tyrosinase HLA-A2 (5), -A24 (5)	iDC + KLH	s.c.	9	1CR, 1SD / 2	1PR / 7	(295)
MAGE-3, GnTV HLA Class I (1) : -A2 HLA Class II (1) : -DP4	INF- β , IL-3, poly I:C ¹	i.d. / s.c.	4		1SD / 4	(296)
gp100, Tyrosinase (idem de Vries, 2003) HLA Class I (5) : -A2 $\pm$ HLA Class II (2) : -DR4	TNF- α , PGE2 KLH	i.v. / i.d. or i.l. (6)	26	1CR, 10SD		(297)
MAGE-3, -1, -4, -10, GnTV, Melan-A, gp100, Tyrosinase HLA Class I (10) : -A1 (3), -A2 (6), -A3 (1) HLA Class II (4) : -DP4 (1), -DR4 (2), -DR13 (1)	TNF- α KLH FLU NPA1, FLU NPA3, FLU MP.A2	s.c.	24	5SD / 10	1CR, 3SD / 14	(298)
MAGE-3, -1, GnTV, Melan-A, gp100, Tyr. HLA Class I (16) : -A1 (3), -A2 (5), -A3 (2), -A24 (4), -B44 (2) HLA Class II (3) : -DR4 (2), -DR13 (1)	TNF- α , IL-1 β , IL-6, PGE2 FLU NPA1, -A3, FLU MP.A2	s.c.	47	2PR, 8SD / 4 / 43		(40)
SUBTOTAL			266	17OR, 18MxR or MR, 54SD		16
Dendritic cells loaded with melanoma cells lysates						
Lysates from autologous melanoma cells	iDC + KLH ²	i.l.	4	1PR / 2	1CR / 2	(293)
	iDC + KLH	i.d.	17	1PR, 1MR / 4	2SD / 7	(299)
	MCM ¹	i.d.	17	1CR, 2PR / 4	2CR, 1PR / 13	(300)
	iDC + KLH	i.l.	19	0 / 2	3PR, 1MxR, 4SD / 17	(292)
Lysate from an allogeneic melanoma cell line	IL-13 ¹ HBs prot. ² , Tetanus Tox. ²	i.d., s.c., and i.l.	15	1CR / 6	1SD / 9	(301)
SUBTOTAL			72	13OR, 2MxR or MR, 7SD		5

(continued on next page)

Table 26 (continued)

Dendritic Cell-based vaccines in patients with detectable melanoma metastases

Type of vaccine Source of tumor antigen(s)	Cytokines ¹ or helper antigens ²	Injection route ³	Patients enrolled ⁴ (n)	Tumor response ⁵ according to maximal extent of disease ⁶		Trials (n) (References)
				non-visceral	visceral	
Genetically modified Dendritic cells						
Transfection of total mRNA from autologous melanoma cells	TNF-α, IL-1β, IL-6, PGE2	i.d. or i.l.	20	0 / 2	1MxR, 1SD / 18	(302)
Dendritic-cell derived vesicles						
Exosomes presenting MAGE-3.A1/B35 and MAGE3.DP4	Tetanus helper peptide	i.d. / s.c.	15	1PR, 1MR, 2SD / 9	1MxR / 6	(303)
TOTAL			373	31 OR, 23 MxR or MR, 64 SD		23

1) In most of the studies, GM-CSF and IL-4 were first used to obtain immature DC (iDC). Pro-inflammatory cytokines or pathogen-associated molecules shown here were used in some studies to induce DC maturation: *IFN- α* = Interferon- α , *MCM* = Monocyte conditioned medium, *PGE2* = Prostaglandin E2, *poly I:C* = Polyriboninosinic polyribocytidylic acid, a double stranded synthetic ribonucleic acid that mimics viral RNA, *TNF- α* = Tumor necrosis factor- α .

2) Helper proteins or recall antigens loaded on DC : *HBs* = Hepatitis B virus surface protein, *FLU* = Influenza peptides; *KLH* = Kehole limpet hemocyanin; *TT* = Tetanus Toxoid; Tuberculin.

3) *Injection route*: i.d. = intradermal, i.l. = intralymphatic, i.v. = intravenous, s.c. = subcutaneous.

4) Patients who received at least 1 vaccination.

5) *Tumor response*: CR = Complete Response, PR = Partial Response, OR = Objective Response = CR and / or PR, MR = Minor Response, MxR = Mixed response, SD = Stable Disease. The other patients had progressive disease.

6) *Melanoma metastases*: non-visceral : American Joint Committee on Cancer 2002 stage III or IV M1a; visceral : AJCC 2002 stage IV M1b or M1c.

priming cytotoxic T cells and were found to mediate rejection of tumors expressing the relevant antigens in the presence of adjuvants (310). A first phase I clinical trial using autologous exosomes pulsed with *MAGE-3* encoded peptides for the immunization of 15 stage III/IV melanoma patients recently highlighted the feasibility of large scale exosome production and the safety of their administration (*Table 26*) (303). Fusion of dendritic cells with autologous tumor cells to obtain cell hybrids called “dendritomas” is described in *Section IV. 1.1.1.2* and in *Table 23*. As it is the case for any autologous cell-based vaccine, the limiting factors to the use DC vaccines are high costs and cumbersome, time-consuming preparation procedures.

Since 1998, several phase I/II clinical trials of immunotherapy with DC-based vaccines were performed in stage III or IV melanoma patients with detectable disease (*Table 26*). Most of them used autologous DC pulsed either with one or several peptides presented by HLA class I or class II molecules, or with autologous tumor lysates (*Table 26*). In 1999, a multicenter, prospective, randomized, open-label phase III trial was designed to compare autologous peptide-loaded DC vaccination with standard dacarbazine (DTIC) chemotherapy in stage IV melanoma patients. DTIC 850 mg/m² was administered i.v. at 4-week intervals (Arm A). DC vaccines loaded with MHC class I and II-restricted peptides were applied s.c. at 2-week intervals for the first 5 vaccinations and every 4 weeks thereafter (Arm B). For patients randomized to the DC vaccination arm, a panel of 19 peptides was used to load their matured DC, each corresponding to an antigen putatively expressed by their tumor, as assessed by HLA typing only. The 19 selected peptides were presented by HLA-A1, -A2, -A3, -A24, -B44, DR4 and DR13 (*Table 26*). The planned accrual per arm was set at 95 patients under the assumption that the response rate would double from 15% for treatment with DTIC to 30% for DC-vaccination to reach significance at 0.05 with 80% power. Accrual started in March 2000 and in December 2003, by the time of the first interim analysis, 55 patients had been enrolled into the DTIC- and 53 into the DC-arm, including 47 who had received at least 1 vaccination. No CR was observed in any of the 2 treatment arms and according to the intention-to-treat analysis, objective response rates (PR only) were low in both treatment arms (DTIC: 5.5%, DC: 3.8%), but not significantly different. Based on these results, the study was prematurely closed to accrual, because of an extremely low probability of reaching the planned study goals. In August 2004, at the time of the final survival data analysis, the median follow-up time was 22.2 months and a total of 75 deaths had occurred. No significant difference in overall survival or in progression-free survival was shown between the 2 arms (40).

IV. 1.2.3.1 Therapeutic efficacy of dendritic cell-based vaccines

DC-based vaccines were tested in patients with detectable stage III or IV cutaneous melanoma. Three hundred and seventy three patients were enrolled in the 23 trials presented in *Table 26*. Patients received at least one vaccination according to the relevant protocol modalities. Two hundred and fifty five patients had tumor progression (69%). Objective tumor responses (CR or PR) were observed in

31 patients (8%) and some tumor regression (MxR or MR) was observed in 23 additional patients (6%). Disease stabilization was observed in 64 patients (17%). Appropriate clinical data obtained from 19 of these trials (83%) indicate that objective tumor response rates were higher in patients with non-visceral metastases only ($14/68 = 21\%$) than in patients who had visceral metastases detected at study entry ($13/183 = 7\%$) (Table 28).

IV. 1.3 Adoptive transfer of T lymphocytes

Adoptive therapy involves the transfer of *ex vivo* expanded immune effector cells to a tumor-bearing host as a means of passively augmenting the antitumor immune response. In selected animal-tumor models, the adoptive transfer of T lymphocytes with antitumor reactivity can mediate the regression of established primary tumors as well as metastases (76, 84, 311, 312). The transfer of activated antitumor lymphocytes to cancer patients has several theoretical advantages compared to the active immunization approaches described above. It can indeed by-pass several tumor- or lymphocyte- related mechanisms that could contribute to tumor escape from immune destruction (313). However, it does not generate any memory T lymphocytes and is therefore also called “passive” immunotherapy, as opposed to the active immunization approaches previously described that are capable to generate both cytolytic- and memory-T cell responses. Lymphocytes for adoptive transfer can be obtained either from PBMC or from tumor-infiltrating lymphocytes (TIL) and can be reinfused to patients either alone, or in combination with other anticancer treatment. T lymphocytes obtained from PBMC or from TIL populations appear to have an equivalent capacity to migrate to tumors (314). Heterogeneous populations or cloned T cells with defined characteristics can be used. Prior to reinfusion, the lymphocytes can be activated *in vitro* with cytokines to exhibit antitumor effector function, or they can be genetically modified to express cytokines such as IL-2, tumor antigen-specific TCRs or antiapoptotic molecules (315, 316). After activation of the T cells *ex vivo*, some cell subpopulations can be selected by *in vitro* testing of their antitumor properties, before being reinfused to the patients. Culture conditions of TIL clones can be optimized *in vitro* to achieve rapid and efficient expansion of functional T cells, in order to obtain the large numbers of selected T lymphocytes required for intravenous infusion (317).

IV. 1.3.1 Lymphokine-activated killer cells

In 1982, Rosenberg and colleagues developed a method for generating cells capable of lysing fresh autologous tumor, by incubating human PBMC with IL-2 (318). These lymphocytes, called lymphokine-activated killer (LAK) cells are different from CTL and NK cells. They are derived from a subpopulation of “null” lymphocytes that bear neither B-cell nor T-cell surface markers and constitute about 5% of all circulating lymphocytes. They may be a part of the innate immune mechanisms aimed at eliminating altered cells such as cancer cells nonspecifically, without having

Table 27

Adoptive transfer of T Lymphocytes in patients with detectable melanoma metastases

Type of vaccine ¹	Combined treatment ²	Injection route ³	Patients enrolled ⁴ (n)	Tumor response ⁵ according to maximal extent of disease ⁶		Trials (n) (References)
				non-visceral	visceral	
<i>Lymphokine-activated killer cells (LAK) with IL-2</i>						
IL-2-activated LAK cells	IL-2 i.v. bolus, intermediate-dose	i.v.	48	4CR, 6PR		(319, 320)
<i>Tumor infiltrating lymphocytes (TIL) with or without IL-2:</i>						
<i>• Heterogeneous populations containing both CD8+ and CD4+ T cells</i>						
IL-2-activated TIL	IL-2 continuous infusion, intermediate-dose + Cyclophosphamide	i.v.	21	1CR, 4PR, 1MR, 6SD		(321)
	IL-2 i.v. bolus, high-dose	i.v.	29	4CR, 5PR		(322, 323)
	IL-2 bolus, high-dose + Cyclophosphamide		57	1CR, 19PR		
		none	i.v.	9	4MR, 1SD	
Anti-CD3-activated TIL	IL-2 i.v. bolus, intermediate-dose	i.v.	18	2CR / 12	1CR / 6	(325)
TIL selected <i>in vitro</i> for their high avidity for Tyrosinase.A2 (AA 369–377)	none	i.v.	10	1PR / 3	1MxR / 7	(326)
<i>• Cytolytic T cell clones obtained from TIL or PBMC</i>						
Anti-gp100.A2 (T210M) (PBMC)	none	i.v.	1		0 / 1	(327)
	IL-2 intermediate-dose s.c.	i.v.	6		0 / 6	
	IL-2 high-dose i.v.	i.v.	6	1MR / 4	1MxR / 2	
Anti-gp100.A2 or Melan-A.A2	IL-2 i.v., low-dose	i.v.	10	2SD / 2	1MxR, 2MR, 3SD / 8	(328)
<i>Adoptive transfer of TIL preceded by lymphodepleting chemotherapy (Cyclophosphamide + Fludarabine)</i>						
Anti-gp100.A2 or Melan-A.A2 T cell clones	none	i.v.	6		0 / 6	(329)
	IL-2 intermediate-dose i.v.	i.v.	3	1MxR, 1MR / 2	0 / 1	
	IL-2 high-dose i.v.	i.v.	6	1MxR, 1MR / 2	1MxR / 4	
Heterogeneous populations of TIL selected for their high avidity for tumor antigens (gp100.A2, Melan-A.A2, other)	IL-2 high-dose i.v.	i.v.	13	5PR, 1MxR / 8	1PR, 3MxR / 5	(330)
	IL-2 high-dose i.v.	i.v.	35	1CR, 6PR	2CR, 9PR	(331)
TOTAL			278			12

1) Type of vaccine: PBMC = peripheral blood mononuclear cells

2) IL-2 = Interleukin-2, high-dose: 720.000 IU/kg every 8 hours to a maximum of 5 days;
intermediate-dose range: 10.000 to 125.000 IU/kg every 8 hours to a maximum of 4 days;
low-dose range: 0.25 to 2 x 10⁶ IU/m²/d for 2 weeks.

3) Cell Injection route: i.v. = intravenous.

4) Patients who received at least 1 infusion of lymphocytes.

5) Tumor response: CR = Complete Response, PR = Partial Response, OR = Objective Response = CR and / or PR,
MR = Minor Response, MxR = Mixed response, SD = Stable Disease. The other patients had progressive disease.

6) Melanoma metastases: non-visceral: American Joint Committee on Cancer 2002 stage III or IV M1a; visceral: stage IV M1b or M1c.

first to recognize defined antigens. Adoptive transfer of autologous LAK cell in conjunction with intermediate-dose IL-2 (10,000 to 100,000 IU/kg, every 8 hours for 4 days per treatment cycle) in 48 patients with advanced melanoma was capable of mediating objective tumor responses in 10 patients (*Table 27*) (319-331). Severe fluid retention related to the use of IL-2 was the major side effect of therapy. Objective response rates observed with LAK + IL-2 (21%) were a little higher than those observed in a series of 270 patients with metastatic melanoma that were treated with high-dose IL-2 alone (720,000 IU/kg, every 8 hours), i.e. 16% (47). Because LAK cells did not appear to home to the tumor sites, further attempts to develop efficient adoptive transfer immunotherapy were focused on tumor-infiltrating lymphocytes (TIL) for their capacity to migrate to tumors and because they were supposed to be enriched in antitumor effector cells.

IV. 1.3.2 Tumor infiltrating T lymphocytes

The adoptive transfer of tumor infiltrating lymphocytes (TIL) expanded in IL-2 to mice bearing various types of tumors showed that TIL have 50 to 100 times more therapeutic efficiency than LAK, when combined with IL-2 and/or cyclophosphamide (312). These experiments provided a rationale for the substitution of LAK cells with TIL in the treatment of patients with advanced cancer, including cutaneous melanoma. Eighty-six patients with metastatic melanoma were treated with autologous TILs plus high-dose i.v. bolus IL-2 (720,000 IU/kg, every 8 hours), with or without the concomitant administration of low-dose cyclophosphamide (25mg/kg, single i.v. dose, 3 days before receiving the first i.v. infusion of TIL plus IL-2 (*Table 27*) (322, 323). IL-2 administration was pursued for a maximum of 5 days per treatment cycle, until grade 3 or 4 toxicity was reached that could not easily be reversed by standard supportive measures. The overall objective response rate in these 86 patients was 34% and was similar in patients receiving TIL and IL-2 alone (31%), or in conjunction with cyclophosphamide (35%). It thus appeared that in patients with metastatic melanoma, this treatment produced higher response rates (34%) than those achieved with LAK plus IL-2 (21%), or with IL-2 alone (16%) (47, 320, 323).

At the beginning of the nineties, the identification of tumor antigens recognized by autologous T cells led to the prospect of treating cancer by adoptive transfer of lymphocytes with high levels of specific activity against defined antigens, that have been first manipulated *in vitro* (332-334). Adoptive transfer of T lymphocytes with high avidity for defined tumor antigens, combined with administration of IL-2, did not only generate anti-tumor immunity and tumor responses, but also mediated immune destruction of normal melanocytes through recognition of shared target antigens, leading to vitiligo and uveitis (335).

IV. 1.3.3 Adoptive transfer of T lymphocytes preceded by lymphodepleting chemotherapy

Because the CD4⁺CD25⁺ T lymphocytes or T regulatory (Treg) cells can suppress the development of appropriate antitumor immune responses after the delivery of an immunotherapeutic agent, the success of cancer immunotherapy may depend on the balance between regulatory and effector T cells (336). In order to shift this equilibrium toward effector T cells, various nonmyeloablative, lymphodepleting chemotherapeutic agents including low-dose cyclophosphamide and fludarabine, have been used prior to adoptive transfer of autologous antitumor lymphocytes. The objective of these trials was to infuse activated antitumor lymphocytes into an appropriate host environment depleted of regulatory T cells. Objective clinical responses have been observed in approximately 50% of patients who received cyclophosphamide (60 mg/kg per day for 2 days) followed by fludarabine (25 mg/m² per day for 5 days) before adoptive transfer of melanoma infiltrating autologous T lymphocytes (*Table 27*) (330, 331, 337). In some patients, these combined treatments lead to the regression of large, vascularized melanoma metastases and the use of heterogeneous TIL populations appeared to be more efficient than that of T cell clones (*Table 27*) (327-331, 337). Tumor regression was correlated with anti-melanocyte autoimmunity (330). In a study carried out on a panel of 25 patients, clinical responses correlated with the *in vivo* persistence of transferred T cells following adoptive transfer (338). Likewise, expression of the costimulatory molecule CD28⁺ and telomere length of transferred lymphocytes correlated with *in vivo* persistence of transferred T cells and with tumor regression, suggesting that the proliferating and differentiation potential of transferred T cells may play a significant role in mediating response to adoptive immunotherapy (339). In addition to vitiligo (37%) and uveitis (14%), toxicities of treatment included hematologic toxicities, mainly anemia (94%), febrile neutropenia (37%), thrombocytopenia (83%) and lymphopenia (100%); toxicities related to high-dose IL-2, opportunistic infections (18%) and Epstein-Barr virus-related lymphomas (3%) (330, 331).

IV. 1.3.4 Therapeutic efficacy of T lymphocyte adoptive transfer

Adoptive transfer of lymphocytes was tested in patients with detectable stage III or IV cutaneous melanoma. Two hundred and seventy eight patients were enrolled in the 12 trials presented in *Table 27*. Patients received at least one treatment injection according to the relevant protocol modalities. One hundred and seventy four patients had tumor progression (62%). After treatment with LAK cells plus intermediate-dose IL-2, objective tumor responses (CR or PR) were observed in 10/48 patients (21%) (*Table 28*). Objective response rates reported after adoptive transfer of TILs, with or without concomitant administration of IL-2, appear to vary greatly with the dose of IL-2 received (*Table 28*). Out of the 30 patients who received no concomitant IL-2 or only low-dose IL-2 with TIL, only 1 had an objective tumor response (3%), compared with 8/45 patients (18%) who received intermediate doses and 29/92 (32%) who received high-dose IL-2 infusions. Likewise, when TIL treatment was preceded by lymphodepleting chemotherapy with cyclophosphamide and fludarabine, none of the 9

patients who received TIL without IL-2 had an objective tumor response, whereas 24/54 patients (44%) who received the same treatment combined with i.v. infusions of high-dose IL-2 had an objective response (*Table 28*). Some tumor regression (MxR or MR) was observed in 20 additional patients (7%) and disease stabilization was observed in 12 patients (4%). Appropriate clinical data obtained from 6 of these trials (50%) indicate that objective tumor response rates were higher in patients with non-visceral metastases only (8/33 = 24%) than in patients who had visceral metastases detected at study entry (2/46 = 4%) (*Tables 27 and 28*).

Although immunotherapy in the adjuvant setting is beyond the scope of this discussion, we would like to mention here the promising results of a prospective monocenter phase II/III randomized trial of adoptive transfer of autologous TIL as adjuvant therapy for 88 patients with stage III melanoma (340). TIL were obtained from the regional metastatic lymph nodes clearance performed for clinically palpable recurrence. After complete tumor resection, the 88 patients were equally randomized to receive either 2 i.v. infusions of autologous TIL with low-dose s.c. IL-2 at 1-month interval, or IL-2 alone. IL-2 treatment consisted of 2 cycles of s.c. injections of 6×10^6 IU/m² per day, 5 days per week for 2 weeks, at 1-month interval. For patients who received the combined treatment, TIL infusion took place the first day of each IL-2 cycle. After a median follow-up of 46.9 months, the statistical analysis of the overall patients' population did not show any significant difference in the relapse-free interval or overall survival between the 2 treatment arms. However, in the subgroup of 34 patients with only one regional metastatic lymph node, the estimated relapse-free rate was lower for the patients treated with TIL + IL-2 (5/15) than for the IL-2 alone group (13/19) ($P = 0.019$). However, there was no significant difference in the median overall survival between the 2 groups. The duration of relapse-free survival was further analyzed, taking into account the immunological specificity of injected TIL (341). A total of 40 patients from whom the autologous melanoma cell line was available were included in the statistical analysis. Twenty-seven of them had received TIL + IL-2 and 13 had received IL-2 alone. In the subgroup of patients who had only one metastatic lymph node, Kaplan-Meier analysis revealed that the injection of tumor-reactive TIL was significantly correlated with prolonged relapse-free survival. Recently, the recognition of a panel of 38 shared TAA by TIL infused to the 88 patients included in this clinical trial was analyzed (342). Results show that the infusion of Melan-A/MART-1 reactive TIL appears to be associated with a longer relapse-free survival for HLA-A2 patients. These results suggest that improved clinical outcome could be obtained after adoptive therapy by selecting appropriate groups of patients and monitoring the specificity of the injected TIL populations.

Table 28

Therapeutic efficacy of various modalities of immunotherapy targeting tumor antigens recognized by T lymphocytes, in patients with detectable melanoma metastases

<i>Type of immunotherapy</i> ¹	Selected trials (n)	Patients enrolled ² (n)	Objective Response Rate ³ (%)			Mixed or Minor Responses Total Rate (%)	Stable Disease Total Rate (%)	Progressive Disease Total Rate (%)	Table	
			Total	According to maximal extent of disease at study entry non-visceral ⁴ visceral ⁴						
<i>Active immunotherapy</i>										
Melanoma cell-based vaccines	21	559	10% <i>58 / 599</i>	14% <i>25 / 179</i>	5% <i>10 / 192</i>	6% <i>36 / 599</i>	14% <i>82 / 599</i>	70% <i>423 / 599</i>	23	
Vaccination with peptides	25	509	7% <i>38 / 509</i>	8% <i>10 / 132</i>	2% <i>3 / 123</i>	5% <i>25 / 509</i>	7% <i>35 / 509</i>	81% <i>411 / 509</i>	24	
<i>Vaccination with a recombinant MAGE-3 protein</i>	3	91	3% <i>3 / 91</i>	7% <i>3 / 41</i>	0% <i>0 / 38</i>	8% <i>7 / 91</i>	3% <i>3 / 91</i>	86% <i>78 / 91</i>	25	
Dendritic cell-based vaccines	23	373	8% <i>31 / 373</i>	21% <i>14 / 68</i>	7% <i>13 / 183</i>	6% <i>23 / 373</i>	17% <i>64 / 373</i>	69% <i>255 / 373</i>	26	
<i>Adoptive transfer of T lymphocytes</i>										
• <i>Lymphokine-activated killer cells</i>										
IL-2 intermediate-dose	1	48	21% <i>10 / 48</i>	NK	NK	none	none	79% <i>38 / 48</i>	27	
• <i>Tumor-infiltrating T lymphocytes</i>										
Without IL-2	3	20	5% <i>1 / 20</i>	- <i>1 / 3</i>	- <i>0 / 8</i>	25% <i>5 / 20</i>	5% <i>1 / 20</i>	65% <i>13 / 20</i>		
+ IL-2 low-dose	1	10	- <i>0 / 10</i>	- <i>0 / 2</i>	- <i>0 / 8</i>	- <i>3 / 10</i>	- <i>5 / 10</i>	- <i>2 / 10</i>		
+ IL-2 intermediate-dose	3	45	18% <i>8 / 45</i>	17% <i>2 / 12</i>	8% <i>1 / 12</i>	2% <i>1 / 45</i>	13% <i>6 / 45</i>	67% <i>30 / 45</i>		
+ IL-2 high-dose	3	92	32% <i>29 / 92</i>	- <i>0 / 4</i>	- <i>0 / 2</i>	2% <i>2 / 92</i>	0% <i>0 / 92</i>	66% <i>61 / 92</i>		
• <i>Tumor-infiltrating T lymphocytes preceded by lymphodepleting chemotherapy (Cyclophosphamide + Fludarabine)</i>										
Without IL-2	1	6	- <i>0 / 6</i>	none	- <i>0 / 6</i>	- <i>0 / 6</i>	- <i>0 / 6</i>	- <i>6 / 6</i>		
+ IL-2 intermediate-dose	1	3	- <i>0 / 3</i>	- <i>0 / 2</i>	- <i>0 / 1</i>	- <i>2 / 3</i>	- <i>0 / 3</i>	- <i>1 / 3</i>		
+ IL-2 high-dose	3	54	44% <i>24 / 54</i>	- <i>5 / 10</i>	- <i>1 / 9</i>	13% <i>7 / 54</i>	0% <i>0 / 54</i>	43% <i>23 / 54</i>		

1) IL-2 = Interleukin-2, *high-dose*: 720.000 IU/kg every 8 hours to a maximum of 5 days;
intermediate-dose range: 10.000 to 125.000 IU/kg every 8 hours to a maximum of 4 days;
low-dose range: 0.25 to 2 x 10⁶ IU/m²/d for 2 weeks.

2) Patients who received at least 1 immunotherapy dosis.

3) Objective Response = Complete Response and / or Partial Response; NA = Not appropriate; NK = not known.

4) *Melanoma metastases*: non-visceral : American Joint Committee on Cancer 2002 stage III or IV M1a; visceral : AJCC 2002 stage IV M1b or M1c.

IV. 1.4 Summary comparison of the efficacy of the immunotherapy modalities

In order to compare the therapeutic efficacy of various modalities of immunotherapy targeting antigens recognized by T lymphocytes on cutaneous melanoma, the tumor response of 1,810 patients with detectable metastatic melanoma was reviewed. These patients were enrolled in 88 selected trials over the last 25 years (*Tables 23 to 28*). Five hundred and fifty nine patients received melanoma cell-based vaccines (31%), 509 received vaccinations with defined peptide antigens recognized by T lymphocytes (28%), 91 received a recombinant MAGE-3 protein (5%), 373 had dendritic cell-based vaccines (21%) and 278 (15%) were treated by adoptive transfer of T lymphocytes, with or without concurrent administration of IL-2. All the patients included in this analysis had received at least one treatment injection according to the relevant protocol modalities.

Although this list of trials is not fully exhaustive and as far as one can conclude from the retrospective comparison of multiple studies, most of which were phase I/II non-randomized trials on small numbers of patients, active immunotherapy approaches targeting tumor antigens recognized by T lymphocytes did not generate any therapeutic benefit in the vast majority of patients. Sixty nine to 86% of them had disease progression (*Table 28*). However, therapeutic benefit was observed in a minority of patients. The objective response rates (3 to 10%) and the mixed or minor response rates (5 to 8%) are low and very similar whatever the antigen presentation and the vaccination schedule used (*Tables 23 to 26*). These results are similar to those obtained in our studies of immunization with peptides or with a recombinant MAGE-3 protein, i.e. 3 to 4% for the objective response rates and 7 to 8% for the mixed response rates (*Table 21*). In a recent review entitled “*Cancer immunotherapy: moving beyond current vaccines*”, similar objective response rates were found by Rosenberg and colleagues, after vaccination with peptides or peptide-loaded DC (3%), or recombinant viral vaccination (2%), in 440 patients with metastatic cancers, most of them with cutaneous melanoma, who were treated at the NCI surgery Branch, Bethesda, Maryland, USA, between 1995 and 2004 (343). Whatever the immunotherapy modality, regression of cutaneous or lymph node metastases was more than 2 times more frequent than that of visceral metastases. This observation is reflected by the higher response rates reported in patients with non-visceral metastases at study entry, as compared with patients presenting with visceral metastases (*Table 28*).

Regarding adoptive transfer of T lymphocytes, objective response rates were much higher when TIL were administered with concurrent high-dose IL-2 (32% and 44% for TIL and TIL preceded by lymphodepleting chemotherapy, respectively) (*Table 28 and Section IV. 1.3.4*) (329-331). Likewise, several years ago, Rosenberg and colleagues reported an objective response rate of 40% in 47 patients who were treated with a gp100.A2 peptide mixed with Montanide and who received concurrently high-dose IL-2 (275, 276). Surprisingly enough, this vaccination approach was not pursued further.

IV. 2 CONCLUSION: PRESENT PLACE OF IMMUNOTHERAPY AND POSSIBLE CAUSES OF FAILURE

Our 13-year clinical research program of vaccination with peptide antigens recognized by T lymphocytes on tumors, or with a recombinant MAGE-3 protein, has included 225 patients with detectable regional or distant melanoma metastases (*Table 21*). Objective tumor responses were observed in 4% of patients who completed the study treatment, and an additional 10% of patients had MxR. Thus, 86% of the patients failed to display any tumor regression. However, the development of appropriate methods to monitor CD8⁺ T cell responses has allowed the detection of - usually weak - CTL responses against the vaccine antigenic peptide(s) in a minority of patients and a better understanding of the role of such immune responses in human tumor rejection (205, 221, 344-349). Because peptide antigens associated with MHC class I or class II molecules are the molecular targets for T-cell recognition of tumors, clinical studies using defined peptide antigens offer special opportunities to improve our knowledge of the mechanisms leading to treatment failure by dissecting the host-tumor relationship in blood, lymph nodes and tumor compartments. Improving the efficacy of therapeutic vaccines will critically depend on their capacity to trigger an appropriate immune response and a better understanding of the mechanisms used by tumors to escape immune attack should hopefully lead to the development of a more effective immunotherapy of cutaneous melanoma and other tumors.

The present place of immunotherapy targeting antigens recognized by T-lymphocytes is discussed in *Section IV. 2.1* and the possible causes of treatment failure are discussed in *Section IV. 2.2*.

IV. 2.1 What is the present place of immunotherapy targeting antigens recognized by T-lymphocytes in the treatment of metastatic melanoma?

The 3-10% objective response rates for the active immunotherapy trials described in the previous Section are lower than those initially observed with DTIC alone (10-20%), which still remains the standard treatment of metastatic melanoma, despite the lack of any evidence that it improves overall survival (30) (chap 31, pp 589-604). However, in recent randomized trials, DTIC produced only 6-12% objective responses (37-40).

Because of their low therapeutic efficacy, we consider that immunotherapeutic approaches involving anti-tumor specific T lymphocytes cannot be validated as such for the standard treatment of melanoma and should remain strictly experimental in the near future. As a first step, small clinical trials should privileged a better understanding of immunology and biology of melanoma, as well as its interactions with the microenvironment. Vaccines should be combined with other treatments, as proposed in *Section IV. 3*. Hopefully, this strategy could lead to the development of large randomized phase III trials in the adjuvant setting using selected combination of treatments including vaccines, in

order to determine their impact and their potential place in the treatment of cutaneous melanoma and other types of cancer (340, 350).

Cell transfer may perhaps be considered the most effective approach to immunotherapy so far (351). Despite the major toxicities of such treatment, the promising response rates (up to 51% in one trial) recently obtained with lymphodepleting chemotherapy followed by adoptive transfer of TIL and concurrent administration of high-dose IL-2 deserve confirmation in large Phase III, multicenter, randomized trials (330, 331). Populations of tumor-reactive T cells could not be expanded from all patients and thus these results represent the outcome of a subset of patients. Introducing high-affinity tumor-reactive TCRs into autologous T cells before their reintroduction into the patient may also improve the quality of their antitumor response.

IV. 2.2 Possible causes of treatment failure

Most of the patients with metastatic melanoma spontaneously develop strong anti-tumor CTL responses, even in the absence of treatment. High numbers of anti-tumor CTL precursors can be found both in the blood and in the metastases of these patients (348). However, most of the time, these T cells do not appear to be sufficiently efficient *in vivo* to eradicate the tumor, either because tumor cells have become resistant to the CTL attack, or because of locally produced immunosuppressive substances. The two main possible causes of vaccination failure are the lack of immunogenicity of the vaccine and the capacity of tumor to escape immune attack. They are not mutually exclusive.

IV. 2.2.1 Lack of immunogenicity of the vaccine

IV. 2.2.1.1 Immunization with peptide-based vaccines

In our first study of monthly immunizations with the MAGE-3.A1 peptide (*Section III. 1.1.1*, study LB 94-001), even though the tumor regressions observed appeared to be linked to the treatment, we found no evidence for any significant increase of anti-MAGE-3 CTL precursors in the blood following repeated immunizations (197). The lack of sensitivity of the MLPC / lysis assays used 10 years ago may have hampered the assessment of the CTL responses induced by vaccination with the MAGE-3.A1 peptide. Nevertheless, it is possible that a weak CTL response was produced in the regressor patients, but that these CTL migrated immediately to the vicinity of the tumor so that they could not be found in the blood. In agreement with this notion, a TCR repertoire study performed on a lesion excised after immunization of patient n°3 who showed a mixed response of cutaneous in-transit metastases, indicated that expansion of a T-cell clone, expressing TCRBV20/TCRAV27 occurred. This T-cell clone was also found, but at low frequency, in a different pre-vaccination metastasis but not in PBL either before or after vaccination (352).

It is also possible that the putative responder lymphocytes were located almost exclusively in lymph nodes. However, we believe that a massive CTL response would have been observed with our tests. Our preferred interpretation is that the peptide injections produced a weak CTL response in some of the patients, including the patients where tumor responses were observed. This interpretation is compatible with the slow pace of the tumor regressions.

Using a much more sensitive method based on tetramer analysis, we have observed a more than hundredfold increase in the frequency of an anti-MAGE-3.A1 CTL in the peripheral blood of a patient who showed a partial regression of a large lymph node metastasis following frequent vaccinations with the MAGE-3.A1 peptide (*Section III. 1.1.4*, Study LUD 97-004, Patient CP-64 had a MxR to treatment) (205). Likewise, a CTL response was found in a minority of patients immunized with the MAGE-3.A1 peptide or with a recombinant canarypox virus coding for this antigen, most of them having displayed OR or MxR after vaccination (347, 353). Most of these CTL responses are weak and are monoclonal or oligoclonal, which means that they result from amplification of a single, or of a few CTL precursors. Even though they provide no information about the effector mechanism responsible for the observed reduction in tumor mass in those patients, they suggest that low-level CTL response can initiate tumor rejection. Despite the facts that we were not able to detect CTL responses in all the patients who displayed OR or MxR and that the amplified anti-MAGE-3.A1 CTL clone was only present at low frequencies, similar to that found in the blood, in the tumor samples of 2 regressor patients immunized with MAGE-3.A1, we believe that such responses might be effective on the basis of the following considerations (349). Mice have been observed to completely reject fast-growing tumors of about 0,5 cm³ in less than one week and these mice appear to display in their blood a level of anti-tumoral CTLp that is approximately 10⁻³ of CD8⁺ T cells (89). Considering that a mouse has a total of about 2 x 10⁷ CD8⁺ T lymphocytes in the blood, lymph nodes and spleen and assuming a uniform distribution of the anti-tumoral CTLp in these locations, a frequency of 10⁻³ in the blood corresponds to a total of 2 x 10⁴ anti-tumoral CD8⁺ T cells in the body. On the same basis, humans, who have a total of about 10¹¹ CD8⁺ T lymphocytes, have a total of 5 x 10⁶ anti-MAGE-3.A1 CD8⁺ when their blood frequency is equal to 5 x 10⁻⁵. If these lymphocytes home to the tumor and attack it as efficiently as observed in the mouse, 5 x 10⁶ CD8⁺ T cells could bring about the elimination of a large volume of tumor, particularly in view of the fact that human tumors progress much more slowly than experimental mouse tumors. It is therefore conceivable that melanoma patients who eliminate small cutaneous metastases, over a period of more than one month, may do so with CD8⁺ T cells that are one or two orders of magnitude lower than those observed here. This would be in line with our inability to detect in other patients, who have shown clinical responses, frequencies of anti-MAGE CTL that are significantly above the level observed in normal individuals. It is possible that even those CTL responses that are below our detection level can trigger a sequence of events that leads to tumor regression. A small number of anti-MAGE-3.A1 CTL activated by the vaccine could reach metastases and resist to the immunosuppressive environment long enough to attack a few tumor cells.

If a small number of anti-vaccine T cells can cause tumor rejection, they probably do so by initiating a rejection process that requires the recruitment of other immune effector cells. It is likely that as a result of an initial attack of the tumor by the anti-vaccine CTL, conditions are created that favor not only further proliferation of the anti-vaccine CTL, but also the activation of T cells directed against other antigens of the tumor. The latter process, initially described for autoimmune responses, has been referred to as "epitope spreading" (354). We observed this phenomenon in a murine tumor (355). The indirect activation by a single epitope-based vaccine of multiple effective CTL directed against many different tumor antigens would considerably decrease the risk of tumor escape and hence, increase the chance of therapeutic success of such vaccines in man.

Therefore, we recently examined whether vaccinated patients develop CTL responses against antigens present on their tumor but different from the vaccine antigen. In a melanoma patient showing a partial response after vaccination with a recombinant canarypox virus coding for the MAGE-3.A1 antigen, we found, in addition to the anti-MAGE-3.A1 vaccine CTLp, other CTLp recognizing antigens encoded by MAGE-C2 and gp100, both genes being expressed by the patient's tumor (215, 221, 348, 349). Although some of these antitumor CTL clones were already present before vaccination, the appearance of new clonotypes after vaccination suggests that a spontaneous antitumor T cell response, which has become ineffective, can be restimulated by vaccination and contribute to tumor rejection.

In addition, the ability of a small number of CTL to cause the destruction of a rather large number of tumor cells may critically depend on the involvement of other, possibly non-specific, effector cells, once the lysis of some tumor cells has been effectuated by the CTL.

In addition to the difficulty in eliciting anti-tumor immune response, it is possible that even when these responses can be induced, they remain weak and rapidly subside, so that even in those patients where tumor regression is initiated, vaccination must be pursued to boost the immune response and render it capable of bringing the regression to completion.

IV. 2.2.1.2 Immunization with protein-based vaccines

The ProtD-MAGE-3-His recombinant protein used without adjuvant is only weakly immunogenic in terms of anti-MAGE-3 CD4⁺ T cell- and antibody responses and these responses are enhanced in the presence of an immunological adjuvant (*Section III.2*) (145, 198, 207, 208, 210). Tumor regression can occur in the absence of a significant anti-MAGE-3 CD4⁺ T cell- and/or antibody response, suggesting that other effector cells, including CD8⁺ T cells, could be involved in the tumor regression process. In order to address this important issue, Pierre van der Bruggen and his colleagues are currently developing a specific and sensitive assay based on the stimulation of PBLs by autologous dendritic cells that would present all the MAGE-3 relevant CD8⁺ and CD4⁺ epitopes for a given patient (Pierre van der Bruggen, personal communication).

Quantitative evaluation of CD8⁺ T cell responses by Tetramer assay in patients receiving anti-tumoral vaccination with a protein is difficult because of the large number of possible HLA-peptide combinations. No such response was detected in the few patients whose PBMC were tested with this approach. Quantitative evaluation of CD4⁺ T cell responses by Tetramer assay is currently under development and such responses have been identified in some of our patients (Pierre van der Bruggen, personal communication).

IV. 2.2.2 Tumor escape to the immune attack

Many properties of the tumor cells could affect the outcome of the treatment. A first set relates directly to the presence of the antigen on the tumor. Tumor cells vary considerably in their degree of expression of genes coding for tumor antigens recognized by T lymphocytes. They can also vary in their general ability to present antigens on class I or class II HLA molecules. This depends on the level of expression of HLA genes and on the integrity of the antigen-processing machinery, which involves the TAP transporter proteins and the proteasome (356-358). These properties are known to differ between tumors of different individuals and between individual metastases of the same patient. Some microheterogeneity may even be present within the same metastasis. Very rare cells having lost the antigen could be selected by the anti-tumoral process and cause subsequent relapse.

Other properties than those affecting the target antigen might affect the outcome of therapeutic vaccination. Some relate to the accessibility of the tumor to the CTL and others, to the regulation of CTL activity by various suppressor pathways.

Tumors have been reported to be fed by microvessels with an abnormal endothelium, which prevents proper migration of T lymphocytes into the tumor (359, 360). This might explain why in some patients some lesions regress completely while others progress: the initial access of lymphocytes to a lesion would be a rare occurrence, but once a small number of CTL had entered a lesion, they could initiate a positive feedback process attracting a large number of other CTL and leading to the complete regression of that lesion.

Once T lymphocytes have gained access to the tumor, their activation may be inhibited by immunosuppressive factors that are produced either by the tumor cells or by normal cells of the microenvironment, such as tumor growth factor- β (TGF- β), IL-4, IL-10, prostaglandins or indoleamine 2,3-dioxygenase (IDO) (361-363). T lymphocytes undergo proliferation arrest when exposed to tryptophan shortage, which can be provoked by IDO, an enzyme that is expressed in placenta and catalyzes tryptophan degradation. IDO is also expressed by cells exposed to IFN- γ and in certain types of activated macrophages and DC, suggesting a role of IDO in the regulation of immune responses. In the mouse model P815, tumors expressing IDO resist immune rejection by T cells.

CTL suppressor pathways include the cytolytic T-lymphocyte antigen-4 (CTLA-4) and the CD4⁺CD25⁺ T lymphocytes or T regulatory (Treg) cells (336). Upon activation, CTLA-4 becomes expressed on the cell surface of both CD8⁺ and CD4⁺ T cells. It is a homologue of CD28 that

competes for B7 binding, blocking the CD28-B7 costimulatory pathway and arresting T cells in the G1 phase of the cell cycle. It induces tolerance to self-antigens and is necessary to maintain T-lymphocyte homeostasis and preventing the development of autoimmune diseases (364). CTLA-4 blockade enhances antitumor responses in mice, either alone in immunogenic tumors, or in combination with vaccines, in less immunogenic tumors. In clinical trials, the inhibition of CTLA-4 activity with anti-CTLA-4 antibodies could therefore allow T-cell expansion to continue after vaccination with a tumor antigen, enhancing the tumor regression process in response to vaccination (*Section IV. 3.2*). Treg cells account for 5% to 10% of CD4⁺ T lymphocytes and constitutively express both CD25 and CTLA-4. They inhibit the activation and proliferation of CD8⁺ and of other CD4⁺ T cells, either directly through cell-cell contact, or indirectly through the secretion of anti-inflammatory mediators such as IL-4, IL-10 and TGF- β . Like other CD4⁺ T cells, Treg cells recognize peptides presented by HLA class II molecules and they require ligand-specific TCR activation and cell-cell contact to exert their suppressive activity on both CD4⁺ and CD8⁺ T cells. In cancer patients, tumor growth promotes the expansion of Treg and increased frequency of Treg is correlated with tumor burden (365-367). Depleting the Treg population by any means could restore the CTL activity raised against tumor antigens and promote tumor regression in response to any associated treatment. The function of Treg is inhibited by IFN- γ and their immunosuppressive activity can be blocked by antibody to glucocorticoid-induced TNF receptor family related protein (GITR), which is present on many cell types. In a mouse model, administration of an anti-GITR agonist monoclonal antibody (DTA-1) inhibited Treg-dependent suppression, enhanced T cell responses, induced tumor rejection in more than 85% of mice challenged with B16 melanoma, but generated only minimal autoimmunity (368). The transcription factor Foxp3 which is essential for Treg cell specification could also represent a useful target for enhancing antitumor immunity. The identification of Treg cells has important implications not only for the treatment of cancer (*Section IV. 3.2*), but also for autoimmunity and organ transplantation (336, 369).

Alternatively, CTL cells could also be killed by FAS ligand expressed by the tumor cells (370). Finally, it is possible that the individual makeup of the tumors, including oncogenes and anti-oncogenes, might affect their sensitivity to immunotherapy.

IV. 2.2.3 Is there any correlation between tumor regression and CTL response?

In order to understand why most of the patients failed to show any evidence of tumor regression, we examined whether there was a correlation between tumor regression and immune responses to the vaccine. This should indeed help us distinguishing between the 2 main causes of vaccine failure, i.e. a failure of the vaccine to induce a significant T-cell response or resistance of the tumor to immune attack (*Sections IV.3.1.1 and IV.3.1.2*). If only those patients who show an OR or a MxR show CTL responses, this will suggest that the induction of CTL responses is limiting. However, since tumor resistance can occur in some patients, it is more reasonable to expect a T cell response in all responder

patients and in some progressor patients. This would also indicate that a limiting factor for success is the induction of a T cell response by the vaccine (344).

In patients vaccinated with the MAGE-3.A1 peptide alone, CTL responses were almost never observed, even with a very sensitive detection method based on *in vitro* restimulation of PBMCs with the antigenic peptide followed by labeling with tetramers. To evaluate CTLp frequencies, these MLPC are performed under limiting dilution conditions. The tetramer-stained cells of the positive microculture are cloned, the lytic specificity of the clones is verified and their diversity is analyzed by TCR sequencing (205). A CTL response was found in 1 out of the 7 patients analyzed who displayed an OR or a MxR and in 0/13 patients who had PD (205, 344, 347). In contrast, a statistically significant correlation was found between tumor responses (OR or MxR) and the specific anti-MAGE-3.A1 CTL response in patients vaccinated with an Albany vaccine (ALVAC) recombinant canarypox virus encoding the antigen and also in patients vaccinated with peptide-pulsed dendritic cells (221, 344-347). Among patients vaccinated with an ALVAC-MAGE recombinant virus, a CTL response was found in the only patient who showed a PR, in 2 out of 3 patients who had a MxR and in 1 out of 11 patients who had PD. Among patients vaccinated with dendritic cells, a CTL response was found in 3 of 3 patients who had OR or MxR and in none of the 3 patients who had PD (347). In patients vaccinated with the recombinant ALVAC virus, the responses appear to be weak and are mainly monoclonal, whereas they are stronger and usually polyclonal in patients immunized with peptide-pulsed dendritic cells (345, 346). Thus, on the one hand, our observation of CTL responses in 7/14 regressor patients and only in 1/27 progressors confirm that there is a correlation between clinical and CTL response (347). This indicates that, under the conditions of vaccination described in this section, the induction of an anti-MAGE T cell response appears to be an important limiting factor for clinical success. It may then be important to find modalities of vaccination that induce better T cell responses.

On the other hand, in an ongoing study, we vaccinated 13 melanoma patients with 8 HLA-A2 peptides mixed with CpG 7909 (Study LUD 03-007, coordinating investigator: Marie Marchand). No objective tumor response was observed. Three patients had a MxR and 1 patient displayed stabilization of a lung metastasis and of mediastinal metastatic lymph nodes lasting for more than 7 months since the beginning of vaccinations. The analysis of the CTL response to individual peptides after 6 immunizations indicates that 6/13 patients display a significant CTL response against at least one of the 8 antigens included in the vaccine (Pierre Coulie, personal communication). Accordingly, there appears to be no correlation between this CTL response and the tumor response to the vaccine. One hypothesis to explain this lack of correlation could be that specific CTL, generated or not by the vaccine, could be present in the tumor, but the action of these CTL would be inefficient at the tumor localization (348, 349). One reason that might explain the lack of efficacy of CTL could be low expression of MHC molecules and therefore of tumor antigens by the tumor cells. It was recently shown in a transgenic mouse model expressing lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP) under the control of the rat insulin promoter, that the presence of IFN- α renders the

pancreatic β -islet cells sensitive to the action of CTL that are present locally and leads to the development of autoimmune diabetes (371). Likewise, the presence of IFN- α in the melanoma metastases of vaccinated patients could sensitize the tumor to *in situ* CTL, trigger cytolysis and cause tumor regression. This is why, in study LUD 03-007, we plan to associate low dose IFN- α (3×3.10^6 IU/week for 14 weeks, s.c.) with the maintenance vaccinations of melanoma patients who will soon be included in the second cohort of 14 patients. These patients will first receive 6 vaccinations with the same HLA-A2 peptides mixed with Montanide ISA 51. Since only one parameter, i.e. the immunological adjuvant varies between the 2 cohorts, results in terms of CTL response and Tumor response after 6 vaccinations will be easily compared. In patients who will receive maintenance vaccinations associated with IFN- α , we also want to determine whether tumor response improves under this association, as compared with vaccinations alone. Association of IFN- α to vaccinations could also indirectly benefit to patients for another reason. It was recently shown that AJCC 2002 stage IIB, IIC and III melanoma patients who developed biological or clinical signs of autoimmunity after treatment with high-dose IFN- α in the adjuvant setting (5×20.10^6 IU/week for 4 weeks, i.v., followed by 3×10.10^6 IU/week, for 48 weeks, s.c.), have statistically significant improvement of relapse-free survival and overall survival, as compared with patients who received the same treatment without developing autoimmune manifestations (372).

IV. 3 PERSPECTIVES: HOW COULD THE THERAPEUTIC EFFICACY OF MELANOMA VACCINES BE IMPROVED?

Active immunotherapy approaches targeting antigens recognized by T lymphocytes on cutaneous melanoma have so far not been associated with significant clinical benefit in patients with metastatic disease (*Section IV. 1*). Improving the immunogenicity of vaccines and combining them with other therapies that selectively target altered genes or proteins belonging to metabolic pathways involved in melanoma pathophysiology could provide more effective treatments.

IV. 3.1 Improving the immunogenicity of vaccines

In order to try to increase the immunogenicity of peptides-based vaccines, several immunological adjuvants can be mixed to the vaccine specific antigen(s), including CpG oligonucleotides and Montanide ISA 51. In an ongoing study, the MAGE-3.A1 peptide is mixed with CpG 7909 (Study LUD 02-001, coordinating investigator: Nicolas van Baren). Likewise, CpG 7909 is mixed with 8 HLA-A2 peptides in another ongoing study (Study LUD 03-007, coordinating investigator: Marie Marchand). Results of both studies will be compared in terms of therapeutic efficacy and immune response. Moreover, the association of low-dose IFN- α to vaccinations could increase the expression of MHC molecules on tumor cells, which would make them more susceptible to the attack of the T lymphocytes that are present in the tumor.

IV. 3.2. Eliminating tumor- and lymphocyte-mediated immune suppressive mechanisms

Once the anti-tumor CTL have migrated into tumors, several strategies can be proposed that should enhance the immune attack of the tumor. Immunosuppressive molecules secreted by the tumor cells or by cells of the microenvironment such as TGF- β , IL-10, IL-13, prostaglandins or indoleamine 2,3-dioxygenase (IDO) could be blocked by various means. Since most human tumors constitutively express IDO, therapeutic vaccinations of cancer patients might be improved by concomitant administration of an IDO inhibitor such as 1-methyl-L-tryptophan (1MT) (363). Other IDO inhibitors are currently under development (B. Van den Eynde, personal communication).

Phase I trials were recently conducted with 2 different anti-CTLA-4 monoclonal antibodies, Ipilimumab and Tivolumab (373). Ipilimumab (MDX-010, Medarex) is a fully human antibody that was tested in 7 patients with metastatic melanoma. Two of them had a PR. Antitumor effects of Ipilimumab included extensive inflammatory, ischemic and hemorrhagic tumor necrosis with lymphocyte and granulocyte infiltrates (374). Ipilimumab (3 mg/kg i.v.) was then associated with s.c. vaccinations with two modified HLA-A2 restricted gp100 peptides mixed with incomplete Freund's adjuvant (IFA) in patients with metastatic melanoma. The trial was intended to accrue 21 patients. Because of the development of grade III/IV autoimmune toxicity in more than 3 patients, accrual ceased after 14 patients were enrolled. There were 3 objective tumor responses (2 CR

and 1 PR). Severe autoimmune toxicity, including dermatitis, enterocolitis, hepatitis and hypophysitis occurred in 6 patients (375). This trial was later extended to evaluate 2 different doses schedules of Ipilimumab in conjunction with peptide vaccination and to explore the relationship between autoimmunity and tumor regression. A total of 56 patients with progressive stage IV melanoma were enrolled. Two patients achieved a CR, 5 had a PR, and 5 of these OR lasted for more than 2 years. Of 14 patients with grade 3/4 autoimmune toxicity, five (36%) experienced a clinical response compared with only two responses in the 42 patients (5%) with no autoimmune toxicity ($P = .008$), showing a clear association between autoimmunity and clinical response. There were no significant differences in response rate or toxicity between the two dose schedules (376). Similar results in terms of tumor response rate and autoimmune toxicity were observed with another anti-CTLA-4 monoclonal antibody, Tivolumab (Pfizer) (377). Ipilimumab has also been combined with DTIC or with high dose IL-2 in metastatic melanoma, and was tested with a multi-peptide vaccine in 44 patients with resected high-risk stage III and IV melanoma (373, 378-380).

Objective response rates observed in these anti-CTLA-4 antibodies trials ranged from 10 to 20% and tumor response, survival and time to relapse were clearly associated with the development of severe autoimmune side effects that were manageable in most cases with steroids. Anti-CTLA-4 antibodies appear to modify the T cell functions either by inhibiting the activity of T regulatory cells or of other inhibitory factors, or by direct action on effector T cells raised against tumor antigens or normal tissue antigens, increasing their activity and concurrently leading to tumor regression and autoimmune side effects (373).

It was recently shown in mouse that cyclophosphamide acts directly on Treg, not only by decreasing their proliferation capacity and making them more sensitive to apoptosis, but also by altering their suppressor functionality (381, 382). The most promising approach is probably the use of low-dose cyclophosphamide that would selectively deplete the Treg population without generating severe toxicities that can be caused by more aggressive myeloablative or lymphodepleting chemotherapies (310). Restoration of CTL activity in clinical trials by depletion of Treg populations after various myeloablative or nonmyeloablative chemotherapy has been discussed in *Section IV. 1.3.3*. Treg cells can also be eliminated by the use of fusion proteins, which link cytotoxic molecules to receptor targets. Recently, a phase I clinical study examined the possibility of a recombinant fusion protein of IL-2 and diphtheria toxin (Denileukin Difitox or ONTAK) to eliminate regulatory T lymphocytes, based on their expression of high-affinity IL-2 receptors (383). ONTAK targets the CD25 component of the IL-2 receptor on the surface of malignant cells and some normal lymphocytes (including Tregs), leading to the death of these cells. It was tested in 12 patients with stage IV metastatic melanoma, to try to eliminate Tregs, break self-tolerance and unleash anti-tumor properties of circulating T cells. No objective tumor response was observed and the number of Tregs, as assessed by *Foxp3* expression, was not decreased.

IV. 3.3 Combining vaccination with new drugs interfering with cell growth, apoptosis or neoangiogenesis

The silencing of tumor suppressor genes as well as the activation of oncogenes have been implicated in the development and progression of melanoma (36). Likewise, angiogenesis, the formation of new capillaries from pre-existing blood vessels is considered essential for tumor growth and metastasis (384).

IV. 3.3.1 Mutated oncogenes and tumor suppressor genes

Inhibition or dysregulation of the apoptotic pathways have been identified in melanoma, including alterations of *p53*, *Bcl-2* and *Bax* or modifications of cell death receptors such as the CD95/Fas receptor (35). Chemoresistance of malignant melanoma and poor survival prognosis are associated to expression of the proto-oncogene *Bcl-2*, an anti-apoptotic mediator (385). Oblimersen (Genasense), an antisense oligodeoxynucleotide targeted against *Bcl-2* mRNA, that can inhibit *Bcl-2* expression, was tested in combination with DTIC in a Phase I-II trial involving 14 patients with advanced melanoma expressing *Bcl-2* as assessed by western blotting (386). The combination regimen was well tolerated and 1 CR, 2 PR and 3 MR were observed. Based on this preliminary observation, a randomized Phase III trial on 771 patients was set up, comparing the therapeutic efficacy of DTIC alone or combined with oblimersen (39). The antitumor response rate was 11.7% for oblimersen/DTIC, compared with 6.8% for DTIC ($p = 0.019$). There was no statistically significant effect on overall survival between the 2 treatment arms (primary endpoint). Patients on oblimersen/DTIC had a median survival of 274 days, compared with 238 days for DTIC alone ($p = 0.18$). However, secondary endpoint did show a significant effect: the progression-free survival was 74 days for oblimersen/DTIC, compared with 49 days for DTIC ($p = 0.003$).

Inhibition of BRAF activity and MAP kinase signaling may provide a new strategy for the prevention and treatment of melanoma. Melanomas arising on skin without chronic sun-induced damage, which represent the most common type of melanoma, frequently have *BRAF* or *N-RAS* mutations (12). Thus, they would be expected to be responsive to treatments targeting the RAS-RAF-ERK and PI3K pathways. In contrast, melanomas that arise on skin chronically exposed to sun or on sites rarely or never exposed to UV light have no such mutations. Thus, they would be less susceptible to drugs such as sorafenib, a tyrosine kinase inhibitor targeting BRAF.

IV. 3.3.2 Interfering with neoangiogenesis

The primary mechanism of action of antiangiogenic agents is the inhibition of tumor growth, rather than cytodestruction. Therefore, their efficacy is generally inversely correlated to tumor burden. Preliminary results of clinical studies indeed suggest that single-agent antiangiogenic therapy is not effective in advanced tumors. However, antiangiogenic agents could have synergistic therapeutic effects in combination with other anticancer therapies. In addition to their direct antiangiogenic effects, they may improve the delivery of chemotherapy to the tumor mass by increasing tumor vasculature permeability and decreasing the elevated intratumoral interstitial pressure. Likewise, they may enhance the sensitivity of tumor cells to cytotoxic drugs by locally increasing the intratumoral oxygen pressure. Combining antiangiogenic agents with chemotherapy should allow a reduction of the doses of chemotherapeutic drugs and hence, their toxicity. Melanoma is a tumor associated with a robust angiogenesis and high microvessel density in primary melanoma is a powerful independent prognostic factor of distant metastasis and overall survival (387). In melanoma patients, the overexpression of angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and IL-8, is associated with increased metastatic potential and poor prognosis (388). Whereas the treatment of melanoma patients with dacarbazine alone could select for more aggressive melanoma phenotype, combination with antiangiogenic agents could potentiate the therapeutic effect of dacarbazine or temozolomide (42, 389). In a randomized phase III trial for patients with metastatic colorectal cancer, the addition of bevacizumab, a monoclonal antibody against VEGF (AvastinTM, Genentech), to a fluorouracil-based combination chemotherapy was recently shown to significantly improve objective response rates, progression-free survival and overall survival, as compared with the same chemotherapy regimen given alone (390). Combination of antiangiogenic agents with radiation therapy can block tumor growth by inhibiting VEGF secretion stimulated by hypoxia. Antiangiogenic agents could also be combined with vaccination.

We conclude that considerable further progress will be required, before immunization with tumor-specific antigens recognized by T cells becomes an effective and generally applicable cancer therapy. The results reported here provide some orientation for future work and suggest that success may be achievable.