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Adoptive Transfer of *Ex Vivo* Activated Memory T-cells With or Without Cyclophosphamide for Advanced Metastatic Melanoma: Results in 36 Patients

J.E. Gold, S.D. Ross, D.J. Krellenstein, F. LaRosa, S.C. Malamud and M.E. Osband

Autolymphocyte therapy (ALT) is the infusion of autologous peripheral blood mononuclear cells (PBMC) activated *ex vivo* by a cytokine-rich supernatant (T3CS) generated from a previous autologous lymphocyte culture using low doses of the anti-CD3 mitogenic monoclonal antibody. The mechanism of action is enhancement of a recall response by CD45RO$^+$ (memory) T-cells (ALT cells) to host tumour without dependence on exogenous interleukin (IL)-2. The existence of anti-tumour-specific T-cells in melanoma patients has been well described, and efforts to utilise them therapeutically have achieved modest tumour response rates. However, few long-term survival data have been reported. From 1986 to 1992, we treated 36 patients with disseminated melanoma using ALT alone (26 patients) or adoptive chemoimmunotherapy using ALT and cyclophosphamide (CY) (10 patients). Over this time period, the cell activation method evolved from using cytokine supernatants derived from a one-way allogeneic mixed lymphocyte culture (MLCS), to the current practice of utilising anti-CD3 and autologous cytokines (T3CS). There were 21 men and 15 women, average age 57 years, range 30–82. 27 had failed prior therapies and 9 had no prior therapy. A total of 161 infusion of ALT cells were given: 65 with cells activated in MLCS and 96 with T3CS. There were no grade 3 adverse events, and an approximate 20% incidence of grades 1 and 2 reactions to ALT-cell infusions. Transient cytopenias were seen in patients receiving CY. Sixty-one per cent (22/36) of patients received the planned six ALT-cell infusions, while 39% did not due to progressive disease. In 33 evaluable patients, there were four complete responses, four partial responses and 6 patients with stable disease (SD). Responding patients and those with SD had prolonged survival compared to historical controls when matched for number of organ systems involved. *Ex vivo* depletion of CD45RO$^+$ T-cells revealed preferential lysis of autologous and HLA-A-matched melanoma targets that was dependent on these memory T-cells. These data suggest that adoptive cellular therapy using *ex vivo* activated memory T-cells with and without CY is active, has low toxicity, is tumour-specific and can result in clinical benefit in patients with disseminated melanoma.

Key words: immunotherapy, adoptive, melanoma, T-cell, memory
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INTRODUCTION

ADOPTIVE CELLULAR therapy of neoplastic disease is the transfer of previously sensitised immune cells to tumour-bearing hosts (TBH) in order to achieve therapeutic effect, and is based on experimental evidence that the cellular arm of the immune system is the crucial factor in mediating tumour rejection [1]. Peripheral blood mononuclear cells (PBMC), obtained from TBH that are cultured short-term in relatively high concentrations of interleukin (IL)-2, results in the generation of cells that are able to lyse fresh, non-cultured, natural killer (NK) cell resistant tumour cells, but not normal cells [2]. Most lymphokine-activated killer (LAK) cells are of NK origin, with some contribution from T-cells and B-cells [2–4]. Because the majority of LAK cells consist of NK cells, they are able to lyse both autologous and allogeneic tumour targets in a non-specific or non-major histocompatibility complex (MHC)-restricted manner *ex vivo*.

It is well-known that mononuclear cells can be found infiltrating various solid neoplasms, and since most of these tumour-infiltrating lymphocytes (TIL), depending on the tumour type, consist predominantly of T-cells [5], it was thought that greater anti-tumour efficacy and tumour specificity would result when these cells were used in adoptive cellular therapy protocols in humans. Rosenberg and co-workers demonstrated that TIL/IL-2 in combination with cyclophosphamide (CY), were 50–100-fold more potent on a per cell basis than LAK cells in mediating anti-tumour effects in murine models [6]. However, when human TIL are expanded with IL-2, many of these TIL, depending on the tumour type, appear to lose their MHC-restricted tumour specificity and become "LAK-like" in their killing of non-autologous tumour targets [7]. Itoh and Balch demonstrated that TIL from melanoma patients possess autologous tumour specificity after IL-2 expansion [8], an observation shared by Kradin and Kurnick who studied TIL from lung cancer patients [9]. Maleckar and co-workers, using their tumour-derived activated cells (TDAC), demonstrated that these TDAC could be made relatively, as opposed to absolutely, tumour specific by frequent restimulation with irradiated, autologous tumour cells [10]. Finke and Bukowski found that repetitive restimulation of renal cell carcinoma (RCC) TIL with autologous tumour generated T-cells that were generally not restricted to autologous RCC targets [11]. It seems that the putative tumour specificity of TIL may vary by tumour type or may be a relative phenomenon in all tumour types.

Autolymphocyte therapy (ALT) is adoptive cellular therapy of cancer using infusions of autolymphocytes (ALT cells) activated *ex vivo* from autologous PBMC. We initially reported ALT as successful anti-tumour-specific adoptive cellular therapy using lymphocytes from murine and human TBH which were immunised *ex vivo* using an autologous 3 M KCl tumour extract as tumour antigen with a mixed lymphocyte culture-supernatant (MLCS) as a stimulator [12, 13]. Antigen-specific secondary responses can be recalled *ex vivo* by non-specific activation without the use of the specific antigen [14]. Based on this, we also demonstrated that non-specific *ex vivo* activation of lymphocytes from murine and human TBH by MLCS or by using a cytokine-rich supernatant (T3CS) generated from a previous autologous (syngeneic) lymphocyte culture stimulated with low dose anti-CD3 in the absence of tumour tissue (antigen), results in successful anti-tumour-specific adoptive cellular therapy [15–17]. ALT consists of monthly infusions of these memory T-cells and has been shown to significantly prolong survival, induce durable tumour responses, and is accompanied by only minimal toxicity in murine TBH with advanced melanoma and carcinoma, as well as human patients with metastatic RCC [15–18]. Analysis of the final T3CS preparation has shown it contains small amounts of anti-CD3, as well as being rich in cytokines such as granulocyte–macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, IL-1, IL-6 and IL-8, although not IL-2 [19]. *Ex vivo* depletion of memory T-cells against syngeneic murine tumours, as well as against autologous human RCC and soft tissue sarcoma (STS) targets, abrogates all anti-tumour activity [20, 21]. Therefore, it is possible to generate tumour-specific recall responses dependent on memory T-cells following non-specific anti-CD3-based *ex vivo* activation.

In a preliminary randomised trial, patients with metastatic RCC who were treated with ALT had a 3–4-fold longer survival with improved quality of life compared to those in the control group [15]. A recent update of the initial study, as well as a new study with additional patients, confirmed both the survival advantage and a response rate of 18% in the ALT-treated group [16]. Patients with advanced malignant melanoma appear to share a number of similarities to patients with metastatic RCC, including sub-optimal responses to conventional chemotherapy or radiotherapy, as well as potential responsiveness to immune manipulation. Based on previous results of ALT in patients with metastatic RCC, as well as in murine TBH with advanced metastatic melanoma, we initiated a pilot study of ALT in patients with disseminated melanoma. As this clinical study progressed, patients with melanoma who had initially responded to and then failed previous chemotherapy regimens (with or without cytokines such as IL-2 or IFN-α) did not appear to respond to ALT alone. We recently demonstrated that adoptive chemoimmunotherapy (ACIT) using subtherapeutic (10^6) numbers of ALT cells together with CY is effective, tumour-specific and curative in murine TBH with advanced metastatic melanoma [22]. Therefore, we treated some chemotherapy-resistant patients with ACIT using ALT cells and CY. The activity and tumour specificity of ALT cells were confirmed using *ex vivo* depletion experiments of memory ($CD45RO^+$) T-cells and NK ($CD56^+$) cells against autologous and allogeneic tumour targets.

PATIENTS AND METHODS

Patients and tumour targets

Adult patients with disseminated melanoma failing radiation therapy, chemotherapy (with or without cytokines), or both were eligible for this study. 36 patients were entered who met the inclusion criteria of a baseline ECOG performance status of 0, 1 or 2; haematological indices suitable for adequate isolation of lymphocytes for *ex vivo* processing (haematocrit > 25%, platelet count < 500000/mm^3 and white cell count > 3000/mm^3); lesions measurable by physical assessment or routine

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imaging studies; negative serological testing for HTLV-I and hepatitis B surface antigen (HBsAg); and no anti-tumour therapy for at least 4 weeks. The study was approved by the relevant institutional review boards and written informed consent was obtained from all patients for both the ALT and ACIT protocol, as well as for HIV testing, before entry into the study. All therapy was given in an outpatient setting. When necessary, certain patients had indwelling central venous catheters placed for access. ALT cells were prepared at a central bioprocessing laboratory in Boston (U.S.A.), where the good manufacturing practice (GMP) regulations formulated by the Food and Drug Administration for the manufacture of pharmaceutical products are maintained. Tumour targets that were evaluated *ex vivo* included human leucocyte antigen (HLA)-A1$^+$ or HLA-A2$^+$ biopsy specimens from two patients treated with ACIT that attained a complete response (CR) (ACIT-1 and ACIT-8) and one with stable disease (SD) (ACIT-10). The HLA-A1$^+$ HT144 melanoma line, the HLA-A2$^+$ osteogenic sarcoma line Saos-2, the HLA-A2$^+$ pancreatic carcinoma line Capan-1 and the K562 myeloid leukaemia line were obtained from the American Type Tissue Collection. ISW-1 is an HLA-A2$^+$ melanoma tumour target derived from the resection of a metastatic pulmonary lesion of a patient who had a near-complete response to chemotherapy and IFN-α. HLA typing of tumour cells or lymphocytes was performed using the complement-mediated microcytotoxicity assay which revealed HLA antigens on tumour cells that were consistent with available autologous lymphocytes or by indirect immunofluorescence using the HLA-A2 monoclonal antibody BB7.2 (American Type Tissue Collection).

Definition of clinical response

The responses to ALT or ACIT were required to last at least 30 days. A CR was defined as disappearance of all measurable disease. A partial response (PR) was defined as a 50% or greater reduction of all measurable lesions with no new lesions developing. SD was defined as a less than 25% decrease in total tumour burden with no new lesions developing.

Preparation of autologous lymphokine mixtures (T3CS and MLCS)

Approximately 2×10^9 PBMC were obtained by use of a Haemonetics V50 apheresis machine. To generate MLCS, the cells were washed twice and suspended in complete medium (CM) which was composed of RPMI 1640 with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, 0.5 μg/ml fungizone (all from Gibco, Grand Island, New York, U.S.A.), and 5×10^{-5} M 2-ME (Sigma, St Louis, Missouri, U.S.A.). Stimulator cells were obtained from healthy volunteers, subjected to 3000 cGy of γ-irradiation, and mixed at a 1:1 ratio with the patient (responding) cells. After 3 days, the culture supernatant or MLCS was harvested. For T3CS, a patient's PBMC were incubated in the presence of OKT3, a mitogenic monoclonal anti-CD3 antibody directed at the antigen non-specific CD3 portion of the T-cell receptor [14, 23]. After 3 days, the culture supernatant or T3CS was harvested. T3CS or MLCS were then ultrafiltered, divided into portions and frozen at −80°C until required for use in cell cultures.

Preparation and infusion of ALT cells (CD45RO$^+$ T cells)

Ex vivo activation of CD45RO$^+$ memory T-cells was performed using MLCS or T3CS as described below and resulted in a consistent 10–20-fold PBMC expansion. Patients' PBMC received 50 cGy of gamma rays to reduce the concentration of radiosensitive suppressor T-cells [24, 25], and were then suspended in medium containing 25% T3CS or MLCS, cimetidine (5×10^{-5} mol/l) and indomethacin (10^{-8} mol/l). Cimetidine and indomethacin were added to reduce tumour-related suppressor cell activity [26, 27]. The cells were cultured for 5 days in a moist-air incubator that contained 5% CO_2 and then washed extensively. Flow cytometric analyses of both the initial PBMC and final ALT cell preparation were performed. Approximately 10^9 ALT cells per patient (mean cell number of all preparations) were infused intravenously over 30 min through a standard blood administration filter. ALT cell infusion was repeated every 28 days for a potential maximum of six cycles. Patients also received oral cimetidine daily at 800 mg three times daily throughout their treatment. For those patients that received ACIT using ALT cells and CY, doses of CY were given intravenously on day 4. The first dose of CY was at 2 g/m^2, with doses at subsequent cycles of 300 mg/m^2. Infusion of ALT cells was performed on day 7. The cycle was repeated every 28 days (see Figure 1). A total of 161 infusions of ALT cells were given: 65 with PBMC activated in MLCS and 96 with T3CS.

Ex vivo *depletion of CD45RO$^+$ T-cells from the ALT cell population*

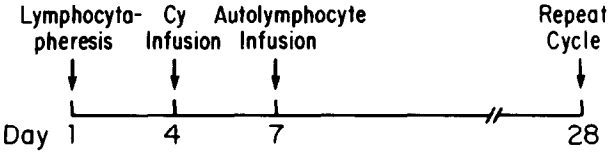
PBMC were activated with T3CS or MLCS to yield an ALT cell population which was then incubated for 30 min at 4°C with phycoerythrin (PE)-labelled anti-CD45RO, washed, and sorted on a FACStar plus cell sorter (Becton-Dickenson, Mountain View, California, U.S.A.) to obtain highly purified CD45RO$^+$ T-cells. The purity of the sorted CD45RO$^+$ T-cell population exceeded 98%. The CD45RO-depleted ALT cells were then used as effector cells against tumour cell targets.

Preparation of NK cell (CD56)-depleted ALT cells

PBMC were incubated for 30 min at 4°C with PE-labelled anti-Leu19 (CD56), washed, and sorted on a FACStar plus cell sorter (Becton-Dickenson) to obtain highly purified CD56-depleted PBMC. The purity of the sorted CD56$^+$ NK cell population exceeded 98%. The CD56-depleted PBMC were then prepared as CD56-depleted ALT cells (see above) and used as effector cells against tumour cell targets.

Measurement of ex vivo *cytotoxicity against tumour targets*

Detection of lytic activity was performed using a 4-h and 18-h ^{51}Cr release assay. ALT cells as well as CD45RO-depleted and CD56-depleted ALT cells were added to U-bottom 96-well plates to achieve effector target ratios (E:T) of 20:1. Prior to cytotoxicity assays, target cells were labelled with 250 μCi of $Na^{51}CrO_4$ (New England Nuclear, Boston, Massachusetts, U.S.A.), washed three times, and then suspended in CM at a



Cy = cyclophosphamide 2 g/m^2 first cycle and then 300 mg/m^2 at successive cycles.

Figure 1. ACIT protocol.

concentration of 10^5 viable cells/ml. Thereafter, target cells (10^4/100 μl) were added to 96-well plates. The plates were incubated for 4 or 18 h at 37°C in 5% CO_2, and the culture supernatants were harvested with the Skatron harvesting system, and the amount of released ^{51}Cr determined in a Beckman Gamma 4000 counter. Maximum ^{51}Cr release was produced by incubation of the targets with 0.1 N HCl. Spontaneous release was measured in target cells to which medium alone was added. In all ^{51}Cr assays, spontaneous release was never greater than 15%. The per cent specific lysis was calculated as:

$$\text{Percent specific lysis} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}} \times 100.$$

All determinations were made in triplicate and the data reported as the mean.

RESULTS

Immunophenotype of ALT cells

The immunophenotypes of the ALT cells from 30 random cell cultures are shown in Table 1. As expected, the majority of the PBMC were $CD3^+$ ($Leu4^+$) T-cells. After *ex vivo* culture, there was a significant increase in the proportion of T-cells expressing the activation marker IL-2R (CD25), as well as in T-cells bearing the memory marker UCHL1/Leu45RO (CD45RO) in the ALT cell population, with a concomitant decrease in CD45RA/leu19$^+$ (naive) T-cells ($P < 0.001$, Student's *t*-test). This expansion was seen regardless of the method (MLCS, T3CS) used to activate PBMC *ex vivo*. No statistically significant difference in the number of *ex vivo* activated memory T-cells was seen between the methods used.

Toxicity

The toxicity of the ALT cell infusion was minimal. Several patients developed brief fever or chills (lasting less than 30 min and relieved by acetaminophen and diphenhydramine) following the infusion of ALT cells. In addition, almost all patients at one point had mild paresthesias of the lips while undergoing lymphocytapheresis, secondary to the binding of serum calcium by the citrate-based pheresis fluid. Patients receiving ACIT using ALT cells together with CY had transient non-life-threatening cytopenias following the first CY dose only. There was no treatment-related morbidity or mortality. Sixty-one per cent (22/36) of patients received the full six courses of ALT cell treatment (Tables 2 and 3). Thirty-nine per cent of patients did not receive the planned full six courses due to progressive disease.

Responses of patients treated with ALT alone

26 patients were treated with ALT alone (Table 2). Of these 26, there were 17 men and 9 women. 17 patients had failed prior therapies and 9 had no prior therapy. Entry ECOG score was 0 in 16, 1 in eight, 2 in one and 3 in one case. The latter 2 patients were excluded from further analysis due to survival times of less than 1 month from rapidly progressive disease. Survival and tumour responses of the 24 evaluable patients treated with ALT alone are shown in Table 2. One patient (ALT-5) with three organ systems involved had a PR to ALT with a survival of 24 months (expected survival of 2 months). The duration of responses and SD were compared to the results obtained by Balch and colleagues in patients with untreated disseminated melanoma, based on the number of organ systems involved [28]. Of the patients in our study, 3 of the 4 with SD actually had some lesions which decreased and/or resolved but by less than 25%. 11 patients had three or more organ systems involved and the median survival of this group was 6 months (expected survival of 2 months). 12 patients had two organ systems involved with a median survival of 14 months (expected survival of 4 months). One of these 6 patients had a CR to therapy that is still ongoing after more than 44 months (expected survival of 7 months) and 1 had SD. One patient with a single organ system involved had a CR to therapy that is still ongoing after more than 64 months (expected survival of 7 months). The two CRs noted were in a patient with a biopsy-proven liver metastasis that measured 2.9 cm at its widest diameter, and in a patient with extensive in-transit melanoma of the lower extremity who also had grossly involved inguinal nodes at presentation (Figure 2).

Response to ACIT using ALT cells and CY

10 patients with disseminated melanoma were treated with ACIT using *ex vivo* activated memory T-cells (ALT cells) and CY (Table 3). One patient was not evaluable following a fatal cerebrovascular accident after being entered into, but before beginning, ACIT. All 9 evaluable patients had three or more sites of disease involved and all had been previously treated with either chemotherapy (+/− cytokines), radiation therapy or both. ECOG score was 0 in five, 1 in three, 2 in one, and 3 in one case. As with the patients who received ALT alone, the duration of responses were compared to the results obtained by Balch and

Table 1. Mean phenotypic percentages of peripheral blood T-cells and ex vivo *activated T-cells (ALT cells)*

	T-cells				
	$CD3^+$	$CD3^+DR^+$	$CD3^+CD25^+$	$CD3^+CD45RO^+$	$CD3^+CD45RA^+$
Peripheral blood T-cells	68	3	2	32	20.1
Ex vivo activated T-cells	83	41*	28*	77*	6.2*

Cell phenotypes based on averages of activation cultures ($n = 30$) using MLCS or T3CS. *$P < 0.001$ compared to unstimulated controls, student's *t*-test.

Table 2. Responses of patients with disseminated melanoma treated by ALT

Patient	Age (years)	Sex	ECOG	Metastatic sites	Prior treatment	Response to ALT	Activation/ no. of cell infusions	Survival (months)
ALT-1	53	m	0	Lu,N	None	Pro	MLCS/4	6
ALT-2	68	m	0	Li	None	CR	MLCS/6	64†
ALT-3	63	f	1	N	CT	Pro	MLCS/6	8
ALT-4	58	f	0	N,Li	CT	Pro	MLCS/3	4
ALT-5	30	m	0	B,Li,Lu	None	PR	MLCS/6	24
ALT-6	42	m	0	B,Lu,N	RT/CT	Pro	MLCS/6	6
ALT-7	73	f	0	Lu,N	CT	Pro	MLCS/6	7
ALT-8	70	m	1	Li,N	CT	Pro	MLCS/2	3
ALT-9	40	m	1	Br,Lu,B,N	CT	Pro	MLCS/2	4
ALT-10	67	m	0	B,Lu,N	CT	Pro	MLCS/6	6
ALT-11	40	m	1	N	RT	Pro	T3CS/2	2
ALT-12	62	m	2	B,Li,S,N	RT/CT	Pro*	T3CS/1	1*
ALT-13	62	f	1	S,N	CT	Pro	T3CS/6	8
ALT-14	32	m	0	Li,Lu,S,N	CT	Pro	T3CS/3	4
ALT-15	60	m	0	Br,N	None	SD	T3CS/6	15
ALT-16	48	m	1	Br,S,N	RT/CT	Pro	T3CS/6	6
ALT-17	38	f	0	Li,Lu,Br,N	RT/CT	Pro	T3CS/1	2
ALT-18	72	f	3	Br,Li,Lu	RT/CT	Pro*	T3CS/1	1*
ALT-19	60	m	0	S,Lu	CT	Pro	T3CS/6	9
ALT-20	45	f	0	Lu,N	None	SD	T3CS/6	13
ALT-21	57	m	0	Li,Lu	None	SD	T3CS/6	16
ALT-22	74	m	0	Li,Lu	CT	Pro	T3CS/2	2
ALT-23	79	m	1	Li,S,N	None	Pro	T3CS/2	2
ALT-24	51	f	0	Li,N	None	SD	T3CS/6	42
ALT-25	67	m	1	B,Br,N	RT/CT	Pro	T3CS/2	3
ALT-26	82	f	0	S,N	None	CR	T3CS/6	44†

m, male; f, female; B, bone; Br, brain; S, skin; Li, liver; Lu, lung; N, lymph nodes; CT, chemotherapy; RT, radiation therapy; SD, stable disease; Pro, progression; CR, complete response. * Not evaluated in final responses due to survival of less than 1 month. † Continues in complete response with no evidence of disease.

Table 3. Responses of patients with disseminated melanomas treated by ACIT (ex vivo *activated memory T-cells and CY*)

Patient	Age (years)	Sex	ECOG	Metastatic sites	Prior treatment	Response to ACIT	Activation/ no. of cell infusions	Survival (months)
ACIT-1	65	f	0	S,N,Lu,Li	RT/CT	CR	MLCS/6	50†
ACIT-2	58	m	0	B,Li,Lu	CT	SD	MLCS/6	14
ACIT-3	60	m	1	Lu,Li,N	CT	PR	MLCS/6	22
ACIT-4	62	f	2	Li,Lu,N,B	CT	Pro	T3CS/3	4
ACIT-5	38	f	1	Br,N,B	CT	Pro	T3CS/6	7
ACIT-6	70	m	3	Br,N,B	CT	Pro*	T3CS/1	1*
ACIT-7	61	f	0	Li,Lu,N,B	CT	PR	T3CS/6	25
ACIT-8	45	f	0	Li,N,Lu	CT	CR	T3CS/6	24†
ACIT-9	33	m	0	Lu,N,A	CT	PR‡	T3CS/6	21
ACIT-10	59	f	1	Li,N,Lu	CT	SD	T3CS/6	18

m, male; f, female; A, adrenal; B, bone; Br, brain; S, skin; Li, liver; Lu, lung; N, nodes; CT, chemotherapy; RT, radiation therapy; SD, stable disease; Pro, progression; CR, complete response; PR, partial response. * Not evaluated in final responses due to survival of less than 1 month. † Continues in complete response with no evidence of disease. ‡ PR followed by no radiographic evidence of progression at 21 months. Has refused surgical evaluation.

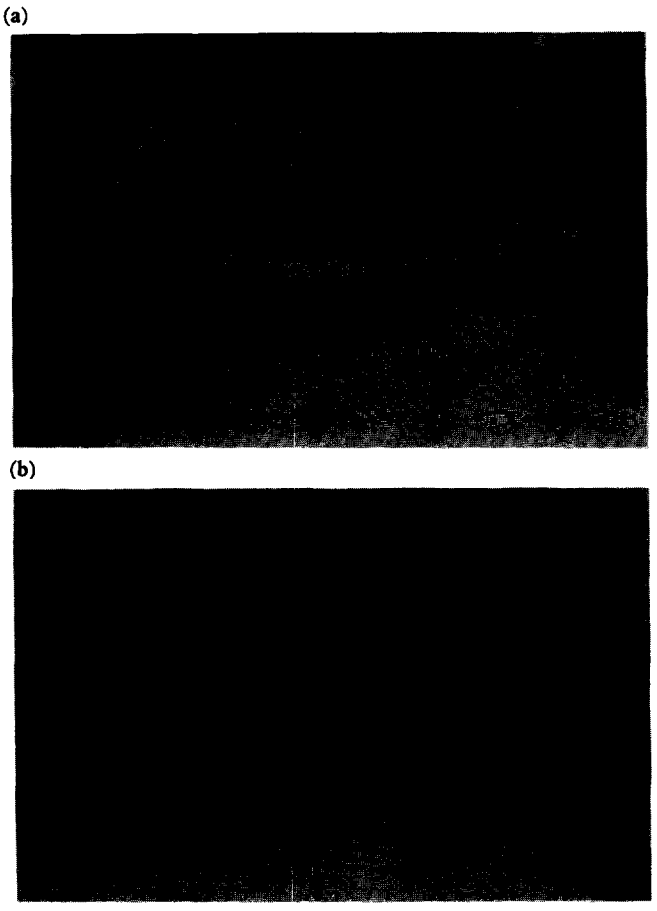


Figure 2. (a) A patient with extensive in transit melanoma of the lower extremity. The inguinal lymph nodes were also enlarged (not shown). (b) Complete clinical response to six cycles of ALT. Response is still ongoing after more than 44 months.

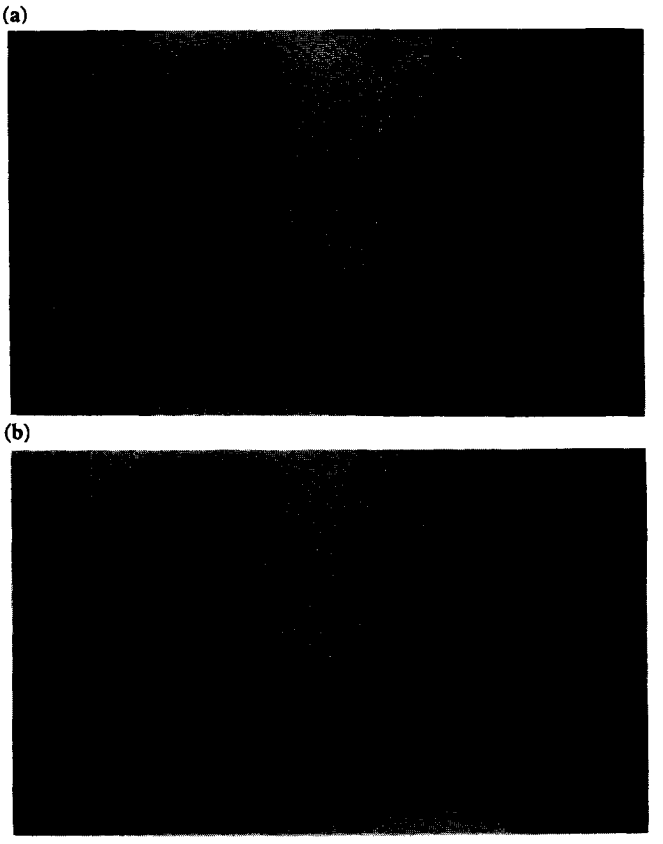


Figure 3. (a) Chest CT of a patient with metastatic melanoma before beginning ACIT showing disease in both lung fields (arrows). (b) CT of the chest after six cycles of ACIT. Complete regression of tumour sites is demonstrated.

colleagues in patients with untreated disseminated melanoma, based on the number of organ systems involved [28]. 2 patients with lung, liver and lymph node metastases had CRs that are still ongoing after more than 50 and 24 months (expected survival of 7 months). As seen in Figures 3 and 4, complete regression of metastatic pulmonary disease was demonstrated. 3 patients had PRs lasting 21, 22 and 25 months and 2 patients had SD for 14 and 17 months (expected survival of 7 months). Patient ACIT-9 following a PR has had no evidence of radiographic change for 21 months. He has refused surgical evaluation of the radiographic abnormalities.

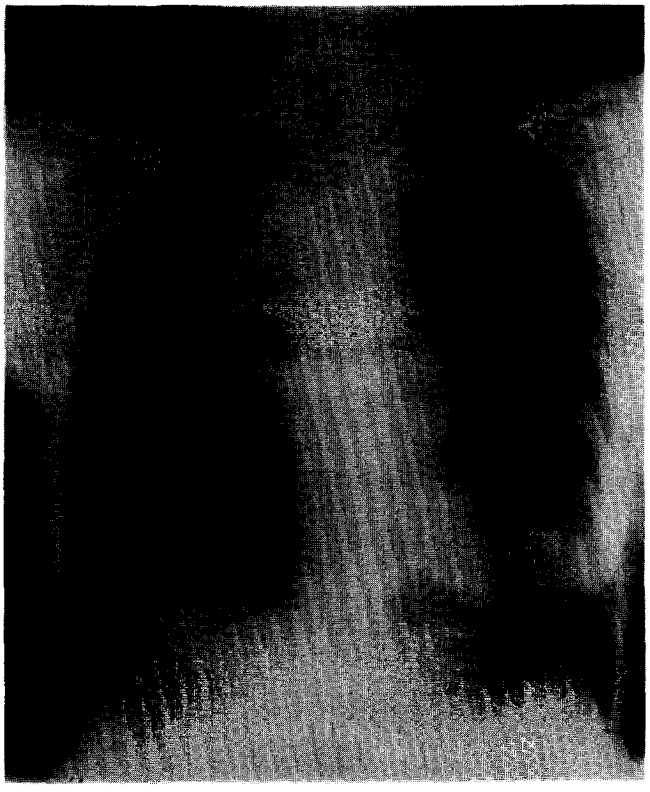
ALT cells demonstrate tumour specificity

ALT cells were derived from 3 patients with melanoma and *ex vivo* cytotoxicity against autologous and allogeneic melanoma including HT144 and ISW-1, as well as leukaemia (K562), pancreatic cancer (Capan-1) and osteogenic sarcoma (Saos-2) targets at an E:T of 20:1 were measured as described above using a 4-h and 18-h ^{51}Cr release assay. As shown in Table 4, ALT cells from patient ACIT-1 demonstrated greater *ex vivo* lysis against autologous melanoma targets with reduced lysis against the allogeneic HLA-matched melanoma target HT144, and not against the other allogeneic melanoma or irrelevant tumour targets. Patient ACIT-8 showed the greatest cytotoxicity against autologous tumour targets with some lytic activity against ISW-1 and ACIT-10, with no lysis of HT144 melanoma or other tumour targets. HLA typing revealed that patients ACIT-8, ACIT-10 and ISW-1, as well as cell lines Capan-1 and Saos-2, were HLA-A2$^+$, while patient ACIT-1 and the allogeneic melanoma cell line HT144 were HLA-A1$^+$. All lysis was significantly increased after 18 h. This demonstrates that relative and absolute *ex vivo* tumour specificity is possible using ALT cells derived from patients with melanoma and appears to be HLA-restricted.

Lysis of tumour targets is dependent on ex vivo *activated memory T-cells*

PBMC, ALT cells, CD45RO-depleted ALT cells, and CD56-depleted ALT cells were derived from 3 patients (ACIT-1, ACIT-8 and ACIT-10) with melanoma and *ex vivo* cytotoxicity against autologous and allogeneic melanoma as well as non-melanoma targets at an E:T of 20:1 were measured as described above using a 4-h and 18-h ^{51}Cr release assay. ALT cells were depleted of CD45RO$^+$ cells following activation of PBMC with T3CS to determine the importance of CD45RO$^+$ cells to the ALT cell population. Similarly, PBMC were depleted of CD56$^+$ NK cells prior to the generation of ALT cells to determine the relative contribution, if any, of NK cells to the *ex vivo* lysis of autologous tumour targets by ALT cells. As seen in Table 5, *ex vivo* lysis of autologous tumour targets by CD56-depleted ALT cells was reduced as measured by the 4-h ^{51}Cr release assay, but was comparable to whole ALT cell lysis at the 18-h assay. When CD45RO-depleted ALT cells were used as effector cells, tumour target lysis was reduced to near-PBMC levels during both the standard (4-h) and delayed (18-h) assay. This demonstrates that *ex vivo* lysis of melanoma tumour targets is mediated mainly by CD45RO$^+$ memory T-cells, is tumour-specific and appears to be HLA-restricted.

(a)



(b)

Figure 4. (a) Chest roentgenogram of a patient with metastatic melanoma before beginning ACIT showing widespread pulmonary disease. (b) Chest roentgenogram after six cycles of ACIT. There has been complete regression of all disease.

DISCUSSION

Clinical trials of adoptive cellular therapy in human patients have focused on lymphocytes activated *ex vivo* by IL-2. As reviewed by Weber and Rosenberg, LAK cells combined with exogenous IL-2 *in vivo* demonstrated some anti-tumour efficacy in human patients with metastatic melanoma with adoptive transfer accomplished by using 10^{10}–10^{11} lymphocytes [29]. IL-2 was administered as a continuous infusion, as bolus doses or as a hybrid programme consisting of a bolus and continuous infusion schedule. Despite manipulation of the IL-2 infusion schedule, the toxicity of IL-2 administration was quite substantial. The overall responses to LAK/IL-2 in patients with disseminated melanoma were approximately 15%. In an attempt to improve LAK/IL-2 responses, Sznol and associates combined LAK/IL-2 with chemotherapy using CY and doxorubicin (DOX) as ACIT in patients with disseminated melanoma [30]. The response rate of 17%, however, was not substantially improved over LAK/IL-2 alone.

The overall responses (31%) in patients with disseminated melanoma treated with TIL/IL-2 were generally superior to the LAK/IL-2 data [31–36]. The studies of adoptively transferred TIL and IL-2 by Rosenberg and associates and Dillman and associates were essentially ACIT trials as TIL/IL-2 were combined with CY and DTIC, respectively [31, 32]. Rosenberg and colleagues were also able to demonstrate responses to TIL in patients who had previously failed IL-2 therapy alone, showing that *ex vivo*, activated adoptively transferred T-cells can potentiate IL-2-based therapy [36]. The putative tumour specificity of human TIL was apparent *ex vivo* in TIL isolated from melanoma specimens, while TIL from RCC and other tumour specimens had broader tumour lytic capabilities. Limiting features of both LAK and TIL therapies include the requirement for labour-intensive generation of cells, as well as the severe toxicities due to the dependence of these cells on constant exogenous IL-2 supplementation. In addition, it has been shown that TIL T-cells are functionally impaired when isolated from tumour environments which may be due to the tumour environment influencing decreased receptor expression of the T-cell receptor ζ chain and $p56^{lck}$ tyrosine kinase so that TIL may not be the T-cells of choice in adoptive cellular therapy [37, 38]. The need for tumour tissue in TIL therapy also makes the potential for widespread use of this therapy somewhat problematic since tumour tissue is not readily available in many patients.

Based on these results and on our own clinical experience using ALT in patients with metastatic RCC, we felt it appropriate to evaluate the efficacy of ALT in another advanced tumour such as melanoma that is relatively resistant to standard anti-neoplastic therapies, yet potentially responsive to immune manipulation. In previous clinical trials of ALT in patients with metastatic RCC, the method of *ex vivo* activation of PBMC also evolved from that of using MLCS to T3CS. Because of greater technical ease generating T3CS as well as comparable ALT cell function using T3CS or MLCS, T3CS-activated PBMC became the standard in experimental and clinical ALT-based melanoma and RCC protocols. No difference in clinical outcome was noted in those whose PBMC were activated *ex vivo* with MLCS or T3CS [15–18]. Analysis of the cytokines in these supernatants revealed high levels of IL-1, IL-6, IL-8, TNF, IFN-γ and GM-CSF, and nearly undetectable levels of IL-2 in the final T3CS preparation, although there are significant levels of IL-2 measured in the first 24–48 h of T3CS generation ([19] and manuscript in preparation). This is consistent with a mechanism of ALT not dependent on exogenous IL-2. The $CD45RO^+$ T-cell

Table 4. Tumour specificity of ALT cells

ALT cell source	Tumour target source	4-hour % lysis	18-hour % lysis
ACIT-1	ACIT-1†	51.4	89.8
	ACIT-8*	3.4	4.4
	ACIT-10*	2.1	2.9
	HT144†	30.2	47.5
	ISW-1*	6.8	8.3
	Saos-2‡	2.9	3.7
	Capan-1§	1.8	2.4
	K562	1.1	2.0
ACIT-8	ACIT-1†	4.0	6.1
	ACIT-8*	57.2	82.2
	ACIT-10*	33.7	50.1
	HT144†	5.2	7.5
	ISW-1*	41.4	57.6
	Saos-2‡	2.2	3.5
	Capan-1§	2.2	4.1
	K562	1.5	1.9
ACIT-10	ACIT-1†	2.4	3.3
	ACIT-8*	25.2	44.3
	ACIT-10*	47.7	81.5
	HT144†	3.5	5.2
	ISW-1*	31.1	63.2
	Saos-2‡	3.6	5.4
	Capan-1§	2.8	4.2
	K562	1.9	2.7

Effector target ratio (E:T) = 20:1. * HLA-A2$^+$ melanoma. † HLA-A1$^+$ melanoma. ‡ HLA-A2$^+$ osteogenic sarcoma. § HLA-A2$^+$ pancreatic carcinoma.

Table 5. Lysis of melanoma tumour targets is dependent on ex vivo *activated memory T-cells*

Patient	MNC source	Tumour	4-hour % lysis	18-hour % lysis
ACIT-1	PBMC	ACIT-1/ACIT-8/ACIT-10	6.2/4.5/5.4	9.4/5.7/5.0
	ALT cells	ACIT-1/ACIT-8/HT144	45.8/5.1/32.4	72.3/5.8/52.1
	CD45RO-depleted ALT cells	ACIT-1/ACIT-8/HT144	3.1/1.5/2.2	8.2/1.2/3.6
	CD56-depleted ALT cells	ACIT-1/ISW-1/HT144	22.8/1.8/17.4	77.7/2.1/48.5
ACIT-8	PBMC	ACIT-1/ACIT-8/ACIT-10	2.6/7.1/4.4	2.5/8.8/6.7
	ALT cells	ACIT-1/ACIT-8/ISW-1/Saos-2	5.6/51.3/39.2/3.5	4.9/90.4/66.3/4.3
	CD45RO-depleted ALT cells	ACIT-1/ACIT-8/ISW-1	1.7/3.8/2.8	1.8/7.8/4.2
	CD56-depleted ALT cells	HT144/ACIT-8/ISW-1/Capan-1	1.1/25.2/12.5/2.4	2.4/81.5/49.2/3.6
ACIT-10	PBMC	ACIT-1/ACIT-8/ACIT-10	3.3/5.5/4.7	3.8/6.2/7.3
	ALT cells	ISW-1/ACIT-8/ACIT-10/Capan-1	35.8/29.4/47.1/5.2	57.2/48.3/88.4/6.6
	CD45RO-depleted ALT cells	ACIT-1/ACIT-10/HT144	2.2/4.1/2.9	1.8/7.6/4.5
	CD56-depleted ALT cells	ACIT-8/ACIT-10/ISW-1/Saos-2	15.2/26.5/19.2/1.9	46.7/79.5/42.8/2.8

Effector target ratio (E:T) = 20.1. MNC, mononuclear cell.

population appears to be the main effector population as *ex vivo* depletion of CD45RO$^+$ T-cells abrogated *ex vivo* auto-tumour lysis of RCC and soft-tissue sarcoma (STS) tumour targets in previous studies [20, 21]. This was also the case here as *ex vivo* depletion of CD45RO$^+$ T-cells also abrogated all lysis of melanoma tumour targets (Table 5). Here again, we demonstrate that memory T-cells form the primary lymphocyte population in ALT cells that contribute to the *ex vivo* cytotoxicity. Depletion of the CD56$^+$ (NK) cells had a marked effect on the 4-h but less so on the 18-h ^{51}Cr release assay (Table 5). It is not known if the memory T-cell population is directly responsible for tumour lysis and/or the initiator of tumour target lysis. Finke and colleagues have reported that separation and comparison of RCC TIL into CD4$^+$, CD8$^+$ and CD56$^+$ populations revealed minimal autologous tumour target lysis by the CD4$^+$ and CD8$^+$ T-cells in a standard 4-h ^{51}Cr release assay [39]. However, it was found that the CD3$^-$CD56$^+$ (NK) cells did mediate significant lysis in a 4-h ^{51}Cr release assay with CD4$^+$ and CD8$^+$ T-cells showing substantial lysis in a delayed (18- and 72-h) assay. It was also noted that similar to ALT cells, the T-cells in the TIL population were CD45RO$^+$, implying that many of these cells were memory T-cells [40]. It would then appear that memory T-cells are primarily effective on a delayed rather an immediate basis, an observation confirmed by Dye and North in murine studies [41].

ALT cells generated from patients with melanoma demonstrated *ex vivo* tumour specificity (Table 4). Patient ACIT-1, whost ALT cells were activated *ex vivo* by MLCS, showed tumour specificity against autologous tumour specimens and some reduced reactivity against the allogeneic melanoma cell line HT144, but not against other melanoma or non-melanoma tumour targets. ALT cells from patient ACIT-8 (activated *ex vivo* by T3CS) showed anti-tumour specificity against autologous tumour targets, as well as some reactivity against allogeneic tumour target ISW-1, but not against HT144 or other non-melanoma tumour targets. HLA tissue-typing revealed that ACIT-1 was HLA-A1$^+$, similar to the melanoma cell line HT144, while ACIT-8 and the metastatic lesion ISW-1, as well as Capan-1 and Saos-2 were HLA-A2$^+$. Autologous lymphocytes from patients in this study confirmed HLA status that matched their tumours. Despite stable disease when treated with ALT cells and CY, patient ACIT-10 nevertheless demonstrated an *ex vivo* cytotoxic effect against autologous tumour that was HLA restricted (A2$^+$). These data demonstrate the limited reactivity and tumour specificity of ALT cells, as well as confirming the results of previous studies where either HLA-A1 or HLA-A2 is a restricting element in *ex vivo* cytotoxicity of lymphocytes on melanoma tumour cells [42–45]. A subsequent study using an IFN-γ release assay as a marker of tumour-specific recognition, in conjunction with antibody blocking studies, confirmed HLA-A2 as a restricting element in *ex vivo* lysis mediated by CD45RO$^+$ memory T-cells (manuscript in preparation).

As shown in Table 2, 2 disseminated melanoma patients treated with ALT alone demonstrated CRs that are still ongoing at 64 and 44 months. Those who demonstrated CR or PR, as well as SD while on ALT had markedly prolonged survivals compared to those patients who were observed by Balch and associates and stratified according to survival by number of disease sites [28]. This is in agreement with previous results in patients with metastatic RCC where a partial response rate of 18% was observed, although 45% of patients had stable disease with a markedly prolonged survival over a carefully matched control group [15, 16]. This would argue strongly that SD is a valuable measure of anti-tumour effect in patients treated with anti-neoplastic therapy, be it chemotherapy, radiation or immune-based therapies. This concept has been confirmed by a recent survey which demonstrated that CR and/or PR to chemotherapy did not correlate with increased survival, and that patients with prolonged survival did not necessarily demonstrate measurable responses to therapy but did have SD [45]. This has been noted by other investigators, particularly in patients treated with adoptive cellular therapies [46, 47].

In murine tumour models, the combination of adoptively transferred T-cells and CY is able to cure significant numbers of TBH with both haematopoietic and solid tumours [48, 49]. Adoptively transferred T-cells and other chemotherapeutic agents such as DOX and cisplatin (CDDP) have also demonstrated significant anti-tumour activity in murine TBH [50, 51]. In a murine model of ACIT using *ex vivo* activated memory T-cells and CY, mice with metastatic melanoma and carcinoma were cured of their disease in a tumour-specific way using subtherapeutic numbers (10^6) of memory T-cells [22]. In human patients, Rosenberg and co-workers used CY together with TIL and IL-2 as an ACIT protocol for human metastatic melanoma patients with demonstrable objective responses [31, 36]. Takayama demonstrated that TIL, together with DOX or mitomycin-C, comprised a promising form of ACIT in human TBH with metastatic or primary liver tumours [52], and ACIT using ALT cells and CY demonstrated initial promising results in patients with various solid tumours [53, 54].

Aside from its direct anti-tumour effects, it is well known that CY possesses potent immunomodulatory properties which are diverse and dose-dependent. As demonstrated by Berd and Mastrangelo in patients with metastatic melanoma, low-dose CY can enhance immune responses in human TBH through interaction with and inhibition of CD45RA$^+$ (naive) T-cells [55]. Oh and co-workers noted that low-dose CY is capable of downregulating the serum concentration of suppressive E-receptor (SER) haptoglobin, an acute phase protein found only in human TBH which inhibits T-cell proliferation as well as IL-1 and IL-2 function [56]. Therefore, low-dose CY was used in this study in order to attempt reversal of tumour-associated immunosuppression prior to administration of ALT cells. High-dose CY is capable of downregulating both cellular and humoral immune responses and so can be used in an attempt to suppress the generation of anti-idiotypic T-cell responses upon infusion of ALT cells [57]. We sought to take advantage of this bi-modal property of CY, by incorporating both high- and low-dose CY into the ACIT study in patients with relapsed and refractory solid tumours [53]. In addition, our preclinical model of mice with advanced melanoma demonstrated *in vivo* synergy between adoptively transferred *ex vivo* activated memory T-cells using either high- or low-dose CY [22]. Since the patients in this study had disseminated melanoma that is not intrinsically sensitive to CY, it was very unlikely that infusions of CY alone would have been effective at any dose and that any anti-tumour effects observed would be due to immunological synergy of the adoptively transferred T-cells and CY.

Based upon these results, and in an effort to improve responses in patients with disseminated melanoma who had failed chemotherapy and who had three or more sites of disease, we elected to treat some patients with disseminated melanoma with ACIT using ALT cells and CY. As seen in Table 3, of 9 evaluable patients, 2 had CR to ACIT and are still disease-free after more than 50 and 24 months. 3 patients had PR and 2 patients had SD with prolonged survival over historical controls, noted in these

3 patients with three or more sites of metastatic disease. Following a PR, patient ACIT-9 had had stable radiographic disease for 21 months and, to date, has refused surgical biopsy to determine if there is residual disease or fibrosis present. Toxicity of ALT and ACIT using ALT cells and CY was minimal, as previously described [53]. As adoptive cellular therapy not dependent on exogenous IL-2, the toxicity profile of ALT or ACIT using ALT cells and CY is significantly less than LAK or TIL therapies.

We have demonstrated that adoptive transfer of *ex vivo* activated memory T-cells with and without CY is safe, biologically active and can result in clinical benefit for patients with disseminated melanoma. Whether the addition of chemotherapeutic agents such as CY or CDDP can significantly enhance the activity of ALT cells in patients with larger tumour burdens or in those with chemotherapy-resistant disease remains to be seen. The previous clinical study of ACIT with ALT cells and CY in patients with solid tumours showed that patients who had relapsed from their previous chemotherapy but were not primarily refractory, demonstrated anti-tumour responses to ACIT using ALT cells and CY [53, 54]. This is in agreement with what was seen in the melanoma patients here and suggests that the synergy demonstrated in murine models of ACIT may also be seen in human TBH with potentiation of immunotherapy by chemotherapy. It is also possible that biotherapy may potentiate chemotherapy, as patients who progress after immunotherapy may demonstrate surprisingly good responses to salvage chemotherapy (Mittleman A, MacLean G, personal communications).

It appears that anti-neoplastic therapy using tumour-specific adoptively transferred T-cells, such as TIL or ALT cells, has the greatest potential for effective cellular immunotherapy of disseminated melanoma as tumour-specific T-cells have been shown to traffic preferentially to sites of neoplastic disease. The manipulation of T-cells *ex vivo* with adoptive transfer of T-cell immunity together with synergistic combinations of cytotoxic and immunomodulatory chemotherapeutic agents, as well as other methods to increase immunogenicity of melanoma targets, holds the promise for the potential success of adoptive cellular therapy in patients with advanced metastatic melanoma.

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