

## Treatment with tumour infiltrating lymphocytes and interleukin-2 in patients with metastatic melanoma: A pilot study

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**Key words:** interleukin-2; metastatic melanoma; tumour infiltrating lymphocytes.

**Abbreviations:** ICAM-1: intercellular adhesion molecule 1; IFN-g: interferon-gamma; IL-2: interleukin-2; IU: international units; LAK: lymphokine activated killer cells; TIL: tumour infiltrating lymphocytes.

### Abstract

Tumour infiltrating lymphocytes (TIL) were isolated and expanded from biopsy samples of 4 patients with metastatic melanoma. The patients were treated with autologous expanded TIL and continuous or bolus infusion of Interleukin 2 (IL-2) at a dose of  $18 \times 10^6$  International Units/m<sup>2</sup>/day for 5 days starting 36–48 hours after administration of cyclophosphamide at a dose of 1 g/m<sup>2</sup>. The number of TIL infused ranged from  $10^{10}$  to  $5,56 \times 10^{10}$  cells. Two patients had stable disease (SD) lasting for 2½ and 4 months respectively and they died 24 and 13 months after therapy. One patient died during therapy due to a pseudomonas septicaemia and another patient developed progressive disease (PD). He died 3 months after the start of therapy. The side effects were substantial but most of them were reversible upon cessation of the treatment.

The majority of the expanded TIL of all patients were of the CD8+ phenotype. Cutaneous metastases from two patients, removed after treatment with IL-2 and TIL, showed moderate lymphocytic infiltration also mainly of CD8+ T cells.

The treatment with IL-2 and TIL is feasible, but further investigations should continue in an attempt to improve the efficacy of the therapy, to reduce toxicity and to diminish the costs and labour of the culture methods.

### Introduction

Metastases of a variety of tumours are infiltrated by lymphocytes [1–3]. These tumour infiltrating lymphocytes (TIL) can be isolated from tumour specimens, expanded *in vitro* with interleukin-2 (IL-2) and administered back to tumour-bearing animals or patients [4–8]. These cells have been shown to have increased specific cytolytic activities against their autologous tumours and they require less IL-2 to support their activity than

lymphokine activated killer (LAK) cells [5, 6]. In contrast to LAK cells, which are often non T, non B, “null” lymphocytes capable of killing a wide variety of tumour cells without major histocompatibility complex (MHC) restriction [9, 10], TIL have been reported to show specific, MHC restricted killing of autologous tumour cells and are generally of the CD3 phenotype [5, 6, 11, 12]. These antigen specific TIL have been more effective in animal tumour models than antigen nonspecific effector cells [5]. TIL, unlike

LAK cells, have the ability to traffic to tumour sites [13], which may be important for their efficacy.

These data have stimulated the initiation of a number of phase I clinical trials utilizing *in vitro* expanded TIL isolated from human solid tumours, which were administered back to the patients together with IL-2 [4, 6–8]. Cyclophosphamide has sometimes been incorporated into these protocols [6] because in animal models it has been demonstrated that pretreatment of the tumour bearing host with either cyclophosphamide or total body irradiation was required for this treatment to be successful [14–16].

We have conducted a pilot study in four patients with metastatic melanoma and present these data with the aim of pointing out some of the potential and problems with this form of adoptive immunotherapy.

#### Patients and methods

This pilot study was approved by the Ethical Committee of the Free University Hospital and all patients had given informed consent.

##### Patients

Four patients (three male, one female) with histologically proven metastatic melanoma entered the study. All patients were evaluable for response by physical or radiographic examination. The Karnofsky performance status was between 80–100%. The patients had normal liver function (bilirubin  $<20 \mu\text{mol/l}$ ; partial thromboplastin time  $\leq 1.3$  times control), adequate bone marrow function (leucocytes  $>3 \times 10^9/\text{L}$ , granulocytes  $>1.5 \times 10^9/\text{L}$ , thrombocytes  $>100 \times 10^9/\text{L}$ ), a life expectancy of more than 3 months and were aged between 34 and 49 years.

The patients had no significant cardiac disease, active infection requiring systemic antibiotic therapy or central nervous system metastases. All four patients had received prior therapy: 2 patients radiotherapy, 1 patient chemotherapy with dacarbazine, and all four patients immunotherapy (1 patient IL-2 alone; 2 patients Interferon-gamma (IFN-g) + IL-2; 1 patient Active Specific Immunization (ASI) + IFN-g). Meta-

static sites included lung (3), bone (2), lymph nodes (2), skin or subcutaneous tissue (3). All patients had 2 or more metastatic sites.

##### Culture methods

Tumour deposits were resected under local anaesthesia. The sources of the TIL were lymph node (patient A), cutaneous (patient C) and subcutaneous (patients B and D) metastases. The excised tumour specimens were transported in a sterile manner from the surgical suite to a laminar flow hood. The dissociation of the tumour specimens was performed as described by Peters et al [17] at a temperature of 37°C with 0.14% collagenase and 0.1% DNA-se (Boehringer Mannheim GmbH).

Upon disaggregation, the cells were put into culture in AIM-V medium (Gibco), containing 50 IU penicillin, 50  $\mu\text{g}$  streptomycin, 50  $\mu\text{g}$  gentamicin, 750 ng fungizone, 5 U heparin and 6000 International Units IL-2 per ml. In addition, the medium was enriched with 20% homologous LAK supernatant. This medium was called "complete" AIM-V. The suspension containing both TIL and tumour cells was placed in gas permeable PL732<sup>R</sup> 3 L bags (Fenwal) at a density of  $2.5 \times 10^5$  cells/ml. After 1 week or when densities of  $1 \times 10^6$  cells/ml were reached, the cells were harvested and replaced in fresh medium at a density of  $2.5 \times 10^5$  cells/ml. Upon further expansion, the cell suspension was diluted periodically to a density of  $2.5 \times 10^5$  cells/ml in complete AIM-V medium. The culture bags were incubated in a flat position at 37°C in a humidified 5% CO<sub>2</sub> air incubator. Before, midway and at the end of the culture period small samples of the TIL culture were drawn for phenotyping and cytotoxicity assays. The viability was checked with the trypan-blue method (equal volumes trypan-blue and cultured cells). Bacteriological cultures were made from each bag at regular intervals to rule out infection in the TIL cultures. The time between the start of the culture and administration of TIL to the patients was  $\pm 25$ –40 days. When enough TIL were grown (patient A:  $3.5 \times 10^{10}$ ; patient B  $8.5 \times 10^{10}$ ; patient C  $5.4 \times 10^{10}$ ; patient D  $3 \times 10^{10}$ ) they were harvested, filtered and administered back to the patient.

### *Treatment plan*

The patients were pretreated with cyclophosphamide  $1\text{ g/m}^2$  intravenously 36–48 hours before the start with IL-2 and TIL. The patients were prehydrated with 2 L 0.9% NaCl over 6 hours. Metoclopramide 40–60 mg i.v. was given before the cyclophosphamide. The cyclophosphamide was administered in 1 hour in 500 ml 0.9% NaCl. The patients A, B and C were treated with  $18 \times 10^6$  International Units (IU) IL-2/ $\text{m}^2$ /day for 5 days as continuous infusion according to the treatment scheme of West [18] and patient D with 3 dd  $6 \times 10^6$  IU IL-2/ $\text{m}^2$ /day as bolus infusion over 15 minutes for 5 days according to the scheme of Eberlein et al [19].

The patients received TIL infusions on the first and second day and the patients C and D also on the third day of the IL-2 administration. The TIL were given in 250 ml 0.9% NaCl over 1 hour. During the TIL infusion the continuous administration of IL-2 was interrupted and restarted again after the end of the the TIL infusion. The number of TIL cells given per infusion ranged from  $2.05 \times 10^9$  to  $2.63 \times 10^{10}$  cells. The total number of the TIL infused was  $10^{10}$  cells in patient A;  $5.56 \times 10^{10}$  cells in patient B;  $3 \times 10^{10}$  cells in patient C and  $3 \times 10^{10}$  cells in patient D. The patients received one TIL infusion per day, except for patient B to whom 2 TIL infusions were administered on the second day of the treatment with IL-2.

The patients received standard supportive care.

### *Toxicity and response criteria*

The toxicity criteria of the WHO were used. The response criteria were as follows: stable disease (SD) constituted a less than 25% change in tumour measurements for more than 4 weeks; progressive disease (PD) was defined as a 25% or greater increase in the measurable disease or the appearance of new lesions.

### *Product description*

Recombinant IL-2 was provided by EuroCetus B.V. (Amsterdam, the Netherlands). The IL-2 had a nominal specific activity of  $3 \times 10^6$  Cetus

U/mg ( $18 \times 10^6$  International Units/mg of protein). The IL-2 was supplied as a lyophilized powder which when reconstituted had a concentration of  $3 \times 10^6$  Cetus U/ml =  $18 \times 10^6$  International Units/ml.

The Units of IL-2 used in this article are International Units.

### *Phenotyping of the TIL cultures*

Quantitative analysis of cell (sub)populations and determinations of the activational state were done by single and dual FACSTAR immunofluorescence analysis (FACSTAR plus, Becton Dickinson) using a panel of commercially available monoclonal antibodies (Becton Dickinson) as marker. Preceding the analyses, a sample of the TIL suspension was fixed by 0.5% formaldehyde in phosphate buffered saline (PBS) during 5 min.

Single and dual immunofluorescence were performed by incubating  $2.5\%10^5$  fixed cells with appropriate dilution of each monoclonal antibody ( $20\ \mu\text{l}$  end volume) during 30 min at room temperature. For indirect fluorescence staining a second step was performed with diluted rabbit antimouse fluorescein isothiocyanate conjugated (FTIC) immunoglobulin (Ig) (Dakopatts) during 30 min. The stained cells were resuspended in  $300\ \mu\text{l}$  PBS and analyzed on the flowcytometer. The percentage of positive cells for each marker were determined at the start, half-way and at the end of the culture period.

### *Cytotoxicity assays*

Cytotoxic activities of the TIL (effector cells) were assessed by using a standard  $\text{Cr}^{51}$  – release assay against K562, a natural killer (NK) and LAK sensitive chronic myelogenous leukemia cell line and against Daudi, a NK resistant, LAK sensitive Burkitt lymphoma line. In addition cytotoxic activities were tested against M14, a malignant melanoma cell line and P815, a malignant mouse mastocytoma cell line, the latter in the presence of anti CD3 [20, 21]. Under these conditions, the P815 cell-line is killed by cytolytic T cells, irrespective of their antigen specificity [20, 21]. The cytotoxic assays were performed as outlined in detail elsewhere [7]. Effector cells

were added to the wells to achieve an effector to target (E/T) ratio of 12.5:1, 25:1, 50:1 respectively.

#### *Handling of excised metastases*

After the treatment with IL-2 and TIL a cutaneous metastasis was removed in the patients C and D. Upon arrival in the pathology laboratory the specimen was halved. One half was snap-frozen in liquid nitrogen for immunohistochemistry. The other half was fixed in buffered formalin and embedded in paraffin for routine histological evaluation. From the paraffin embedded material a Hematoxylin-Eosin stained section was prepared. Immunohistochemistry was performed on 5 µm thick sections using an alkaline phosphatase-anti alkaline phosphatase method, developed with new fuchsin in order to prevent problems in evaluation of the staining results in heavily pigmented lesions. Monoclonal antibodies used for immunohistochemistry were purchased from Becton Dickinson (Sunny Vale, California) and Ortho (Raritan, New Jersey).

## **Results**

#### *Toxicity and response*

The cyclophosphamide was well tolerated by 3 of the patients, with the exception being patient D who experienced grade III nausea and vomiting in spite of intravenous administration of metoclopramide and lorazepam. Side effects directly related to TIL infusions were chills and WHO grade II-III fever in all patients. During the treatment with IL-2 all patients suffered from nausea, vomiting, anorexia, erythrodermia, fever (WHO grade II), chills, hypotension (grade III-IV) and neuropsychiatric disturbances, ranging from drowsiness (patient D), confusion, lethargy (patients B and C) to disorientation and psychosis (patient A). Two patients developed diarrhoea (grade I-II) and three patients stomatitis (grade I-II). The objective toxicities consisted of anaemia requiring blood transfusions (patients B, C), thrombocytopenia  $<50 \times 10^9/l$  (patients A, B, C), WHO grade I-II rise in creatinine (patients A, B, C), WHO grade I rise in bilirubin (patient B), WHO grade III-IV rise in bilirubin (patient A, C) and a WHO grade I-II rise in G-GT (patients A, C, D). Weight gain as % of baseline was 2,9% in patient D; 6,4% in patient A and 14,4% in patient C.

Patient B developed a severe septicaemia with *Pseudomonas aeruginosa* on the second day of treatment, which caused the death of the patient 2 days later in spite of intensive care treatment. The source of the septicaemia was never found. All culture bags were negative for *Pseudomonas aeruginosa*. At autopsy the patient had multiple lung metastases, several subcutaneous metastases and one metastasis in the distal left femur.

Patient C suffered from an anterolateral myocardial infarction (CPK max. 1096 U/L, MB89%, LDH max 1371 U/L), one day after the end of treatment. He needed vasopressor support with dopamine and dobutamine. Four days later, the patient developed an adult respiratory distress syndrome and artificial ventilation was necessary. Antibiotics were administered, because it was thought that the patient might have a sepsis, but all cultures remained sterile. The patient recovered from this period with an adequate myocardial function. He had SD for 4 months. Thereafter he developed new cutaneous metastases and haemoptysis, probably due to recurrence of the intrabronchial metastasis, which had been treated one year previously with laser therapy. Twelve months after the therapy with TIL and IL-2, it became apparent that the patient had cerebral metastases, which caused his death one month later.

Patient A had a SD for 2½ months. Thereafter he experienced PD in his lung- and lymph node metastases. He died 24 months after the start of therapy due to respiratory insufficiency caused by a pneumonia in combination with lung fibrosis. Autopsy was not performed.

Patient D experienced progressive disease after the therapy with IL-2 and TIL. He died 3 months after the start of this therapy due to his malignant disease. At autopsy, wide-spread metastatic disease was found.

#### *Characteristics of the TIL and growth curves*

The growth curves of the TIL cultures are shown in fig. 1. After an initial decline of the cell

number due to death of tumour cells, the TIL started to grow with a lag phase of 7–20 days after the start of incubation. The cells could be harvested for administration to the patients after a culture period of 3–5 weeks.

The majority of the TIL of all patients consisted of cytotoxic T lymphocytes (CD3+, CD8+) at the end of the culture period. Monocytes (CD14+), B cells (CD19+) and lymphocytes of the LAK/NK phenotype (CD56+) only accounted for more than 5% of the total cultured cells in the end culture of patient D, but this may, however, have been due to non-specific immunofluorescence.

The expression of HLA-DR antigens was up-regulated in all cultures, reaching a peak level at 14–34 days after incubation, with a slight decline towards the end of the culture period.

The expression of homing receptors (CD44) and the Leucocyte Function Antigen 1 (LFA-1) increased during the whole culture period. The same holds true for expression of CD45RO (a marker for activated T cells and memory T cells). The TIL culture of patient A showed a decline of the expression of CD45RO by the end of the culture period.

Upregulation of CD25 (P55 chain of the IL-2 receptor) could not be detected in either culture. The expression of intercellular adhesion molecules (ICAM-1, CD54+ cells) showed a different pattern in the cultures. The ICAM-1 expression on day 1 was probably due to positive tumour cells and at the end of the culture due to positive lymphocytes.

The cytotoxic properties (E/T ratio 50:1) of the TIL at the end of the culture period are shown in Table 1. Except for the TIL of patient A, low cytotoxicity was observed for the K562, Daudi and M14 cell lines.

Overall T cell cytotoxic activity, as measured by

*Table 1.* Cytotoxicity assays. Percentage of target cells killed during 4 hours at an E/T ratio of 50:1 by effector cells from the final TIL culture.

Target	Patient A day 21	Patient B day 35	Patient C day 39	Patient D day 35
K562	42%	11%	3%	2%
Daudi	35%	1%	2%	2%
P815	43%	28%	79%	82%
M14	28%	3%	3%	3%

P815 killing in the presence of antiCD3 was most pronounced in the cultures of the patients C and D.

#### *Immunohistochemistry*

In both cases (patients C and D) the cutaneous metastases removed after therapy showed the same pattern. The number of infiltrating cells was low to moderate. Most cells were seen at the interface of the tumour and the surrounding tissue. Cells within the tumour were mostly present in small aggregates and rarely as a diffuse infiltrate throughout the tumour.

The infiltrates contained virtually no B lymphocytes. The T cells were mostly CD8 positive cells with a predomination over CD4 positive cells in a ratio of approximately 10:1. At least some of these cells were HLA-DR positive, but this phenotype was always in the minority. Only few scattered cells were CD25 positive and similarly Leu7 positive (natural killer and T cell subsets) cells were very scarce. Macrophages were found in the same distribution as the T lymphocytes, the majority being at the margins of the tumour.

Langerhans cells (CD1 positive) showed the same distribution pattern as the macrophages. The tumour cells of the cutaneous metastasis of patient C partly expressed HLA-DR.

#### **Discussion**

Interleukin-2 with or without adoptive cellular therapy can induce responses in 15–30% of the patients with metastatic melanoma [6, 8, 18, 19, 22]. Encouraging experiments employing adoptive immunotherapy with cytotoxicity T cells in animal models [5, 23] have prompted the initiation of a number phase I clinical studies in melanoma patients [4, 6, 8]. After the initial report of Rosenberg and colleagues [6], further publications relating to this treatment approach have been rather scarce and results were often disappointing [4, 8]. The four patients that we have treated with IL-2 and TIL highlight some of the problems that can be encountered during this treatment and provide some insight into the

reasons why this therapy has not been studied as extensively as might have been expected based upon the first encouraging reports.

The culturing of TIL in bags is expensive (\$21,000) and laborious requiring a considerable incubator space, large volumes of culture medium (80–120 l), IL-2 and a lot of technician time. Strict working is required in order to prevent bacteriological or fungal infections in the TIL cultures [6]. Recent technological advances may offer the possibility of replacing the culture bags with a hollow fibre bioreactor [24]. This equipment is compact, uses considerably less medium (4.3 l/10<sup>10</sup> TIL) and IL-2 and is less expensive (\$6000). In addition, an estimated 80% decrease in technical time can be obtained when this methodology is used [24]. The system is completely closed and replacing the culture medium is easily and quickly performed which means that the risk of infection is small.

In our patients the time taken to generate sufficient TIL for therapy varied between 25 and 40 days. This relatively long time scale before therapy can be initiated, means that a proportion of patients will develop progressive disease during the culture period. This leads to the inevitable fact that some patients will no longer be suitable for such treatment or even have died by the time that sufficient cells are available [6]. Exposure of the TIL to monoclonal antibodies such as anti CD3 and anti CD28 which are capable of providing an activation signal to the TIL may shorten the initial lag phase before exponential growth begins [25] (Figure 1).

The TIL cultures of the patients B, C, D showed low cytotoxicity against K562, Daudi and M14 cell-lines, which are preferentially killed in a MHC unrestricted way by NK or/and LAK cells [9, 10]. T cells can have antigen aspecific cytotoxic capacities (TIL culture of patient A), but the physiological role of this phenomenon is unclear [26]. Modifying the culture conditions by adding other cytokines such as Interleukin-4 [27] or tumour necrosis factor [28] might enhance the specific autologous cytotoxicity of TIL. Re-exposure to autologous tumour cells during culture could help generate a broad repertoire of cytotoxic T cell clones capable of recognising a number of different tumour associated antigens thus overcoming the problem of tumour cell hetero-

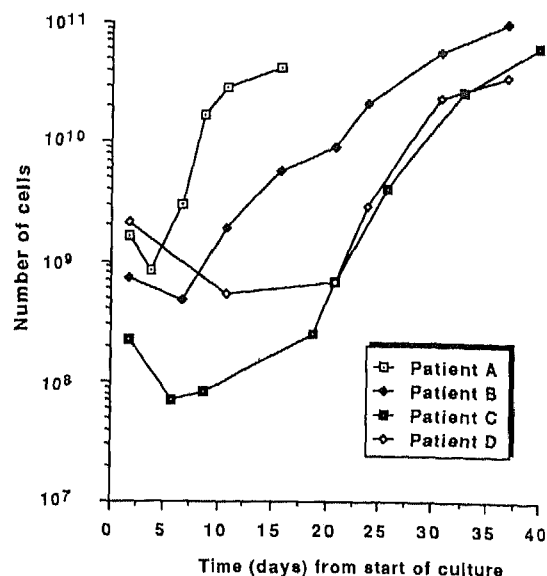


Fig. 1. Growth curves of the TIL over time. After initial decline in cell numbers due to death of tumor cells the TIL start to grow after a lag phase of 7–20 days.

geneity and maximising the possibility of achieving tumour eradication [29, 30]

The complex interaction of T cells with their target cells is not only determined by the expression of antigen and antigen-specific receptors, but also by the mutual expression of adhesion molecules [31]. This is illustrated by the fact that the susceptibility of melanoma cells to cytotoxic T cells is influenced by the expression of HLA-antigens [31–33] which play a role in antigen-specific recognition by T cells. On the other hand it is also determined by the expression of adhesion molecules such as ICAM-1 [31, 34, 35], LFA-1 (CD11a, CD18), the ligand for ICAM-1 [31] was upregulated during the culture period of the TIL of our patients. The same holds true for the expression of CD44, which might play a role in T cell activation, the extravasation of TIL from blood vessels and their ability to travel to metastatic sites [36]. Factors, governing homing, might determine at which sites anti-tumour response occurs. Sites, such as the central nervous system, which are not preferential homing places for recirculating lymphocytes, might be prone to relapse of metastatic disease after successful immunotherapy [37].

Furthermore, it was striking that after therapy

the infiltrates around the metastases of patients C and D were found at the margins of the deposits and thus were rather tumour "observing" than truly "infiltrating" lymphocytes. More knowledge and clinical experience have to be developed in order to evaluate the contribution of adhesion molecules to anti-tumour response. In addition, the role of specific T cell subsets in the induction of anti-tumour responses has to be further elucidated. Although anti-tumour activity of a bulk population of TIL is usually contained within the CD8+ subset of T cells [29], also TIL with the CD4+ phenotype has been shown to be capable of contributing to anti-tumour response in some patients [4, 6].

The inability of fresh TIL from most human solid tumours to kill tumour cells [29] and the long lag phase of *in vitro* TIL cultures before they start to proliferate in spite of stimulation with a high-dose of IL-2 indicate that tumour tissue might be the site of active suppression of lymphocyte function [8, 38]. The tumour cells can produce immune-suppressive factors such as prostaglandin E2 [39], transforming growth factor- $\beta$  [40] gangliosides and lipoprotein antigens [41]. CD4+ lymphocytes from melanoma invading lymph nodes can induce CD8+ suppressor cells to inhibit the anti-tumour activity exerted by peripheral blood T effectors [42]. In mice, pretreatment with cyclophosphamide enhanced the anti-tumour efficacy of this treatment approach, probably by eliminating suppressor cells, facilitating lymphocyte "homing" and enhancing the growth, cytotoxicity and phenotypic expression of cytotoxic T cells [5, 7, 43]. The value of this pretreatment of patients receiving IL-2 and TIL is, however, still not clear [4,44]. Rosenberg et al [44] have demonstrated the feasibility of gene transfer in TIL, offering the possibility of inserting the genes of cytokines into T cell effectors. This might result in the local production of cytokines thus counteracting tumour related suppressive influences without the toxicity of systemically administered agents.

The side-effects of IL-2 treatment can be very serious [6, 18, 19, 22] as was observed in our patients. Patient B died because of a septicaemia during therapy and patient C developed a myocardial infarction. These toxicities are recognised complications of IL-2 treatment [45] and

more investigations have to be done to explore the mechanisms by which IL-2 induces toxicities in the hope of managing them more effectively.

The optimal dose and schedule for IL-2 therapy has still not been found. Treatment of the patient with a combination of IL-2 and other cytokines such as Interferon- $\alpha$ , which was very effective in the animal models [46] might improve anti-tumour responses.

In summary, the potential of TIL in anticancer therapy depends on the successful generation of tumour-reactive or tumour-specific cytolytic cell lines of clones and their effective delivery to tumour sites. Human TIL, unlike those in murine tumours, are not readily triggered by autologous tumour cells and IL-2 [29]. Additional activation signals and synergistic effects of several cytokines may have to be combined to assure that tumour specific T cells can be derived in the hope of improving treatment strategies for patients with metastatic melanoma.

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