Adoptive Cell Therapy for Metastatic Melanoma Patients: Pre-clinical Development at the Sheba Medical Center

Michal J. Besser PhD[1], Avraham J. Treves PhD[2], Orit Itzhaki PhD[1], Izhar Hardan MD[3], Arnon Nagler MD[3], Moshe Z. Papa MD[4], Raphael Catane MD[5], Eyal Winkler MD[6], Bruria Shalmon-Zifroni MD[7] and Jacob Schachter MD[1]

[1]Ella Institute for Treatment and Research of Melanoma, [2]Sheba Cancer Research Center, [3]Institute of Hematology, [4]Department of Surgical Oncology C, [5]Oncology Division, [6]Department of Plastic & Reconstructive Surgery and [7]Institute of Pathology, Sheba Medical Center, Tel Hashomer, Israel

Key words: melanoma, immunotherapy, tumor-infiltrating lymphocytes, interleukin-2, adoptive cell therapy

Abstract

Background: Metastatic melanoma is an aggressive and highly malignant cancer. The 5 year survival rate of patients with metastatic disease is less than 5% with a median survival of only 6–10 months. Drugs like dacarbazin (DTIC) as a single agent or in combination with other chemotherapy agents have a response rate of 15–30%, but the duration of response is usually short with no impact on survival. Interleukin-2-based immunotherapy has shown more promising results. The National Institutes of Health recently reported that lymphodepleting chemotherapy, followed by an adoptive transfer of large numbers of anti-tumor specific tumor-infiltrating lymphocytes, resulted in an objective regression in 51% of patients.

Objectives: To introduce the TIL technology to advanced metastatic melanoma patients in Israel.

Methods: We generated TIL cultures from tumor tissue, choosing those with specific activity against melanoma and expanding them to large numbers.

Results: TIL cultures from nine patients were established and examined for their specific activity against the patients' autologous tumor cells. Twelve TIL cultures derived from 5 different patients showed the desired anti-tumor activity, making those 5 patients potential candidates for the therapy.

Conclusions: Pre-clinical studies of the TIL technology in a clinical laboratory set-up were performed successfully and this modality is ready for treating metastatic melanoma patients at the Sheba Medical Center's Ella Institute.

IMAJ 2008;6:164–168

Melanoma is the most malignant form of skin cancer and the fifth most common cancer type in both men and women [1]. Australia and Israel have the highest occurrence of melanoma incidences in the world [1]. According to the Israel Ministry of Health, almost a thousand new cases of melanoma were reported in Israel in 2002. Statistical data demonstrate that of 100,000 people in Israel, 16 men and 14 women develop melanoma each year [2]. Fortunately, in the majority of patients, melanoma is diagnosed at an early stage and can be cured by surgical resection. For those patients who either present or recur with an advanced disease, the prognosis remains poor and the median survival is in the range of 6–10 months.

Chemotherapy with single drugs such as DTIC (dacarbazine) or cisplatin can induce objective tumor regressions in only 15–20% of patients, with no influence on survival [3]. Combination chemotherapy can achieve a response rate of about 30% but has not shown a survival advantage over single-agent chemotherapy.

The spontaneous regression of certain cancers, such as melanoma or renal cell cancer, supports the idea that the immune system is sometimes capable of delaying tumor progression and on rare occasions eliminating a tumor altogether. These observations have led to research interest in a variety of immunologic therapies designed to stimulate the immune system. Immunotherapy based on interferon-alpha or interleukin-2 has elicited a similar response rate to that of chemotherapy, with some of the patients achieving a complete response [4]. As a result, the need to improve treatment for advanced melanoma patients is of vital importance.

The term adoptive immunotherapy describes the transfer of immunocompetent cells to the tumor-bearing host. The major research challenge in adoptive immunotherapy is to develop immune cells with specific anti-tumor reactivity that could be generated in large enough quantities for transfer to cancer patients.

In 2002 Rosenberg et al. [5] described a new technology to generate autologous tumor-infiltrating lymphocyte cultures from patients with metastatic melanoma. Tumor-infiltrating lymphocytes that specifically recognize tumor cells were selected, and then expanded to large numbers of activated anti-tumor effective T cells [5]. These T cells were adoptively transferred back to the pre-treated patient and had the ability to mediate the rejection of large vascularized tumors. This novel therapeutic approach consists of two major parts – laboratory and clinical [4]: The first involves large-scale production of melanoma-specific T cells, derived from tumor tissue surgically removed from patients with metastatic melanoma. The second consists of lymphodepleting chemotherapy, performed prior to TIL administration, which provides the proper environment for the anti-tumor lymphocytes and the addition of a high dose of IL-2 administered with the TIL in order to maintain the activity of the cells *in vivo*. A further report of this study published in the *Journal of Clinical Oncology* [6] and

TIL = tumor-infiltrating lymphocytes

IL = interleukin

the *Proceedings of the National Academy of Sciences* [7] demonstrated that using the above-mentioned technology an objective response rate of 51% (18 out of 35), including three complete responders, could be achieved in patients with advanced melanoma. All those patients were heavily pre-treated with chemotherapy and/or immunotherapy (including high dose IL-2). Such a high response rate cannot be accomplished with any other known technology. This promising therapy was offered at the NIH only, until today. A new facility (the Ella Institute) was established at the Sheba Medical Center in Israel and is devoted to adopting this promising technology in a clinical compatible processing laboratory and providing it to metastatic melanoma patients in Israel. The Ella Institute will also work on methods to simplify the adoptive cell transfer technology and uses this platform to extend its practice to other malignancies. The laboratories of the Ella Institute work under Good Laboratory Practice regulations and offer three clean laboratories (ISO 7, class 10,000) for the generation of anti-tumor potent T cells as well as additional cell therapy modalities for future use. In the present study we provide the data of the pre-clinical development work of the TIL production at the Ella Institute.

Patients and Methods

Patients

Melanoma patients at stages III and IV (except for patient #1 who had a stage IIB melanoma) undergoing a surgical procedure for clinical reasons signed an informed consent to provide tumor tissue and participate in the study. They were all negative for human immunodeficiency virus as well as hepatitis B and C infections.

Methods

The generation of TIL with specific reactivity against tumor antigens was adopted precisely as published by Dudley et al. [8], attempting to include clinically compatible reagents and good laboratory practice in a class 10,000 clean laboratory. It consists of the following stages:

- Isolation of TIL from metastases or primary tumor.
- Selection of T lymphocytes with high activity against the tumor, by determining IFNγ secretion levels after co-culture of TIL and autologous melanoma cells.
- E*x vivo* expansion and activation of the selected melanoma-reactive T lymphocytes by using anti-CD3 antibody, IL-2 and irradiated peripheral blood mononuclear cells from non-related donors as feeder cells. Basically, melanoma tissue from metastatic or primary tumor is surgically removed. After a pathologist divides and examines the fresh tissue it is sent to the laboratories of the Ella Institute for isolation of TIL and tumor cells. Five different methods are used to obtain TIL and melanoma cultures: a) cutting from the tumor tissue small fragments, 1–2 mm^3 of size and culturing each of them in different wells of tissue culture plates; b) tissue homogenization with Medimachine (BD Biosciences, Rockville, MD, USA); c) fine-needle aspiration; d) tissue remnant culture, i.e., the remnants of all the above-mentioned methods are collected, centrifuged and then placed in culture plates.

Each TIL culture obtained from any of those methods is maintained independently. In all cases IL-2 (Proleukin, 6000 IU/ml) is frequently added to the culture medium. When sufficient TIL numbers are available, only those with high activity against the tumor are selected for continuous growth. TIL anti-tumor activity and specificity are determined by analyzing the IFNγ secretion after an overnight co-culture of TIL with melanoma cells derived from the patient him/herself. Autologous melanoma cell lines are generated in the same way as TIL cultures, except for the absence of IL-2 in the culture medium. If a patient is HLA-A2 class I-positive and autologous melanoma cells are not available, HLA-A2 class I-positive melanoma cell lines are used for co-culture instead of autologous melanoma cells. The reason is that T cells recognize short peptide fragments (8–18 amino acids) that are presented on surface class I or II major histocompatibility molecules of antigen-presenting cells. Recent studies have identified genes that encode melanoma tumor antigens, such as MART-1 (melanoma antigen recognized by T cells-1) and gp100 which are recognized by TIL in the context of the HLA-A2 class I molecule [9–11].

About 40% of the Jewish population in Israel is HLA-A2 phenotype-positive [12]. For co-cultures we can use MART-1, gp100 expressing HLA-A2-positive melanoma lines, as well as HLA-A2-negative cell lines as controls for those patients. After 14–16 hours the supernatant is removed and the secreted IFNγ levels are determined by sandwich enzyme-linked immunosorbent assay. TIL are considered effective if IFNγ secretion after co-culture with autologous melanoma line or HLA-A2 melanoma lines (only for HLA-A2-positive patients) is at least 200 pg/ml and twofold higher than the highest negative control (either HLA unmatched melanoma line or medium only). Potent TIL cultures are then expanded to treatment levels by using anti-CD3 antibody (Orthoclone OKT-3, Janssen-Cilag, 30 ng/ml), IL-2 (Proleukin, Chiron BV, 6000 IU/ml) and irradiated feeder cells (5000 rad, 200-fold more feeder cells than TIL). Feeder cells are allogeneic peripheral blood mononuclear cells derived from at least three healthy donors who are negative for HIV, hepatitis B and hepatitis C. Within 2 weeks, cultures expand by approximately 1000-fold at a final volume of 40–60 L of medium. We use 20–40 gas-permeable bags of 3 L (Life-cell tissue culture flask, Miltenyi Biotec) to expand the cells. On the last day of expansion, cells must reach a cell count of at least $5x10^8$. On this day (the potential day of infusion), the volume of the culture medium (40–60 L) is reduced drastically by pheresis machine (Cobe Spectra, Gambro BCT, Denver, CO, USA). After concentrating the tumor-specific TIL and resuspending them in 200 ml infusion media containing 2.5% human albumin and 300 IU/ml IL-2 in 0.9% sodium chloride, the cells will be

NIH = National Institutes of Health
IFNγ = interferon-gamma

HIV = human immunodeficiency virus

Time Scale	Process		Quality and SterilityTesting #
Day 0	**Surgery**	Tumor processing (Fragmentation, Homogenization, TRC, FNA)	
Day 1-40	**Growth of TIL to moderate numbers**	Growth of TIL and melanoma cultures Testing of tumor reactivity (IFNγ release)	IFNγ ELISA
Day 41-54	**(Small-scale rapid expansion)* recommended**	Small-scale expansion with irradiated feeder cells, anti-CD3 antibody and IL-2 Testing of tumor reactivity (IFNγ release)	IFNγ ELISA Cell Count
Day 55-72	**Rapid expansion**	Large-scale expansion with irradiated feeder cells, anti-CD3 antibody and IL-2 Testing of tumor reactivity (IFNγ release)	IFNγ ELISA, Mycoplasma, Microbiology
Day 65-71	**Non-myeloablative lymphodepleting chemotherapy**	Clinic: administration of cyclophosphamide and fludarabine into patient	
Day 72	**Day of infusion**	Drastic reduction of media volume from 50l to 200 ml Clinic: Intravenous administration of TIL into patient Patient starts receiving high dose IL-2	IFNγ ELISA, Cell Count, Endotoxin, Microbiology

Figure 1. Schematic flow chart of cell processing, *ex vivo* expansion, non-myeloablative lymphodepleting chemotherapy and quality testing

* Small-scale rapid expansion: Same procedure as large-scale rapid expansion, just in small amounts. The purpose of the procedure is to estimate the proliferation capacity of the TIL before starting the large-scale rapid expansion. This step is recommended, but not a necessity.

Acceptance criteria for quality and sterility tests are specified in Table 1.

Table 1. Acceptance criteria of final product

Test	Method	Limits
Cell viability	Trypan blue exclusion	> 70%
Total viable cell number	Microscopic cell count	> $5x10^8$
Tumor reactivity before and after rapid expansion	IFNγ release vs. control	> 200 pg/ml
Microbiologic examination	Gram stain	Undetectable
Aerobic culture	Direct inoculation	No growth
Anaerobic culture	Direct inoculation	No growth
Fungal culture	Direct inoculation	No growth
Mycoplasma	PCR	Undetectable
Endotoxin	LAL	< 5 EU/kg

Cell count and viability is determined after microscopic cell count and exclusion of dead cells after staining with trypan blue. Tumor reactivity: IFNγ ELISA after overnight culture of TIL with melanoma cells. Direct inoculation: addition of diluted product sample to bacterial growth media followed by 14 days incubation (according to USP 28). Specific polymerase chain reaction for detecting mycoplasma genome. LAL is a chromogenic endotoxin assay utilizing a modified limulus amoebocyte lysate and a synthetic color-producing substrate.

Table 2. Melanoma biopsies processed to establish TIL for potential adoptive therapy

Patient no.	HLA-A2	Site of biopsy	Processing method	Initiated TIL	Established TIL*	Specific activity**	Potential candidate
1	Negative	Skin	Fragments	13	10	1	Yes§
			Homogenize	1	1	0	
			TRC	1	1	0	
2	Negative	Muscle	Fragments	10	5	ND	No§§
		Gluteus	Fragments	5	4	ND	
		Mus.+Glut.	Homogenize	1	0	–	
3	Negative	Skin	Fragments	23	12	3	Yes
		LN	Fragments	8	7	0	
			TRC	1	0	–	
4	Positive	Lung	Fragments	8	1	0	No
			Homogenize	1	0	–	
			TRC	1	0	–	
		LN	Fragments	11	5	0	
			Homogenize	1	0	–	
			TRC	2	2	0	
5	Negative	LN	Fragments	7	5	1	Yes
			FNA	1	1	0	
			Homogenize	1	1	0	
6	Positive	LN	Fragments	12	5	5	Yes
			TRC	1	1	1	
7	Negative	LN	Fragments	8	2	0	No
8	Positive	LN	Fragments	12	5	1	Yes
			TRC	1	1	0	
			FNA	1	0	–	
9	Positive	SC	Fragments	8	3	0	No
			FNA	1	0	–	
			TRC	1	0	–	
Total				145	72	12	

* TIL expanded sufficiently

** TIL culture demonstrates specific IFNγ release after co-culture with tumor cells (defined as IFNγ release at least 200 pg/ml and at least twice the value of any negative control line).

§ Yes: One or more potentially effective TIL cultures were generated

§§ No: no potentially effective TIL cultures were generated.

LN = lymph node, SC = subcutaneous, Mus = muscle, Glut. = gluteus, FNA = fine-needle aspiration, TRC = tissue remnant culture

administered intravenously into the patient. The schematic flow chart of cell processing, *ex vivo* expansion and quality testing is shown in Figure 1. The acceptance criteria for the final product, as set by the NIH laboratory, is given in Table 1.

Results

Tumor tissues from nine melanoma patients were received in the laboratories of the Ella Institute between June 2004 and January 2005. The tissues were derived from different sites such as lymph node, skin, lung and muscle [Table 2]. Eight of the nine tissues were of metastatic origin and one was from a primary lesion. Four of the nine patients were classified as HLA-A2 phenotype.

Different techniques for tissue processing were utilized, such as fine-needle aspiration, fragmentation (Fragment), homogenization (Medimachine) and tissue remnant cultures. T cell cultures were set up in 24-well plates and IL-2 was added frequently.

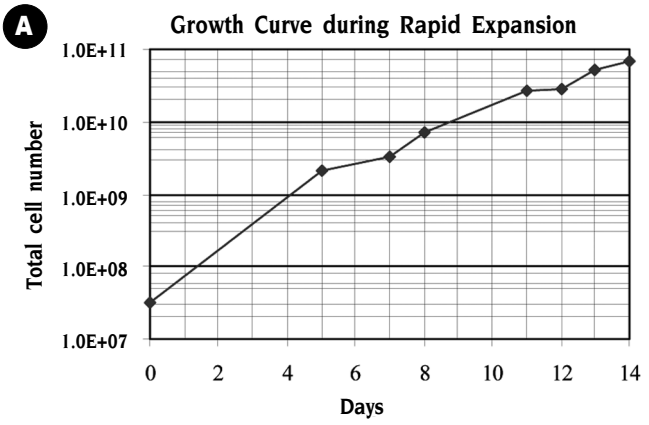
Successful establishment of T cell cultures was obtained in 72 of 145 initiated TIL (50%) [Table 2], compared to 74% of 860 at the NIH [7]. Basically, TIL cultures were successfully generated from 51% of 125 fragment-derived cultures (compared to 69.9% of 710 at the NIH), 40% of 5 Medimachine cultures (compared to 90.3% of 31 at the NIH), 33% of 3 FNA -derived cultures and 63% of 8 TRC cultures.

We further tested the established cultures for specific activity against tumor antigens by co-culture of TIL with their autologous melanoma cells or HLA-A2-positive melanoma control lines and measuring IFNγ release. Twelve of 72 TIL cultures (17%) demonstrated the required melanoma-specific activity and were considered potentially effective for clinical use. One or more potentially effective TIL cultures were generated from five of the nine patients (# 1, 3, 5, 6 and 8). Thus, more than half of the patients tested could potentially be recruited for the clinical study. No specific activity was detected in TIL cultures isolated from patients # 4, 7 and 9. These patients are therefore not qualified for the therapy. The same is true for patient # 2 who was HLA-A2-negative and for whom we could not establish melanoma cultures, making it impossible to test his TIL for specific anti-tumor activity [Table 2].

After selecting TIL with specific anti-tumor reactivity, the T cells were further expanded. The rapid expansion of TIL cultures with anti-CD3, IL-2 and feeder cells was performed for nine different TIL cultures. Twice the rapid expansion was performed in full-scale, starting with $30x10^6$ TIL (derived from TRC culture of patient # 1) and $18x10^6$ TIL (derived from fragment of patient # 3) and expanding them within 14 days to $6.6x10^{10}$ TIL (2213-fold expansion) and $1.08x10^{10}$ cells (600-fold expansion) respectively. According to Rosenberg and Dudley of the NIH [7], a total number of at least $5x10^8$ TIL are required on the day of infusion. The growth curve for TIL derived from patient # 1 during the 14 days of rapid expansion is shown in Figure 2A. Seven times the expansion was performed in small-scale dimension starting with less than $1x10^6$. The purpose of the procedure is to estimate the proliferation capacity of the TIL before starting the large-scale rapid expansion. The cells expanded at an average of 1392-fold (range 208- to 4271-fold) [Figure 2B] compared to the NIH with an average expansion of 1320-fold (range 181- to 2623-fold) [7].

One complete dry run was successfully performed for melanoma patient # 3, starting from the day of tissue processing until the cell infusion product was ready for submission, including all quality tests such as IFNγ ELISA and cell count. The tumor-reactive TIL, ready for infusion, secreted 4449 pg/ml IFNγ after co-culture with autologous melanoma cells (acceptance criterion: at least 200 pg/ml). TIL cultures proliferated 600-fold during the 14 days of rapid expansion, obtained a final cell count of $8.1x10^9$ cells (acceptance criterion: at least $5x10^8$) and a cell viability of 79% (acceptance criterion: at least 70%). (For acceptance criteria see Table 1)

Microbiologic examinations – such as gram stain, anaerobic, aerobic and fungal cultures, mycoplasma testing and endotoxin testing – were performed during the culture period and in the final infusion product. All cultures and the final product were found negative.



B

Patient no.	TIL	Fold expansion	Scale
1	TRC	2213	Full-scale
3	Fragment 4	600	Full-scale
1	Fragment 5	778	Small-scale
1	Fragment 8	208	Small-scale
1	Medimachine	925	Small-scale
1	TRC	4271	Small-scale
3	Fragment 4	2049	Small-scale
3	Fragment 1	485	Small-scale
8	Fragment 6	1001	Small-scale

Figure 2. Results of rapid expansion. **[A]** Growth curve: Cell number (logarithmic) during 14 days of rapid expansion of TIL with anti-CD3 antibody, IL-2 and irradiated feeder cells. TIL were derived from TRC culture of patient # 1. **[B]** Fold expansion of different TIL cultures after 14 days rapid expansion

Discussion

Melanoma is an immunogenic tumor, which is usually infiltrated by T lymphocytes. However, in most cancer patients, tumor-infiltrating lymphocytes fail to destroy the tumor. Among various possible reasons for this occurrence are insufficient lymphocyte numbers, insufficient activation status, the existence of regulatory T cells which suppress the cytotoxic effect of TIL, and modulation of tumor and histocompatibility antigens [13,14]. As a result, a new method to generate a very large number of activated, specifically reactive TIL has been developed [6,7].

A proposed method to enhance the success of the TIL treatment is conditioning the patient with chemotherapy before the cell transfer. Lymphodepleting chemotherapy provides an altered environment for the transferred cells, including the elimination of regulatory suppressive lymphocytes and lymphocytes that compete with the transferred cells for homeostatic cytokines such as IL-7 and IL-15 [15]. The patients receive highly potent TIL which, due to lymphodepletion, persist in the blood at > 70% of the total lymphocyte population for many months after transfer [6-8,16]. Thus, the combination of anti-tumor activity of the T

FNA = fine=needle aspiration
TRC = tissue remnant culture
ELISA = enzyme-linked immunosorbent assay

cells and the modified immunologic environment contribute to the effectiveness of the treatment [6,7].

At the Ella Institute, TIL cultures were established from 50% of 145 initiated cultures and examined for their specific activity against the patients' autologous tumor cells. In order to be a potential candidate for the treatment, at least one tumor-reactive TIL culture must be isolated for each patient. This was the case in 56% of nine of our melanoma patients (compared to 81% of 62 at the NIH [7]). Rapid expansion with anti-CD3, IL-2 and feeder cells was carried out for nine different TIL cultures. The cells expanded at an average of 1392-fold (1320-fold at the NIH [7]).

The obtained results indicate that such comparable pre-clinical studies will enable the Ella Institute laboratory to pursue the clinical phase of this promising therapy modality. A clinical trial for patients with stage IV metastatic melanoma is pending the approval of the National Review Board of the Israel Ministry of Health.

Acknowledgment. We are very grateful for the technology transfer support and the cell lines provided by Steve A. Rosenberg, Mark Dudley and their team. We are also thankful for the expert cell culture and technical assistance provided by Dragoslav Zikich, Yulianna Sandov and Rina Sarely.

References

1. Pal SP. Melanoma is fifth most common cancer. US P*harmacist* 29 April 2004:
2. www.health.gov.il/pages/default.asp?maincat=22&catId=183&PageId=1595
3. Tsao H, Atkins MB, Sober AJ. Management of cutaneous melanoma. N E*ngl* J *Med* 2004;351:998–1012.
4. Rosenberg SA, Yang JA, Nicholas PR. Cancer immunotherapy: moving beyond current vaccines. N*at Med* 2004;10:909–15.
5. Dudley ME, Wunderlich JR, Robbins PF et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. S*cience* 2002;298:850–4.
6. Dudley ME, Wunderlich JR, Yang JC, et al. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. J C*lin* O*ncol*. 2005;23:2346–57.
7. Rosenberg SA, Dudley M. Cancer regression in patients with metastatic melanoma after transfer of autologues antitumor lymphocytes. P*roc* N*atl* A*cad* S*ci* 2004;101(Suppl 2):14639–45.
8. Dudley ME, Wunderlich JR, Shelton TE, Even J, Rosenberg SA. Generation of tumor-infiltrating lymphocyte cultures for use in adoptive transfer therapy for melanoma patients. J I*mmunotherapy* 2003;26:332–42.
9. Kawakami Y, Eliyahu S, Sakaguchi K, et al. Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2 restricted tumor infiltrating lymphocytes. J E*xp Med* 1994;180:347–52.
10. Kawakami Y, Eliyahu S, Delgado CH, et al. Cloning of the gene for a shared human melanoma antigen recognized by autologous T-cells infiltrating into tumor. P*roc* N*atl* A*cad* S*ci* USA 1994;91:3515–19.
11. Kawakami Y, Eliyahu S, Delgado CH, et al. Identification of a human melanoma antigen recognized by tumor infiltrating lymphocytes from a patient responding to immunotherapy. P*roc* N*atl* A*cad* S*ci* USA 1994;54:3124–6.
12. Amar A, Zohar M, Tiwari J, Brautbar C. HLA and schizophrenia in Israel. I*sr* J *Med Sci* 1988;24:28–31.
13. Bubenik J. MHC class I down-regulation: tumour escape from immune surveillance? [Review]. I*nt* J O*ncol* 2004;25:487–91.
14. Antony PA, Piccirillo CA, Akpinarli A, et al. CD8+ T-cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells. J I*mmunol* 2005;174:2591–601.
15. Klebanoff CA, Finkelstein SE, Surman DR, et al. IL-15 enhances the in vivo antitumor activity of tumor-reactive CD8+ T cells. P*roc* N*atl* A*cad* S*ci* USA 2004;101:1969–74.
16. Powell DJ, Rosenberg SA. Phenotypic and functional maturation of tumor antigen-reactive CD8+ T lymphocytes in patients undergoing multiple course peptide vaccination. J I*mmunother* 2004;27:36–47.

Correspondence: Dr. J. Schachter, Ella Institute for Treatment and Research of Melanoma, Oncology Division, Sheba Medical Center, Tel-Hashomer 52621, Israel.
Phone: (973-3) 530-4907
Fax: (972-3) 530-4934
email: jacob.schachter@sheba.health.gov.il