# Combination of Transient Lymphodepletion With Busulfan and Fludarabine and Peptide Vaccination in a Phase I Clinical Trial for Patients With Advanced Melanoma

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**Summary:** Taking advantage of homeostatic mechanisms to boost tumor-specific cellular immunity is raising increasing interest in the development of therapeutic strategies in the treatment of melanoma. Here, we have explored the potential of combining homeostatic proliferation, after transient immunosuppression, and antigenic stimulation of Melan-A/Mart-1 specific $CD8^+$ T-cells. In an effort to develop protocols that could be readily applicable to the clinic, we have designed a phase I clinical trial, involving lymphodepleting chemotherapy with Busulfan and Fludarabine, reinfusion of Melan-A specific $CD8^+$ T-cell containing peripheral blood mononuclear cells (exempt of growth factors), and Melan-A peptide vaccination. Six patients with advanced melanoma were enrolled in this outpatient regimen that demonstrated good feasibility combined with low toxicity. Consistent depletion of lymphocytes with persistent increased CD4/CD8 ratios was induced, although the proportion of circulating $CD4^+$ regulatory T-cells remained mostly unchanged. The study of the immune reconstitution period showed a steady recovery of whole T-cell numbers overtime. However, expansion of Melan-A specific $CD8^+$ T-cells, as measured in peripheral blood, was mostly inconsistent, accompanied with marginal phenotypic changes, despite vaccination with Melan-A/Mart-1 peptide. On the clinical level, 1 patient presented a partial but objective antitumor response following the beginning of the protocol, even though a direct effect of Busulfan/Fludarabine cannot be completely ruled out. Overall, these data provide further ground for the development of immunotherapeutic approaches to be both effective against melanoma and applicable in clinic.

**Key Words:** immunotherapy, melanoma, $CD8^+$ T-cells, lymphodepletion, vaccination

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Current therapeutic options for advanced stage melanoma, including surgery, chemotherapy, and radiotherapy have only a limited impact on disease outcome. Despite these treatments, only 6% to 15% of patients with stage IV melanoma are alive after 5 years.[1] New strategies to fight this cancer are needed and immunotherapy has the potential to provide an alternative treatment. Observations of spontaneous melanoma regression in immunocompetent patients and increased cancer incidence in immunocompromised patients support the hypothesis that an immune control of cancer development is possible. However, although melanomas are antigenic, they are often poorly immunogenic, so that the immune response is weak and only low numbers of tumor-specific T-cells can be detected. On the basis of the assumption that large numbers of effective T-cells would be the key to control tumor growth, considerable enthusiasm has been directed towards the discovery of tumor antigens and the induction of potent tumor-specific cytotoxic $CD8^+$ T-cell (CTL) responses in humans.[2] Two main approaches of immunotherapy have been explored over the years. The first one is based on boosting tumor-specific CTL through vaccination strategies.[3] The other approach consists of reinfusing in patients [adoptive cell transfer (ACT)] antigen-specific CTL populations expanded in vitro to provide high numbers of effector lymphocytes.[4,5] The latter approach has shown good results in the treatment of cytomegalovirus or Epstein-Barr virus–related pathologies.[6–8] Nonetheless,

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neither approach alone has led so far to significant clinical effects in the treatment of melanoma patients.[9]

In recent years, the concept that the in vivo proliferation of transferred cells may be favored in an "empty immunologic space" through homeostatic mechanisms, has lead to the rationale for combining ACT with transient lymphodepletion.[10] For instance, in a lymphopenic environment, the competition for limited amounts of cytokines [such as interleukin-7 (IL-7) or IL-15] may be decreased, so that the different lymphocyte subpopulations (eg, naive and/or stimulated memory) may respond in a different way to persist and also to replenish their number through homeostatic proliferation. Even if the exact immunologic process is not yet well understood, several reports have documented that significant tumor control could be achieved in mice using ACT after lymphodepletion. In these models, large monoclonal expansion of melanoma antigen-specific T-cells could be found more than 4 months after reinfusion.[11–13] This may be attributed to homeostatic T-cell proliferation occurring in a T-cell depleted environment.

On the basis of these encouraging preclinical results, Rosenberg and colleagues, who have pioneered these studies in humans, tested a regimen of lymphodepleting chemotherapy and consecutive infusion of autologous in vitro expanded tumor-specific TILs (tumor infiltrating lymphocytes) combined with high dose IL-2 to treat advanced melanoma patients. They reported a significant antitumor efficacy with an objective response rate of 50% in stage IV melanoma.[14,15] Not only did this study come out as the most successful T-cell–based immunotherapy protocol against melanoma tested to date, but it also provided the proof of principle that immune control of tumor development is possible in humans. The main drawback of this strategy may currently lie in its lack of general applicability to the clinic, hence to the treatment of a large number of patients. First, it implies the harvesting of autologous tumor-specific TILs from biopsies, and their consecutive in vitro expansion that is only feasible in good manufacturing practice-certified facilities.[16] Furthermore, high doses of IL-2, used to enhance in vivo expansion of the reinfused TILs, are associated with considerable toxicity and consecutive intensive medical care.

Here, we present data from a phase I clinical trial designed to take advantage of lymphodepletion/ACT strategy in a protocol that would be noninvasive for the patient. This protocol aimed at the exploitation of homeostatic proliferation (after lymphodepleting chemotherapy) in combination with antigen-specific proliferation (after Melan-A peptide vaccination) of Melan-A specific $CD8^+$ T-cells contained in reinfused autologous peripheral blood mononuclear cells (PBMCs). Lymphodepletion was achieved through administration of Busulfan and Fludarabine, agents that are generally used in nonmyeloablative immunosuppressive conditioning regimens before allogeneic transplantation to favor the graft versus malignancy effect.[17] This outpatient protocol was exempt from the administration of recombinant hematopoietic growth factors (eg, granulocyte-colony stimulating factor) or cytokines (eg, IL-2), which represents therefore an interesting model for the study of the immune reconstitution period after lymphodepletion. To avoid the delicate step of tumor-specific T-cell in vitro expansion, we aimed at boosting in vivo proliferation of such cells using peptide vaccination. For this purpose, melanoma patients received several rounds of vaccination before the beginning of the treatment. Patients' PBMC containing vaccine-induced Melan-A specific $CD8^+$ T-cells were then spared by lymphocytapheresis before the initiation lymphodepletion, and subsequently reinfused as vaccination was reinitiated. In addition to feasibility and toxicity, we report on the analysis of lymphocyte subsets and Melan-A specific $CD8^+$ T-cells and the clinical outcome of the patients.

## MATERIALS AND METHODS

### Patients and Study Design

Stage IV melanoma patients who previously participated in one of the vaccination trials of the Ludwig Institute for Cancer Research, Lausanne branch, consisting of Melan-A/Mart-1 peptide (ELAGIGILTV) admixed in Incomplete Freund's Adjuvant ISA-51 (IFA) and CpG oligodeoxynucleotide 7909 were selected for this study.[18] Patients had to have progressive disease (PD) during vaccination and a detectable immune response (ie, 3 times expansion accompanied with differentiation phenotype) as measured by ex vivo tetramer analysis of circulating Melan-A specific $CD8^+$ memory T-cells. Adequate bone marrow, hepatic and renal function was required for study entry. Furthermore, tumors had to express the melanoma antigen (Melan-A) and patients had to be HLA-A2 positive. All patients gave written informed consent to the protocol approved by the Ethical and Regulatory Authorities.

This phase I clinical study investigated the toxicity and feasibility of a nonmyeloablative conditioning regimen using Busulfan and Fludarabine before PBMC reinfusion and peptide vaccination. In the present study, the dose of Busulfan was reduced from standard 8 to 4 mg/kg to avoid prolonged myelosuppression. After lymphocytapheresis patients were given immunosuppressive chemotherapy on an outpatient basis with oral Busulfan 2 mg/kg for 2 days, followed by intravenous Fludarabine 30 $mg/m^2$ for 3 days (Fig. 1A). Three to 5 days after the end of chemotherapy, PBMCs were reinfused on the day of maximal lymphodepletion (defined as ACT on day 0). The following day, subcutaneous vaccination with Melan-A analog peptide (Melan-A derived decapeptide spanning aa residues 26-35 and incorporating the A27L amino acid substitution, (Merck Biosciences AG, Läufelfingen, Switzerland) and IFA (Seppic, France) was restarted and continued in a monthly schedule. The administration of CpG oligodeoxynucleotide 7909 was not maintained in this second round of vaccination owing to the lack of toxicity data regarding its administration to immunosuppressed

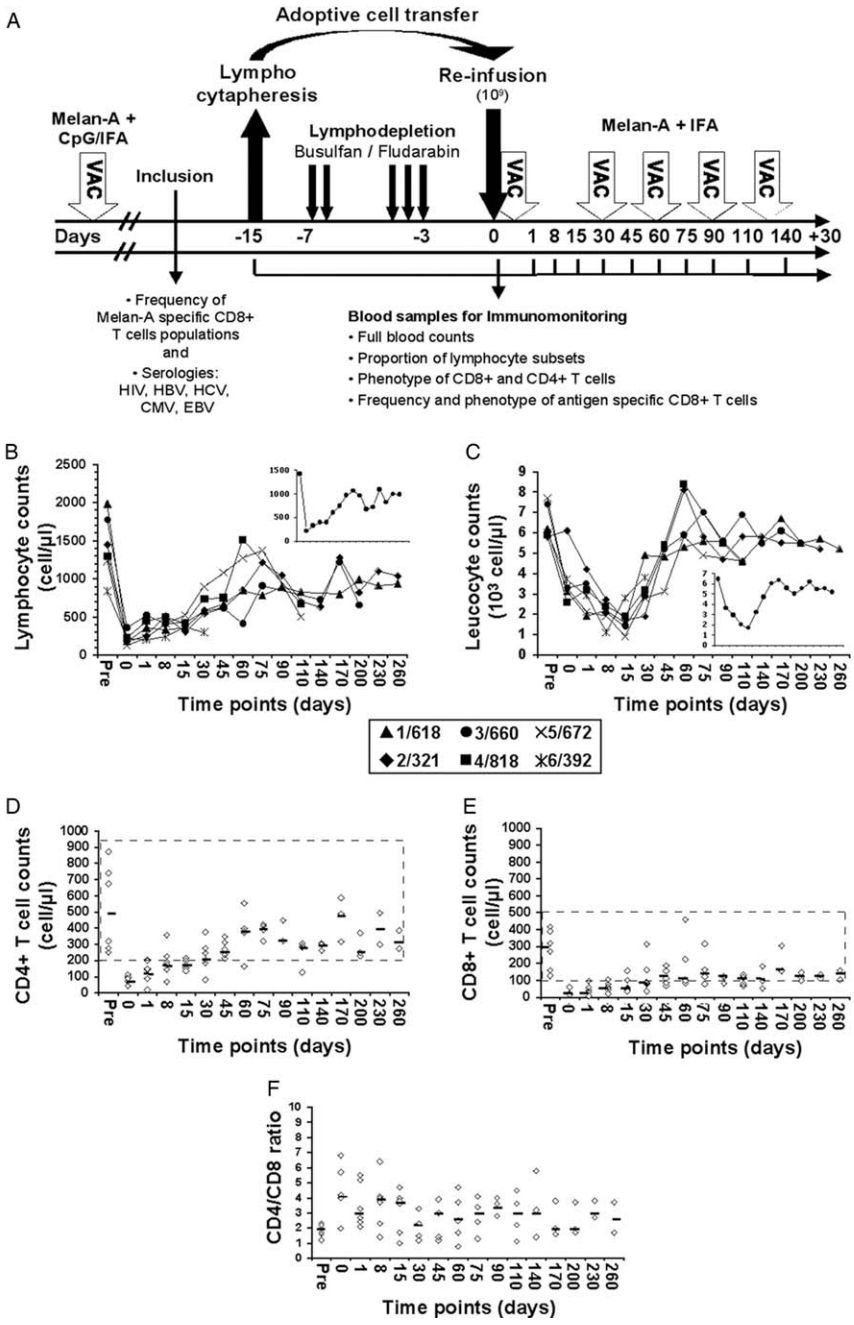


**FIGURE 1.** Study design and leukocyte and lymphocyte counts overtime. A, Design of the protocol with treatment and immunomonitoring schedule. VAC indicates subcutaneous Melan-A peptide vaccination; CpG, CpG oligodeoxynucleotide 7909. B, Total lymphocyte and (C) leukocyte counts are shown overtime (reduced size graphs show mean values of all patients). D, $CD4^+$ lymphocyte counts, E, $CD8^+$ lymphocyte counts, and F, CD4/CD8 ratios are shown overtime, each diamond representing 1 patient and horizontal bold bars representing median values with dotted lines indicating the normal range. X-axis timepoints correspond to days after PBMC reinfusion (day 0).

individuals. No recombinant growth factors were given. Toxicity was graded according to the common toxicity criteria version 2.0. We monitored lymphocyte population frequency and phenotype during the immune reconstitution period.

## Lymphocytapheresis and Freezing of PBMCs

Lymphocytapheresis was performed with a Cobe Spectra Cell separator (Gambro BCT, Inc, Labewood, CO). PBMCs were prepared for freezing within 3 hours of collection and distributed in 2 to 3 bags (cryocyte, Nexell Therapeutics INC, Irvine, CA), diluted in a solution containing Human Albumin (ZLB Behring AG, Bern, Switzerland) and Cryosure—dimethyl sulfoxide (WAK-Chemie Medical, Gmbh, Steinbach, Germany) at a concentration of 10%. The final freezing cell concentration was 25 to $45 \times 10^6$/mL and the volume of the suspension was 60 to 100 mL per bag. The bags were frozen in an advanced control rate freezer (Kryo 10/16, Planer Products Ltd, Sunbury, Middlesex, England) and

stored in liquid nitrogen. The bags were thawed in a water bath just before PBMC reinfusion in the patient.

## Follow-up Visits and Assessments

During the hematopoietic reconstitution period, patients had a physical examination and full blood count twice weekly until complete hematologic recovery. Immunologic monitoring was performed on 50 mL whole blood samples on the day of inclusion, days 0 (ACT), 1, 8, 15, 30, 45, 60, 75, 90, 110, and then monthly. Even though this phase I study was not designed to assess clinical tumor response, all patients underwent radiologic examination with computed tomography before treatment and then every 3 months. Evaluation of antitumor responses was performed according to World Health Organization (WHO) criteria[19] and confirmed with a second examination after 4 weeks. A complete response was defined as the disappearance of all metastases and partial response as a reduction of $\geq 50\%$ in the sum of the products of perpendicular diameters of all measurable metastases. A reduction of $\leq 50\%$ or an increase of $< 25\%$, and an increase of $\geq 25\%$ in the sum of the products of perpendicular diameters of all measurable metastases were considered as stable disease and PD, respectively.[20]

## Reagents and Flow Cytometry

Melan-A-ELAGIGILTV HLA-A2 tetrameric complexes (''tetramers'') were produced as previously described.[21] Anti-CD8-PerCP (Peridinin chlorophyll protein), anti-CD3-PerCP, anti-CD4-PE-Cy7 (phycoerythrin cyanine 7), anti-CD28-FITC (Fluorescein isothiocyanate), anti-CD27-APC (Allophycocyanin), anti-CD45RA-FITC, anti-CCR7-Alexa 648, anti-CD38-APC, anti-Ki67-FITC, anti-HLADR-APC, anti-Granzyme A-FITC, anti-Granzyme B-Alexa 648, anti-CD25-PE, and anti-CTLA-4-PE antibodies were purchased from Becton Dickinson Pharmingen (San Diego, CA). Anti-FoxP3-FITC antibodies were purchased from eBiosciences (San Diego, CA) and used according to the manufacturer's recommendations. Most analyses were performed directly on whole blood.[22] Briefly, tetramers (PE conjugated) were added to 300 μL of heparinized blood, followed by addition of a panel of antibodies. The lymphocytes were then fixed and the red blood cells lysed using FACS lysis solution (BD). Cells were washed, fixed, and permeabilized in FACS Permeabilization buffer (BD). After washing, intracellular staining was performed using titrated antibodies. Cells were then washed and stored in Cell Fix buffer (BD) at 4°C until analysis. Samples were analyzed on a BD FACSCanto, after compensation was checked using freshly stained PBMC.

# RESULTS

The characteristics of the 6 patients enrolled in the study are shown in Table 1. Median age was 49 years (range 24 to 75 y) and all patients had progressive stage IV metastatic disease while on peptide vaccination before study entry. All patients displayed a measurable immune

**TABLE 1.** Patient Characteristics and Hematotoxicity

| UPN | Age/Sex | Site of Metastases | Prior Treatment | Time After Last Vaccination | Disease Status at Study Entry | Reinfused[9] PBMC×10 | NADIR | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Plt/day | ANC/day | ALC | CD4 | CD8 | CD4/CD8 Ratio |
| 1/618 | 75/F | Ln, Sk | IF, ILP, I | 2 mo | PD | 4.3 | 89/15 | 800/15 | 190 | 71 | 17 | 4 |
| 2/321 | 70/M | Ln, P | I, ILP, IF, I, ILP | 6 mo | PD | 3 | 25/22 | 700/22 | 180 | 102 | 18 | 6 |
| 3/660 | 24/F | Ln, Cns† Sc | S, I | 2 wk | PD | 5 | 44/19 | 760/15 | 360 | 116 | 58 | 2 |
| 4/818 | 59/M | Ln | S, I | 3 wk | PD | 10 | 58/14 | 1200/14 | 230 | 94 | 23 | 4 |
| 5/672 | 39/M | Ln, Sk, P, H, B* | S, ILP, R, I, C | 12 mo | PD | 5.2 | 20/22 | 200/16 | 130 | 44 | 21 | 2 |
| 6/392 | 37/F | Ln, P, B | S, R, I | 2 mo | PD | 9 | 58/14 | 210/12 | 168 | 20 | 6 | 7 |

*Patient was in near complete remission after 8 cycles of chemotherapy at study entry.
†A single cerebral metastasis was resected before study entry.
ALC indicates absolute lymphocyte count at day 0 (cells/mm$^3$); ANC, abolute neutrophil count (cells/mm$^3$), except 6/392 in whom the nadir was at day 1; B, bone; C, chemotherapy; Cns, cerebral; CD4, CD4$^+$ T-cells at day 0 (cells/mm$^3$); CD8, CD8$^+$ T-cells at day 0 (cells/mm$^3$); H, hepatic; I, immunotherapy with peptide vaccination; IF, interferon α; ILP, isolated limb perfusion (Melphalan, interferon γ); Ln, lymph node; P, pulmonary; Plt, platelets (G/L); R, radiotherapy; S, surgery; Sc, subcutaneous; Sk, skin; UPN, unique patient number.

response against the Melan-A epitope after CpG/IFA/Melan-A vaccination with frequencies of 0.2% to 0.6% Melan-A specific cells in the whole $CD8^+$ T-cells at the time of inclusion. These cells showed good functional capacities.[18] A median number of $5.1 \times 10^9$ of autologous PBMCs (range 4.3 to 10) was reinfused in the patients (Table 1). The follow-up of the patients at the time of analysis was 260 and 140 days (1/618, 2/321, and 4/818, respectively). Patients 6/392, 5/672, and 3/660 went off study due to PD at days 30, 110, and 200, respectively.

## Low Hemato and Nonhematotoxicity

Hematotoxicity was mild with a median neutrophil nadir of 730/mm$^3$ (range 200 to 1200) and a platelet nadir of 51 G/L (range 20 to 89) (Table 1). The low values of absolute neutrophil count in the last 2 patients have to be interpreted in the context of prior chemotherapy with an alkylating agent in one patient (5/672) and pelvic radiotherapy in another (6/392). Of note, the myelotoxicity induced by the present regimen was of relatively late onset with a nadir occurring after a median of 15 and 19 days for absolute neutrophil count and platelets, respectively. No patient required transfusional support and no patients experienced fever neither during neutropenia nor during the immune reconstitution period. Nonhematologic toxicity was mainly fatigue grade 1 in 4 patients and grade 3 in 2 patients, lasting for 1 to 2 months. Reversible alopecia grade 2 and 3 occurred in 2 patients. Mild infections grade 1 to 2 were observed in 3 patients and 1 patient experienced cutaneous allergic reaction to Fludarabine. Overall toxicity was low and patients did not have to remain hospitalized (apart from the day of PBMC reinfusion).

## Consistent Lymphodepletion and Slow T-cell Recovery

A single cycle of Busulfan $2 \times 2$ mg/kg and Fludarabine $3 \times 30$ mg/m$^2$ induced a rapid onset immunosuppression with a median absolute lymphocyte count of 205 cells/mm$^3$ (range 130 to 360) 8 days after treatment start (range 7 to 10 d). Although depletion was successful, resulting in low lymphocyte counts, the recovery of whole lymphocytes remained slow overtime (Fig. 1B). For instance, median pretreatment T-lymphocyte counts were still not reached after 3 months, whereas median total leukocyte numbers normalized within 30 to 45 days after ACT (Fig. 1C). Compared with other whole lymphocyte populations, natural killer and B cells were particularly depleted with the latter not starting to recover before 2 months (data not shown).

Interestingly, the $CD8^+$ T-cell population was more depleted than the $CD4^+$ population with median numbers of 20/mm$^3$ for $CD8^+$ T-cells and of 83/mm$^3$ for $CD4^+$ T-cells, corresponding to a median CD4/CD8 ratio of 4 (Table 1). This observation is also reflected by initially increasing $CD4^+$ T-cell proportions and decreasing $CD8^+$ T-cell proportions, as compared with whole lymphocytes (data not shown). $CD4^+$ T-cells recovered to values above 200/mm$^3$ after 1 to 2 weeks, whereas $CD8^+$ counts remained suppressed below or at 100 cells/mm$^3$ for up to 2 months (Figs. 1D, E). It is of note that the limit of 200/mm$^3$ for $CD4^+$ is critical, because the incidence of opportunistic infections is known to increase dramatically when $CD4^+$ counts fall below 200/mm$^3$ or when CD4/CD8 ratios are less than 1.[23] The relatively long-lasting suppression of $CD8^+$ T-cells over several weeks, compared with the faster recovery of $CD4^+$ T-cells within the first month, resulted in a persisting increase of CD4/CD8 ratios (Fig. 1F).

## Phenotypic Analysis of Whole Lymphocyte Subsets During Reconstitution

To further characterize the reconstitution of the T-cell compartment, the activation status and subset distributions along the differentiation pathway of both $CD8^+$ and $CD4^+$ T-cells were analyzed using different combinations of phenotypic markers. The expression of CD38 reflects the activation status of lymphocytes, whereas Ki67 provides information regarding cell cycling and proliferation. CD45RA, CCR7, CD27, and CD28 are widely used markers that are associated with cellular differentiation (Fig. 2A).[24] On average, a modest increase of CD38 expression was observed over the first 2 months after reinfusion, particularly within the $CD8^+$ compartment (Fig. 2B). It was associated with a small increase in Ki67 expression (Fig. 2C). These observations are in line with the slow expansion of the whole T-cell population. The lymphodepleting chemotherapy resulted in a moderate rise of the proportions of naive $CD8^+$ T-cells early after reinfusion (mean increase of 12% as characterized by a $CD45RA^+/CCR7^+$ phenotype), but not of naive $CD4^+$ T-cells (Fig. 2D), suggesting that the memory subset of $CD8^+$ T-cells may be particularly sensitive to the present chemotherapy. The proportion of highly differentiated cells (eg, $CD28^-/CD27^-$) remained stable over time (Fig. 2E). We also monitored the level of circulating regulatory T-cells, because their depletion with chemotherapy is part of the strategy to favor the proliferation of reinfused tumor-specific lymphocytes. Despite lymphodepletion, proportions of $CD25^+/FoxP3^+$ cells within the $CD4^+$ T-cell population remained mostly unchanged (if not increasing slightly) (Fig. 3A), likely due to nondepleted remaining cells and also cells reinfused with the PBMC. These cells expressed consistent levels of the inhibitory molecule CTLA-4 (Fig. 3B).

## Different Patterns of Melan-A Specific $CD8^+$ T-cell Evolution

The monitoring of the Melan-A specific $CD8^+$ T-cells included the assessment of their percentages within the whole $CD8^+$ T-cell population (Figs. 4A, B), kinetics of recovery (Fig. 4C), activation level (on the basis of the expression of CD38) (Fig. 4D), as well as changes in differentiation status [through staining of CD45RA, CCR7, CD27, CD28, and of Granzymes A and B (GrA, GrB) as markers of cytolytic potential] in comparison with whole $CD8^+$ T-cell populations

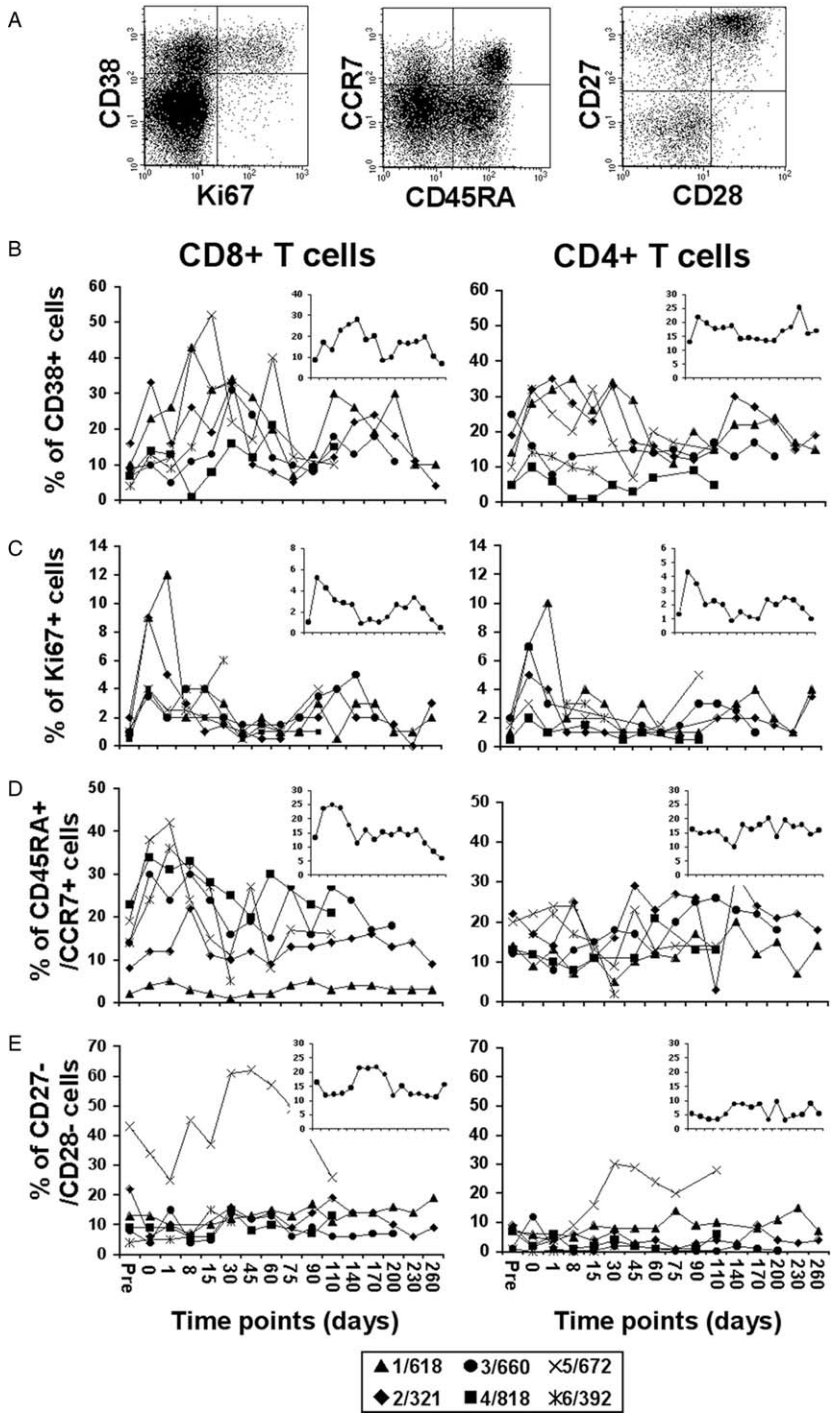


**FIGURE 2.** Kinetics of activation and subset variation in the $CD8^+$ or $CD4^+$ T-cell populations. A, Representative examples of stainings for markers of activation (CD38 and Ki67), of naive versus antigen-primed cells (CD45RA and CCR7) and of differentiation (CD28 and CD27) on whole $CD8^+$ T-cells. Percentages of $CD8^+$ and $CD4^+$ T-cells that are (B) activated ($CD38^{high}$), (C) cycling ($Ki67^+$), (D) of naive phenotype ($CD45RA^+/CCR7^+$), or (E) highly differentiated ($CD28^-/CD27^-$) are shown overtime. Reduced size graphs show mean values of all patients. X-axis timepoints correspond to days after PBMC reinfusion (day 0).

(Figs. 4E–G). Apart from the low expression of CD45RA and CCR7 on Melan-A specific $CD8^+$ T-cells that remained mostly stable (suggesting that the cells were antigen experienced overtime), the analysis of Melan-A specific $CD8^+$ T-cell populations revealed diverse patterns specific to particular patients, contrasting with the observations made on whole lymphocyte subpopulations, consistent between patients. Data are shown for 3 different patients. In 1 patient (3/660 shown in Fig. 4), a modest expansion of Melan-A specific $CD8^+$ T-cells was observed (from 0.2% to 0.4% of $tetramer^+$). This was accompanied by a higher activation level and faster recovery kinetics compared with the whole $CD8^+$ T-cell population, along with further differentiation of the antigen-experienced Melan-A specific $CD8^+$ T-cells (increased percentages of $CD28^-/CD27^-$ cells and granzyme expression) in the sense of a genuine antigen-driven response. Of note, this immunologic response

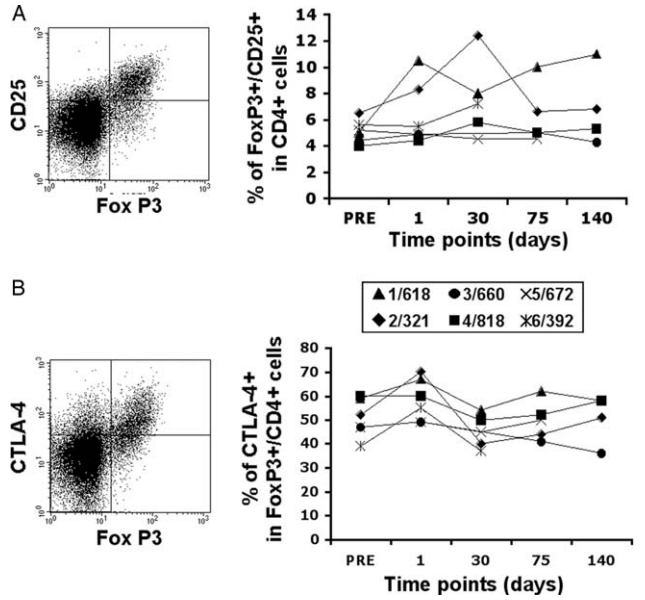


**FIGURE 3.** Longitudinal analysis of regulatory T-cells. A, Representative example of FoxP3/CD25 staining on circulating CD4+ T-cells and changes in the patients overtime. B, Representative example of FoxP3/CTLA-4 staining on circulating CD4+ T-cells and changes in the patients overtime.

lasted for up to 4 months, before Melan-A specific $CD8^+$ T-cell frequency and phenotype started returning towards baseline values. Instead, 2 patients (2/321 shown in Fig. 4 and 4/818) experienced a reduction of Melan-A specific $CD8^+$ T-cells within the whole $CD8^+$ T-cell population after the start of the protocol and a slow recovery after 3 to 4 months. The phenotype of the Melan-A specific $CD8^+$ T-cells remained mostly unchanged over the study period, apart from signs of activation during the first 2 days after ACT (which may be related to pretreatment activation). In 2 other patients (1/618 shown in Fig. 4 and 5/672), circulating Melan-A specific $CD8^+$ T-cell percentages started to rise slightly (from 0.3% to 0.7% of $tetramer^+$ for 1/618, and 0.1% to 0.5% for 5/672) after day 30 and an initial decline. However, the kinetics of recovery and the activation level were no different from those of the whole $CD8^+$ T-cell population. Unexpectedly, the Melan-A specific $CD8^+$ T-cell population became less differentiated overtime (although remaining $CD45RA^-/CCR7^-$ antigen experienced), as shown by a steady decrease of double negative CD28, CD27 and reduction in GrA and GrB expression, suggesting that highly differentiated cells had died or migrated to peripheral sites. The follow-up of the sixth patient (6/392) was suspended after day 22 owing to PD. During this short observation period, no change in the percentage of the Melan-A specific $CD8^+$ T-cells was observed.

## Clinical Follow-up of the Patients

Three patients have experienced disease stabilization for 2, 5, and 10+ months (5/672, 3/660, and 1/618, respectively). One patient (4/818) with a single axillar lymphadenopathy at study entry who had remained stable underwent surgical resection 2 months after the start of treatment; he has been disease-free since (4+ mo). One patient had rapid disease progression at day 22 after the start of treatment. One patient (2/321) who suffered from progressing lung metastasis and hilar lymphadenopathy during the 6 months before study entry experienced an objective partial response 3 months after the start of treatment which evolved into close to complete remission 10 months after ACT (94% reduction) (Fig. 5).

## DISCUSSION

T-cell–based vaccines are now able to induce consistent and measurable immune responses to tumor antigens (eg, Melan-A) in vivo,[18] however with limited clinical benefit. Instead, preclinical and now clinical data indicate a synergistic activity of ACT and lymphodepletion leading to an effective antitumor activity. However, the implementation of described procedures can represent a heavy burden (ie, high technical expertise and toxicity) to be realistically applied in the general clinic. We have worked towards the development of a protocol that could be more applicable, combining lymphodeletion with an antigen delivery via peptide vaccination to provide 2 driving forces for the expansion of tumor-specific CTLs: triggering of homeostatic mechanisms and antigen driven proliferation. Here, we have demonstrated that a conditioning regimen using Busulfan at $2 \times 2\,mg/kg$ and Fludarabine at $3 \times 30\,mg/m^2$ in the absence of a hematopoietic graft or growth factors was well tolerated. It induced a rapid onset lymphodepletion combined with acceptable hematotoxicity and mild nonhematologic toxicity in 6 patients who had progressive stage IV melanoma. The low toxicity profile and convenient mode of administration of the drugs enabled a complete outpatient protocol without the requirement of specialized facilities. The exploitation of peptide vaccination procedures associated with the reinfusion of untouched PBMC containing tumor-specific $CD8^+$ T-cells offers good feasibility to a strategy combining homeostatic mechanisms and antigen-driven proliferation of antigen specific $CD8^+$ T-cells.

The present study represents a unique setting in which to study physiologic mechanisms of lymphocytic homeostasis due to the absence of any cytokine support. Thus, the repopulation of the "immunologic space" was mostly because of the expansion of reinfused and remaining lymphocytes. Interestingly, the analysis of the immune reconstitution period revealed that a single cycle of Busulfan and Fludarabine particularly depleted $CD8^+$ T-cells, resulting in increased CD4/CD8 ratios followed by a slow recovery compared with the $CD4^+$ subset. This is in contrast with previous reports of marked depletion $CD4^+$ T-cells for several months with decreased CD4/CD8 ratios $< 1$ and clinical immunosuppression,[15,25–29] although lymphodepletion in these studies was usually induced by the use of cyclophosphamide (CTX) in a

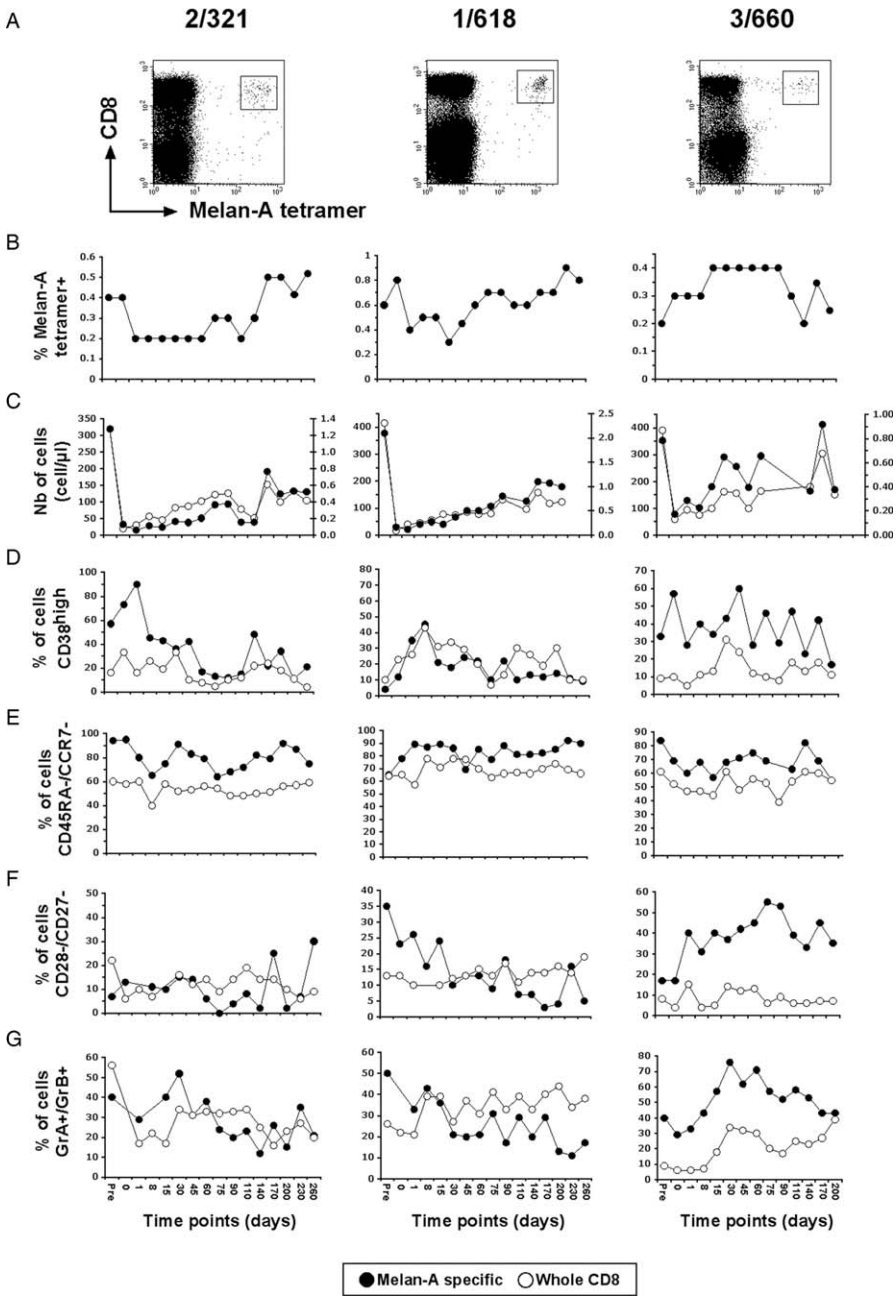


**FIGURE 4.** Longitudinal analysis of circulating Melan-A specific $CD8^+$ T-cells in 3 patients. A, Representative examples of Melan-A tetramer staining. B, Percentages of Melan-A tetramer positive cells within the $CD8^+$ T-cell population. C, Evolution of tetramer positive cell frequency (right y-axis) in comparison with whole $CD8^+$ T-cell counts (left y-axis). Longitudinal followup of Melan-A specific $CD8^+$ T-cells that are (D) activated (CD38high), (E) antigen primed CD45RA/CCR7 cells, (F) highly differentiated (CD28/CD27), and (G) granzymes A and B positive cells.

transplant setting. Data from preclinical, in vitro analyses indicate that the treatment of PBMCs with Fludarabine leads to a higher level of apoptosis in $CD8^+$ T-cells compared with $CD4^+$ T-cells.[30] These in vitro findings and our clinical results in patients who did not receive hematopoietic transplant or growth factor, support the hypothesis of a preferential depletion of the $CD8^+$ T-cell subset through Fludarabine. The analysis of whole lymphocyte populations revealed a remarkably similar pattern of depletion in all patients. However, the kinetics of recovery were unexpectedly slow. Our observations differ from previous studies which report a more rapid lymphocyte recovery,[31,32] although these concerned allogeneic or autologous stem cell transplantation settings and thus cannot be directly compared. Overall, homeostatic proliferation of T lymphocytes to repopulate an empty/depleted immunologic space seems to be relatively weak, in the absence of growth factors, with no particular subset taking over from the others.

In contrast to the remarkably similar depletion and recovery of whole lymphocyte populations, the evolution of the Melan-A specific $CD8^+$ T-cell population during

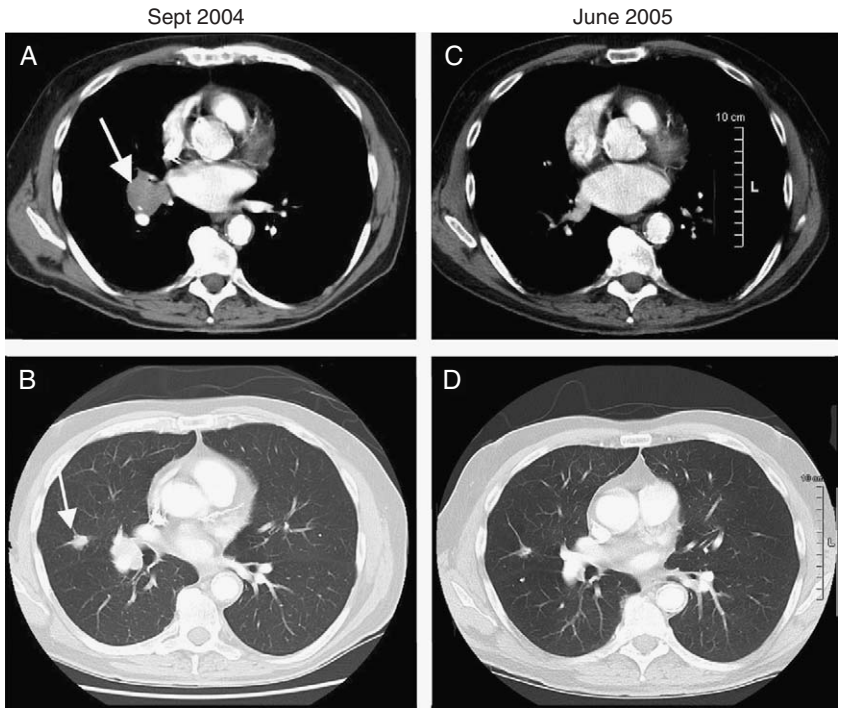


**FIGURE 5.** Objective tumor response in patient 2/321. The figure shows computed tomography of a clinical response (94% tumor regression). A, Right hilar lymphadenopathy before treatment start (white arrow). B, Right pulmonary metastasis before start of treatment (white arrow). C, Complete regression of right hilar lymphadenopathy after 10 months. D, Residual lesion of pulmonary metastasis after 10 months.

the immune reconstitution period showed differences between patients. On average, expansion of Melan-A specific $CD8^+$ T-cells, as measured in peripheral blood, was mostly inconsistent despite Melan-A peptide vaccination. Increase in the percentages of circulating Melan-A specific $CD8^+$ T-cells could be observed in 3 patients during the protocol, although these remained limited. These expansions were accompanied with changes in cellular phenotype, but no general phenotypic pattern could be drawn, owing to interpatient variability. A lack of consistent Melan-A specific $CD8^+$ T-cell expansion was rather unexpected, because IFA + peptide vaccine usually drives significant T-cell expansion when repeated vaccination is provided.[33,34] The antigenic stimulus offered by this formulation may be too weak to drive a robust expansion of Melan-A specific $CD8^+$ T-cells in our setting. The addition of CpG oligodeoxynucleotides to the second round of vaccination is being considered. It is also possible that a more pronounced lymphodepletion may be necessary to fully trigger homeostatic mechanisms and to induce a more consistent T-cell proliferation, as observed in other studies.[15] For this purpose, CTX may replace Busulfan. Furthermore, the late nadir of neutrophils and platelets in the present trial reflects a typical late recovery-of-myelotoxicity profile of Busulfan. This is likely due to the toxicity in early hematopoietic bone marrow precursors, which potentially include lymphoid precursors interfering with homeostatic T-cell proliferation.[35] In contrast, CTX displays a more rapid onset toxicity in cells of the myeloid and lymphoid lineage with more profound nadirs followed by a faster recovery within 2-3 weeks.[36] Additionally, CTX does not affect early hematopoietic bone marrow precursors. Lastly, CTX has been associated with a strong depletion of $CD4^+$ T-cells[37] and particularly regulatory T-cells[38,39] that may participate in inhibiting homeostatic mechanisms. In our study, we could observe the presence of remaining regulatory T-cells, which may indeed have a role in the slow lymphocyte recovery and/or lack of consistent Melan-A specific $CD8^+$ T-cell expansion. Further improvements might include the use of recombinant IL-7, a cytokine known for its crucial role in driving homeostatic proliferation, or the inhibition of regulatory T-cell activity (persisting at day 0 or contained in reinfused PBMCs) using anti-CD25 or anti-CTLA-4 antibodies. However, the use of any additional factors should be balanced with their potential toxicity and side effects.

Although the analysis of clinical outcome was not a main end point in this trial and the small series studied does not allow the drawing of definite conclusions, the finding of a long-lasting tumor regression in 1 patient may be encouraging. Considering that the responding patient had PD before study entry and tumor shrinkage occurred within a few weeks on study and continued over several months, this response is unlikely to be due to spontaneous remission. To our knowledge, there is no evidence that Busulfan has inherent activity against melanoma, but alkylating agents (like Melphalan) in general are known to display a modest activity against melanoma. Chemotherapy has to be given repetitively to exert

potent antitumor activity, and in the present setting, a single administration of Busulfan was given to the patient. Nonetheless, one cannot rule out completely a direct antitumor effect of the treatment with Busulfan and Fludarabine, in particular because the patient had not previously failed this chemotherapy.

The discrepancy between this potential clinical response and the apparent lack of Melan-A specific T lymphocyte expansion in the blood in this patient stresses nonetheless the question whether the immunomonitoring of peripheral blood is adequate. Although this remains purely speculative, effective Melan-A specific T lymphocytes may have migrated from the circulation to peripheral areas, such as the tumor sites. This would be in keeping with our observation of an initial decrease in circulating Melan-A specific T lymphocytes, accompanied with a decrease in highly differentiated cell proportions. The study of tumor infiltrating antigen-specific T-cells (TILs) may be more adapted to monitor the efficacy of immunotherapeutic strategies. However, this is often limited by the difficulty to access tumor material and detect TILs. We were able to analyze tumor biopsies from 3 patients, but we could not detect any significant numbers of TILs ex vivo. The clinically responding patient did not have tumor accessible for biopsy. Further immunologic analyses of blood of this patient revealed no detectable immune responses against other common antigens expressed by melanomas, such as gp100, Tyrosinase, NY-ESO-1, Camel, and SSX-2. These observations emphasize the importance of refining the monitoring of immune-based therapies, including the definition and standardization of surrogate markers.

To conclude, we have performed a phase I clinical trial combining lymphodepleting chemotherapy and Melan-A peptide vaccination, and demonstrated the feasibility of this approach for the development of protocols that would be applicable to the treatment of melanoma patients. This approach is a complete outpatient and nontoxic regimen. However, the use of Busulfan and Fludarabine for the lymphodepletion combined with IFA + Melan-A vaccination did not seem to provide the optimal conditions to boost tumor-specific $CD8^+$ T-cells. Further developments of a strategy combining homeostatic and antigen-driven proliferation of tumor-specific T-cells are necessary to produce a therapy which will make the balance between effectiveness in melanoma and applicability in the clinic.

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## REFERENCES

1. Balch CM, Buzaid AC, Soong SJ, et al. Final version of the American Joint Committee on Cancer staging system for cutaneous melanoma. *J Clin Oncol*. 2001;19:3635–3648.
2. Rosenberg SA. Progress in human tumour immunology and immunotherapy. *Nature*. 2001;411:380–384.
3. Ribas A, Butterfield LH, Glaspy JA, et al. Current developments in cancer vaccines and cellular immunotherapy. *J Clin Oncol*. 2003; 21:2415–2432.
4. Dudley ME, Wunderlich J, Nishimura MI, et al. Adoptive transfer of cloned melanoma-reactive T lymphocytes for the treatment of patients with metastatic melanoma. *J Immunother*. 2001;24: 363–373.
5. Yee C, Thompson JA, Byrd D, et al. Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. *Proc Natl Acad Sci USA*. 2002;99:16168–16173.
6. Moss P, Rickinson A. Cellular immunotherapy for viral infection after HSC transplantation. *Nat Rev Immunol*. 2005;5:9–20.
7. Walter EA, Greenberg PD, Gilbert MJ, et al. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *N Engl J Med*. 1995;333:1038–1044.
8. Rooney CM, Smith CA, Ng CY, et al. Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients. *Blood*. 1998;92: 1549–1555.
9. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. *Nat Med*. 2004;10:909–915.
10. Klebanoff CA, Khong HT, Antony PA, et al. Sinks, suppressors and antigen presenters: how lymphodepletion enhances T cell-mediated tumor immunotherapy. *Trends Immunol*. 2005;26: 111–117.
11. Pardoll D. T cells take aim at cancer. *Proc Natl Acad Sci USA*. 2002;99:15840–15842.
12. Dummer W, Niethammer AG, Baccala R, et al. T cell homeostatic proliferation elicits effective antitumor autoimmunity. *J Clin Invest*. 2002;110:185–192.
13. Maine GN, Mule JJ. Making room for T cells. *J Clin Invest*. 2002; 110:157–159.
14. Dudley ME, Wunderlich JR, Robbins PF, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science*. 2002;298:850–854.
15. 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 Clin Oncol*. 2005;23:2346–2357.
16. Ho WY, Yee C, Greenberg PD. Adoptive therapy with CD8(+) T cells: it may get by with a little help from its friends. *J Clin Invest*. 2002;110:1415–1417.
17. Slavin S, Nagler A, Naparstek E, et al. Nonmyeloablative stem cell transplantation and cell therapy as an alternative to conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases. *Blood*. 1998;91:756–763.
18. Speiser DE, Lienard D, Rufer N, et al. Rapid and strong human CD8+ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909. *J Clin Invest*. 2005;115:739–746.
19. WHO handbook for reporting results of cancer treatment. World Health Organization; 1979.
20. Oken MM, Creech RH, Tormey DC, et al. Toxicity and response criteria of the Eastern Cooperative Oncology Group. *Am J Clin Oncol*. 1982;5:649–655.
21. Romero P, Dunbar PR, Valmori D, et al. Ex vivo staining of metastatic lymph nodes by class I major histocompatibility complex tetramers reveals high numbers of antigen-experienced tumor-specific cytolytic T lymphocytes. *J Exp Med*. 1998;188: 1641–1650.

 

22. Appay V, Rowland-Jones SL. The assessment of antigen-specific CD8+ T cells through the combination of MHC class I tetramer and intracellular staining. *J Immunol Methods*. 2002;268:9–19.
23. Hughes MA, Parisi M, Grossman S, et al. Primary brain tumors treated with steroids and radiotherapy: low CD4 counts and risk of infection. *Int J Radiat Oncol Biol Phys*. 2005;62:1423–1426.
24. Appay V, Rowland-Jones SL. Lessons from the study of T-cell differentiation in persistent human virus infection. *Semin Immunol*. 2004;16:205–212.
25. Hakim FT, Cepeda R, Kaimei S, et al. Constraints on CD4 recovery postchemotherapy in adults: thymic insufficiency and apoptotic decline of expanded peripheral CD4 cells. *Blood*. 1997; 90:3789–3798.
26. Mackall CL, Fleisher TA, Brown MR, et al. Distinctions between CD8+ and CD4+ T-cell regenerative pathways result in prolonged T-cell subset imbalance after intensive chemotherapy. *Blood*. 1997;89:3700–3707.
27. Mackall CL. T-cell immunodeficiency following cytotoxic antineoplastic therapy: a review. *Stem Cells*. 2000;18:10–18.
28. Dudley ME, Wunderlich JR, Yang JC, et al. A phase I study of nonmyeloablative chemotherapy and adoptive transfer of autologous tumor antigen-specific T lymphocytes in patients with metastatic melanoma. *J Immunother*. 2002;25:243–251.
29. Fagnoni FF, Lozza L, Zibera C, et al. Cytotoxic chemotherapy preceding apheresis of peripheral blood progenitor cells can affect the early reconstitution phase of naive T cells after autologous transplantation. *Bone Marrow Transplant*. 2003;31:31–38.
30. Gamberale R, Galmarini CM, Fernandez-Calotti P, et al. In vitro susceptibility of CD4+ and CD8+ T cell subsets to fludarabine. *Biochem Pharmacol*. 2003;66:2185–2191.
31. Guillaume T, Rubinstein DB, Symann M. Immunological recovery and tumour-specific immunotherapeutic approaches to post-autologous haematopoietic stem cell transplantation. *Baillieres Best Pract Res Clin Haematol*. 1999;12:293–306.
32. Savage WJ, Bleesing JJ, Douek D, et al. Lymphocyte reconstitution following non-myeloablative hematopoietic stem cell transplantation follows two patterns depending on age and donor/recipient chimerism. *Bone Marrow Transplant*. 2001;28:463–471.
33. Monsurro V, Nagorsen D, Wang E, et al. Functional heterogeneity of vaccine-induced CD8(+) T cells. *J Immunol*. 2002;168:5933–5942.
34. Powell DJ Jr, Rosenberg SA. Phenotypic and functional maturation of tumor antigen-reactive CD8+ T lymphocytes in patients undergoing multiple course peptide vaccination. *J Immunother*. 2004; 27:36–47.
35. Tannock IF, Hill RP. *The Basic Science of Oncology*. 3rd ed. McGraw Hill; 1998.
36. Tannock IF. Conventional cancer therapy: promise broken or promise delayed? *Lancet*. 1998;351(suppl 2):SII9–SII16.
37. Mackall CL, Fleisher TA, Brown MR, et al. Lymphocyte depletion during treatment with intensive chemotherapy for cancer. *Blood*. 1994;84:2221–2228.
38. Ghiringhelli F, Larmonier N, Schmitt E, et al. CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. *Eur J Immunol*. 2004;34:336–344.
39. Lutsiak ME, Semnani RT, De Pascalis R, et al. Inhibition of CD4(+)25+ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. *Blood*. 2005; 105:2862–2868.