Reprogramming T Lymphocytes for Melanoma Adoptive Immunotherapy by T-Cell Receptor Gene Transfer with Lentiviral Vectors

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Abstract

T-cell receptor (TCR) gene transfer for cancer immunotherapy is limited by the availability of large numbers of tumor-specific T cells. TCR α and β chains were isolated from a highly lytic HLA-A2–restricted cytotoxic T lymphocyte (CTL) clone recognizing the melanoma-associated Melan-A/MART-1 antigen and inserted into a lentiviral vector carrying a bidirectional promoter capable of robust and coordinated expression of the two transgenes. Lentiviral vector–based gene delivery systems have shown increased transfer efficiency and transgene expression compared with the widely used γ-retroviral vectors. This vector performed more efficiently than a γ-retrovirus–based vector containing the same expression cassette, resulting in a T-cell population with 60% to 80% of transgenic TCR expression with mainly $CD8^+$ intermediate effector phenotype. Transgenic T cells specifically produced cytokine in response to and killed antigen-expressing melanoma cells, retained an overlapping functional avidity in comparison with the TCR donor CTL clone, and exerted significant therapeutic effects *in vivo* upon adoptive transfer in melanoma-bearing severe combined immunodeficient mice. Optical imaging showed their accumulation in the tumor site. Overall, our results indicate that lentiviral vectors represent a valid tool for stable and high-intensity expression of transgenic TCR and support clinical exploitation of this approach for therapeutic application. [Cancer Res 2009;69(24):9385–94]

Introduction

A major obstacle to the clinical diffusion of adoptive cell transfer (ACT) is represented by technical factors limiting the availability of adequate numbers of tumor-specific T cells to infuse (1). T-cell receptor (TCR) engineering may provide a valid tool to overcome such limitations, leading to the rapid generation of large amounts of tumor-specific T cells endowed with the desired specificity and obtained in an environmental milieu not constrained by the homeostatic control of suppressive mechanisms based on cytokines or regulatory T cells. Data from the first phase I clinical trial of TCR gene transfer showed the feasibility of this approach in humans as well as evidenced its intrinsic limitations (2). Indeed, a sizable and durable presence of modified T cells in the circulation of most of the patients in the absence of therapeutic effects suggested both the failure of these cells to reach the tumor and/or a functional deficiency due to a decreased retroviral transgene expression (3). Such results solicit further protocol improvements, relying on alternative gene transfer methods and appropriate *in vitro* expansion techniques, capable to preserve the desired cellular properties.

Lentiviral vector (LV)–based gene delivery systems, as opposed to the widely used γ-retroviral vectors (RV), could be used to increase vector performances in terms of transfer efficiency and transgene expression extent and stability. Self-inactivating LV contain constitutive internal promoters that drive transgene expression, similarly to wild-type long terminal repeat–mediated expression (4), but independent of transduced T-cell activation status, and *cis*-regulatory sequences added to increase gene transfer efficiency and transgenic RNA stability (5, 6). Moreover, the LV genome structure significantly reduces the risk of silencing phenomena in embryonic stem cells and preimplantation embryos (7) and integrates in resting cells (8), thus being more effective for gene transfer in slowly dividing T lymphocytes. Indeed, it has been shown that the proliferative potential of transferred cells influences the persistence of tumor-infiltrating lymphocyte (TIL)–derived T lymphocytes collected from the peripheral blood of patients treated by ACT protocols (9–11), an aspect ultimately correlated with a positive clinical response (12). Thus, LV could allow to shorten the *ex vivo* manipulation phase and to generate large numbers of scarcely differentiated central memory-like T cells (13), which are considered optimal for therapeutic purposes.

On these grounds, we aimed at developing a reliable, reproducible, and clinically oriented approach for redirecting human lymphocytes against melanoma by LV-mediated transfer of a TCR specific for the Melan-A/MART-1_{26-35} antigen (hereafter called Melan-A), derived from a HLA-A2–restricted CTL clone. Here, we describe the use of a LV carrying a synthetic bidirectional promoter (14) to drive the simultaneous and coordinated expression of both α and β TCR chains in transduced T lymphocytes.

Materials and Methods

Cells and reagents. The following melanoma cell lines were used: A375 (Melan-A^-/HLA-$A2^+$), SK-23 MEL (Melan-A^+/HLA-$A2^+$), PDO-328 MEL

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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(Melan-A$^-$/HLA-A2$^-$), and Mel3/3 (Melan-A$^+$/HLA-A2$^+$). Other cell lines were 293T, 293gp, T2 (HLA-A2$^+$), Jurkat (15), and HT-29 (colorectal adenocarcinoma, Melan-A$^-$/HLA-A2$^-$). The human Melan-A_{26-35}–specific, HLA-A2–restricted CD8$^+$ SELA-A/64 CTL clone was derived from a metastatic melanoma patient, as described elsewhere (16). Cell surface markers were labeled using fluorescein isothiocyanate– or phycoerythrin-conjugated antibodies to CD4 and CD127 (Becton Dickinson); CD8 and CCR7 (BD Pharmingen); CD27, CD28, and CD62L (Immunotech); and CD45RO (Caltag Laboratories). Phycoerythrin-conjugated Melan-A/A2 tetramer (16) and anti-TCR Vβ14 (Immunotech) were used for evaluating TCR expression on T cells. The negative control setting for the Melan-A/A2 tetramer and each monoclonal antibody (mAb) was determined by using a non-correlated phycoerythrin-conjugated tetramer (BMLF1/A2) and the corresponding isotypes, respectively. Intracellular cytokine staining was performed using a Cytofix/Cytoperm Plus Fixation/Permeabilization kit (BD Biosciences) and a fluorescein isothiocyanate–conjugated anti–IFN-γ (Immunotech) mAb.

LV plasmids. LV pCMV-TCRα and pCMV-TCRβ plasmids encoding the TRAV12-2/J35/C and TRBV27/D2/J2-7/C2 cDNA derived from the SELA-A/64 CTL clone, respectively, were generated from the self-inactivating HIV-1–based vector pRRL.sin18.cPPT.CMV.GFP.Wpre (pCMV-EGFP), as reported previously (15). The bidirectional LV (BD I-TCR and BD V-TCR) are derivative of the pCCL.sin.cPPT.SV40polyA.CTE.WPRE construct (5) and carry a minCMVPGK (14) bidirectional promoter driving TCR α- and β-chain expression in antisense orientation (Fig. 1). Vesicular stomatitis virus (VSV)–pseudotyped lentiviral particle production is described elsewhere (17). Concentrated LV stocks were measured by HIV-1 p24 protein ELISA (Innogenetics).

Peripheral blood mononuclear cell and Jurkat cell transduction. Peripheral blood mononuclear cells (PBMC) were isolated from anonymous healthy donors. For transduction, PBMC were activated at 1×10^6 per well in 24-well tissue culture plates for 48 h in complete RPMI added with 10 μg/mL phytohemagglutinin (PHA; Sigma) or anti-CD3/anti-CD28–coated beads (Dynabeads CD3/CD28; Dynal, Invitrogen) and 100 units/mL recombinant human interleukin-2 (rhIL-2; Proleukin; Chiron). Unless otherwise stated, all PBMC transduction experiments were performed by incubating 10^6 activated lymphocytes with 200 μL of 100-fold concentrated LV-containing supernatant in the presence of protamine sulfate (Sigma; 8 μg/mL). After 1 h at 37°C, transduction suspension was added with 1 mL fresh complete RPMI, supplemented with IL-2 at 100 units/mL, and finally plated in 24-well plates for an additional 72 h. Cotransduction experiments were performed by sequential infections with pCMV-TCRα and pCMV-TCRβ. Immediately after transduction, and weekly thereafter, PBMC were harvested, washed, and restimulated with irradiated (60 Gy) T2 cells, loaded with the Melan-A_{26-35} analogue peptide (5 μmol/L; ELAGIGILTV), at a ratio of 5:1. Jurkat cells were transduced by incubating 2×10^5 with 1 mL standard viral-containing supernatant for 6 h at 37°C in the presence of protamine sulfate (8 μg/mL).

Cytotoxicity assay. Cytotoxicity against peptide-loaded or unloaded T2 cells and melanoma cells was measured by a standard ^{51}Cr-release assay (18). For antibody-blocking experiments, target cells were preincubated for 30 min at 37°C with 10 μg/mL anti-CD8 mAb (UCHT-4 clone; Sigma). Cytotoxicity was expressed either as percentage of lysis or as $LU_{30}/10^6$. For peptide titration assays, ^{51}Cr-labeled target T2 cells were incubated with serial dilution of Melan-A peptide for 1 h at 37°C and finally mixed with TCR transduced cells at a ratio of 1:1.

Mice and adoptive immunotherapy. *In vivo* experiments were performed using 6- to 8-wk-old female severe combined immunodeficient (SCID) mice (n = 5-11 for all groups) purchased from Charles River Laboratories (Calco) and housed in our specific pathogen-free animal facility. Procedures involving animals and their care were in conformity with institutional guidelines (D.L. 116/92 and subsequent implementing circulars). Winn assay was performed by injecting s.c. 1×10^6 SK-23 MEL or A375 tumor cells per animal mixed with either RPMI or TCR-transduced T-cell suspension (5×10^6 per animal). For therapeutic experiments, tumor-bearing mice were repeatedly treated by s.c. or i.v. injections with 10×10^6 TCR-transduced T lymphocytes, starting from day 5 after tumor inoculation, and up to a maximum of three injections. Where indicated, six doses of 30,000 units/dose of rhIL-2 were injected i.p., 6 to 8 h apart, into control and treated tumor-bearing animals.

***In vivo* T-cell tracking and optical imaging.** TCR-transduced T lymphocytes were labeled with the fluorescent membrane dye DiR (Invitrogen) for 60 min at 37°C. To induce tumor growth, SCID mice received 10×10^6 Mel3/3 cells in 200 μL PBS s.c. in the right flank. One month was required to get *in vivo* established tumors. At that time point, $30 \times 10^6/60 \times 10^6$ DiR$^+$ TCR-transduced T cells were adoptively transferred by i.v. injection. *In vivo* monitoring of cell migration was performed on anesthetized animals before injection and daily for 4 d following adoptive transfer, using the eXplore Optix imager (ART, Canada). TIL were obtained as cell suspension by the enzymatic digestion of minced pieces of Mel3/3 melanoma tumors (19) and analyzed cytofluorimetrically.

Statistical analysis. Kaplan-Meier product-limit method was performed to estimate the survival curves, and comparison of survivals between groups was performed using the log-rank test. Medians were calculated and reported with their P values based on a two-sided testing. Statistical analyses were carried out with the MedCalc statistical package (version 8.1).

Results

TCR selection and development of efficient bidirectional LV. The previously described (16) Melan-A–specific SELA-A/64 CTL clone was selected as donor of TCR α and β chains (TRAV12-2/J35/C and TRBV27/D2/J2-7/C2, respectively).

Before proceeding with the construction of a viral gene delivery system for TCR, we sought to compare the gene transfer efficiency of either lentiviral (pCMV-EGFP) and retroviral (RV LESN; see Supplementary Materials and Methods) constructs expressing the *EGFP* reporter gene. Vector performances were assessed in highly transducible Jurkat cells, characterized by low expression levels of the endogenous TCR (15) and in PBMC. Standard viral preparations disclosed strong differences in virus particle titers (2×10^6-5×10^6 and 2×10^5-4×10^5 transducing units/mL for LV and RV, respectively), consistent with previous data (20). This reflected on transduction outcome, as a single round of infection with a equal amount of nonconcentrated virus supernatant led to *EGFP* expression in almost 80% and 30% of Jurkat cells after LV and RV addition, respectively (Supplementary Fig. S1, *top*). A similar difference was also observed in PHA-activated PBMC.[6] Nonetheless, infection experiments carried out on PBMC using the same multiplicity of infection (MOI) confirmed a transduction advantage for LV compared with RV (Supplementary Fig. S1, bottom). Based on these results, we therefore decided to proceed with LV development. To optimize TCR transduction, we adopted a single LV containing a bidirectional promoter (14), which assures a robust and coordinated expression of two transgenes (Fig. 1). Two bidirectional LV (BD I-TCR and BD V-TCR) were produced, differing for the relative α and β transgene orientation with respect to the LV elements. An identical vector production system led to VSV-pseudotyped monocistronic LV coding for the separated α (pCMV-TCRα) or β (pCMV-TCRβ) TCR chains (Fig. 1). Bidirectional and monocistronic LV were compared cytofluorimetrically by Jurkat cell staining with the specific tetramer at 72 hours after transduction with nonconcentrated viral supernatants (Fig. 1, *right*). Although cotransduction with monocistronic vectors was followed by detectable levels of exogenous TCR expression in about one third of cell population, bidirectional vectors significantly enhanced surface expression of the specific anti–Melan-A

[6] Unpublished data.

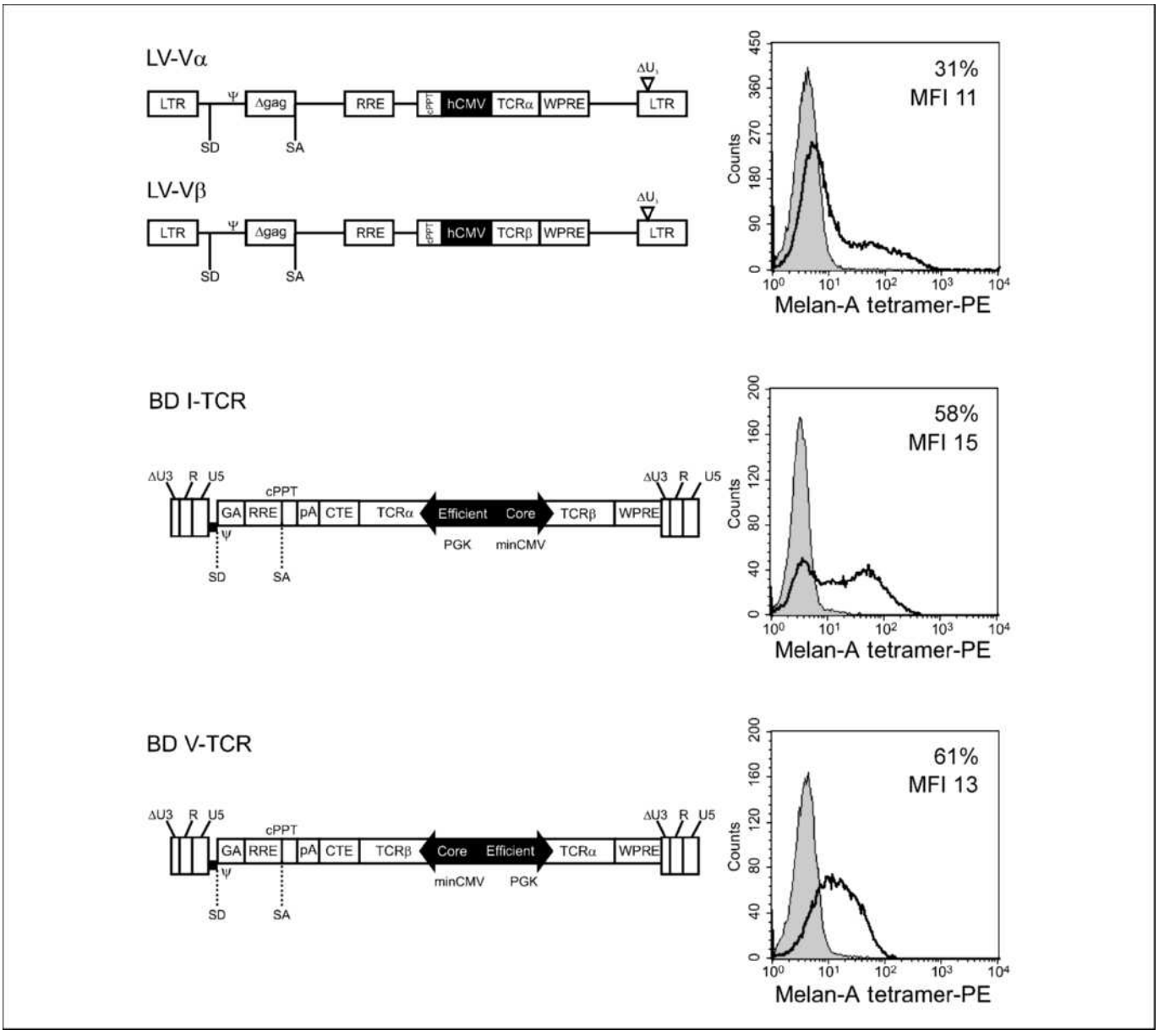

Figure 1. LV for TCR transduction. *Left,* linear maps of the recombinant proviral vectors developed; *right,* cytofluorimetric profiles of tetramer-stained transduced (*dark lines*) and nontransduced (*shaded curves*) Jurkat cells. The percentage and mean fluorescence intensity (*MFI*) of tetramer$^+$ cells within the total population are indicated in the top right corner of each histogram.

TCR. Moreover, as the twin bidirectional vectors did not differ for TCR surface expression efficiency, we selected BD I-TCR (hereafter simply called BD-TCR) for further work.

Generation of Melan-A–specific T-cell populations by TCR-encoding LV transduction of activated PBMC. In activated PBMC transduced with the BD-TCR, a small fraction of T lymphocytes was detected as tetramer$^+$ events at 72 hours postinfection without any previous selection,[6] consistent with previous data (14, 21). A rapid expansion protocol was then developed (Fig. 2*A*). PBMC obtained from two HLA-A2$^-$ donors were either transduced at a high MOI with BD-TCR or cotransduced with the two monocistronic TCR vectors or left untransduced (mock). Cultures were then restimulated weekly with antigen-loaded T2 cells and serially analyzed for growth and transgenic TCR expression by tetramer staining. BD-TCR–transduced PBMC increased up to 100-fold in 30 days on antigenic stimulation, whereas cotransduced cultures expanded with a slower kinetics (Fig. 2*B*). Moreover, transgenic T lymphocytes could be easily frozen and resumed an even more elevated expansion rate after one or more rounds of defrosting.[6] During the first two rounds of stimulation, both cultures accumulated a detectable tetramer$^+$ T-cell component.[6] By the third stimulation round, the expansion became faster and more sustained for the cultures transduced with BD-TCR compared with those undergoing cotransduction. Values of T cells expressing the transgenic TCR peaked at 6 weeks and reached ~60% to 80% to remain constant thereafter (Fig. 2*C*). No significant expansion of the Melan-A–reactive endogenous T-cell component was detected in mock control cultures, although stimulation conditions overlapped those described to generate and expand the original TCR-donor lymphocytes (16).

The efficacy of LV as TCR gene transfer strategy was ultimately validated by a comparative analysis on activated PBMC with a self-inactivating RV containing the entire bidirectional cassette. All transduction experiments were performed with concentrated supernatants at a MOI of 5. Upon infection, RV disclosed a very low proviral DNA integration compared with LV (Supplementary Fig. S2*A*), supporting data already seen with EGFP. Following stimulation with antigen-loaded T2 cells (Fig. 2*A*), LV-transduced PBMC progressively expanded, whereas the RV-infected and the mock populations underwent a contraction after a peak of expansion around day 12 probably due to the terminal effects of PHA activation (Supplementary Fig. S2*B*). These phenomena are consistent with the differential TCR expression induced by either viral systems and hence with the capacity of responding and expanding to the antigen encounter. Indeed, a tetramer$^+$ population readily accumulated in LV-transduced PBMC and was maximal in correspondence of growth peak. Conversely, the transgenic TCR-expressing population was negligible or apparently absent in the RV-infected cultures (Supplementary Fig. S2*C*). These data are also paralleled by results obtained by staining the cultures with an anti-Vβ14 mAb (Supplementary Fig. S2*C, inset*). To exclude that the RV was not functional, PBMC cultures were then transduced with virus preparations concentrated at a MOI of 100. In this case, a TCR-positive fraction, albeit very low, was clearly detectable by day 20 (Supplementary Fig. S2*D*); on the other hand, when using a MOI of 5, only a very long period of antigenic stimulation (~2 months) allowed for the emergence of a well-defined tetramer$^+$ subset (Supplementary Fig. S2*E*). Overall, the present data prompted us to select LV for further work.

Coculture of BD-TCR–transduced PBMC with antigen-loaded T2 cells promotes the generation of functional effector memory T lymphocytes. *In vitro* phenotypic properties and functions of transduced T lymphocytes were studied by flow cytometry assays and functional tests at the time of *in vivo* experiments. Immunophenotypically, the engineered T lymphocytes progressed toward an intermediate and late effector stage. Nevertheless, the CD27 stimulatory molecule, an index of a less differentiated state of T lymphocytes, was still highly expressed on approximately all cells (Fig. 3*A*). Transferred T cells were CD8$^+$ lymphocytes for >99%, due to a progressive exhaustion of the CD4$^+$ fraction in culture (Fig. 3*B*), likely resulting from CD8 dependency of the transgenic TCR (see below). Functionally, TCR-transduced T cells produced IFN-γ in response to antigen stimulation (Fig. 3*C*) and selectively and efficiently killed T2 cells loaded with Melan-A peptide but not T2 cells alone. Moreover, BD-TCR–transduced T cells were also lytic against SK-23 MEL cancer cells at very low effector:target ratios but not against control PDO-328 MEL melanoma cells (Fig. 3*D, left*). Specific recognition was CD8-dependent

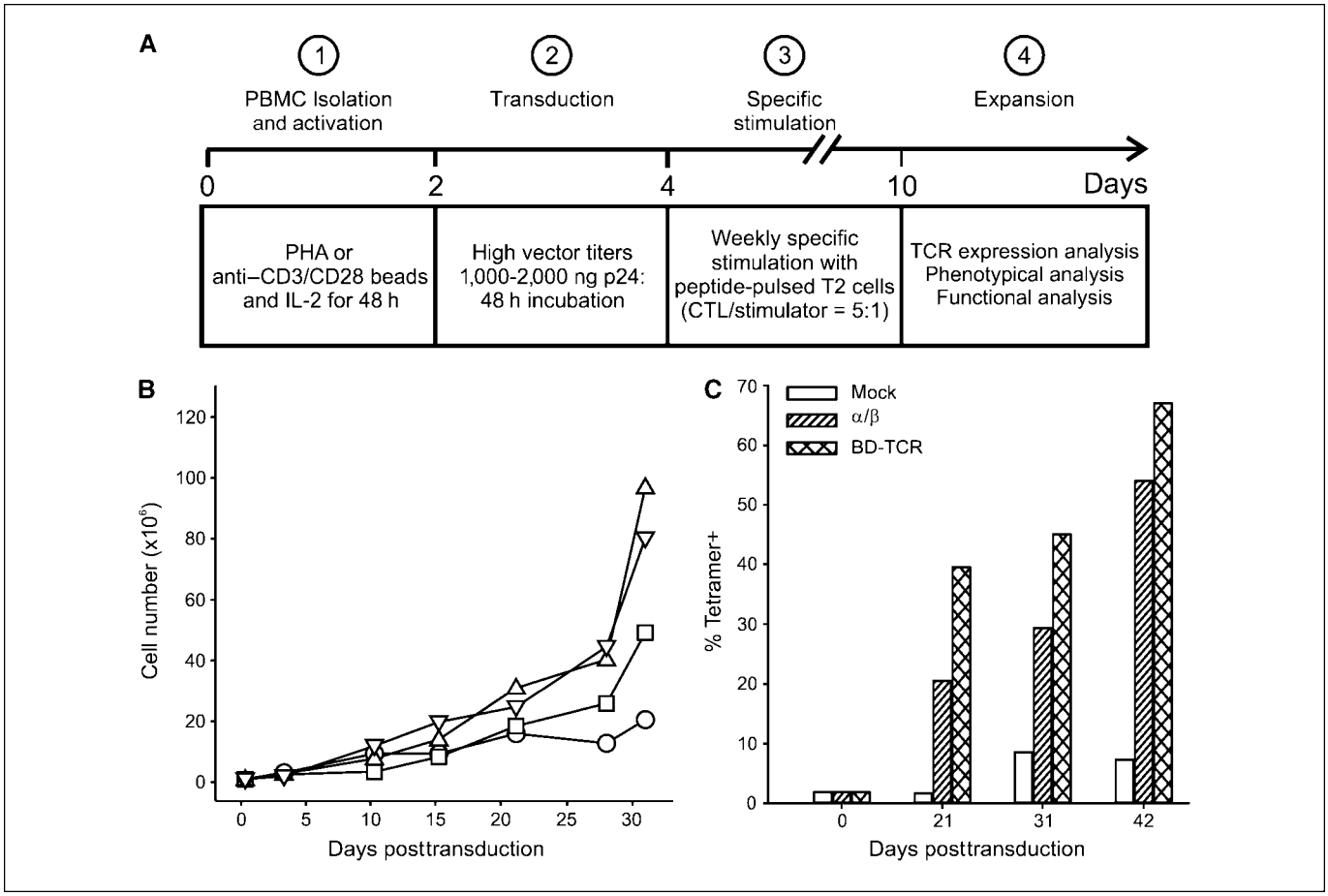

Figure 2. *A,* schematic representation of PBMC stimulation and expansion protocol. *B,* growth kinetics of T-cell cultures generated by transducing activated PBMC with BD-TCR (donor 1, △; donor 2, ▽) or with two monocistronic vectors (α/β; □). A nontransduced, antigen-expanded culture is reported as control (mock; ○). *C,* transgenic TCR expression kinetics in transduced T cells. A comparison between PBMC transduced with BD-TCR and cells cotransduced with two single-chain–expressing monocistronic vectors (*α/β*). Nontransduced cells are shown as a negative control (*Mock*).

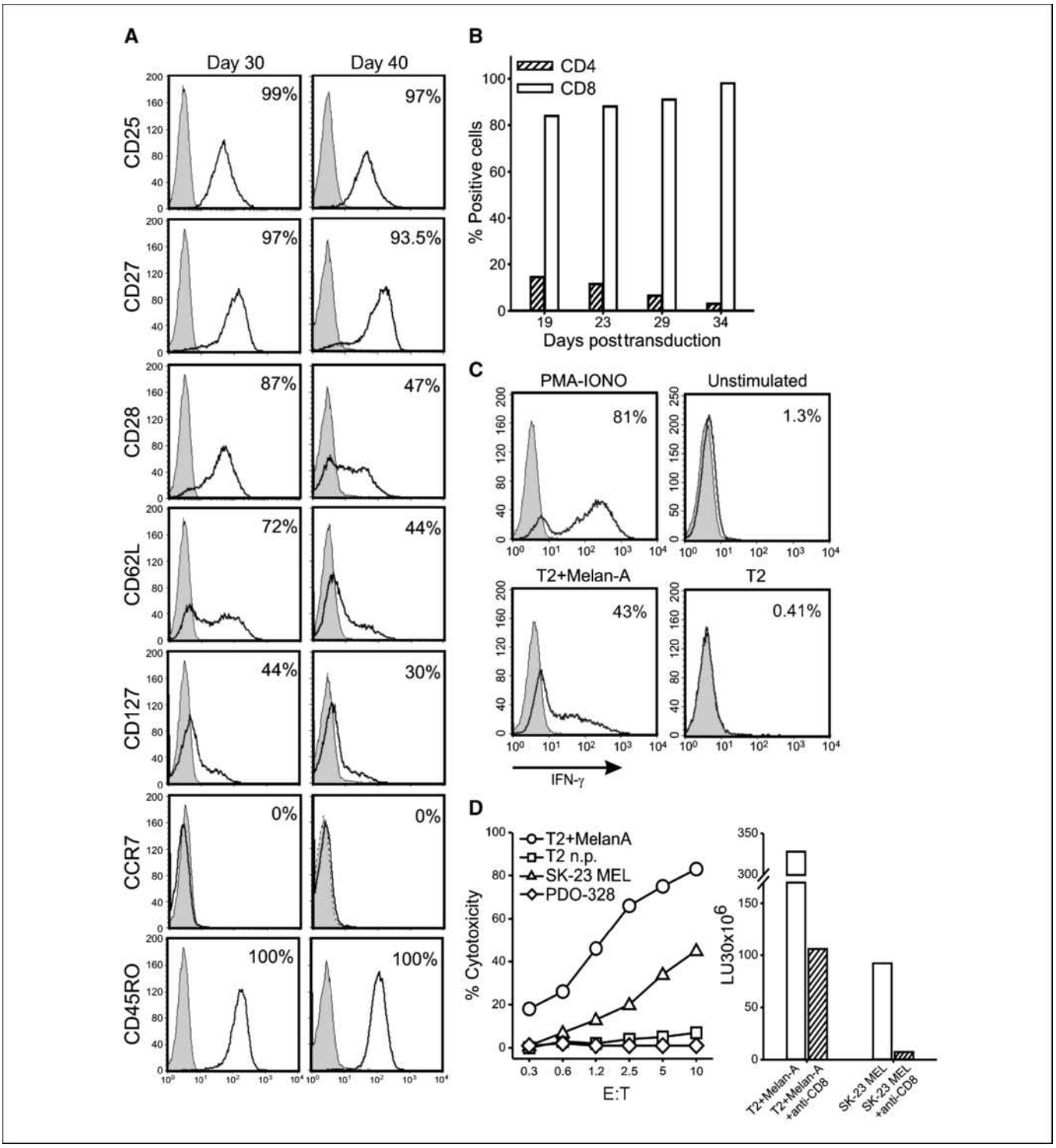

Figure 3. Phenotypic and functional properties of LV-transduced T cells. *A,* surface phenotype of cultured T cells 30 d (*left*) and 40 d (*right*) posttransduction. The expression of each molecule on total gated T cells is reported (*shaded curves,* isotype controls). *B,* kinetics of CD4 and CD8 coreceptor expression. *C,* antigen-induced IFN-γ production upon stimulation of transduced T cells. Incubation with phorbol 12-myristate 13-acetate/ionomycin (*PMA-IONO*) is shown as the positive maximal control stimulus. *D,* lytic activity and specificity of T lymphocytes from the transduced population (*left*). Cytotoxicity depended on CD8 coreceptor as shown by reduction in the presence of anti-CD8 blocking antibody (*right*).

(Fig. 3*D, right*), supporting previous observation about $CD8^+$ T-cell selection in culture on stimulation with pulsed T2 cells.

Transduced T lymphocytes acquire and maintain the specificity and functional activity of the original CTL clone. Next, we assessed whether the clonal culture (99% $CD8^+$/$tetramer^+$ with a mean fluorescence intensity for the tetramer of 23) and an unsorted transgenic population (70% $CD8^+$/$tetramer^+$ with a tetramer mean fluorescence intensity value of 18; Fig. 4*A*) had similar ligand

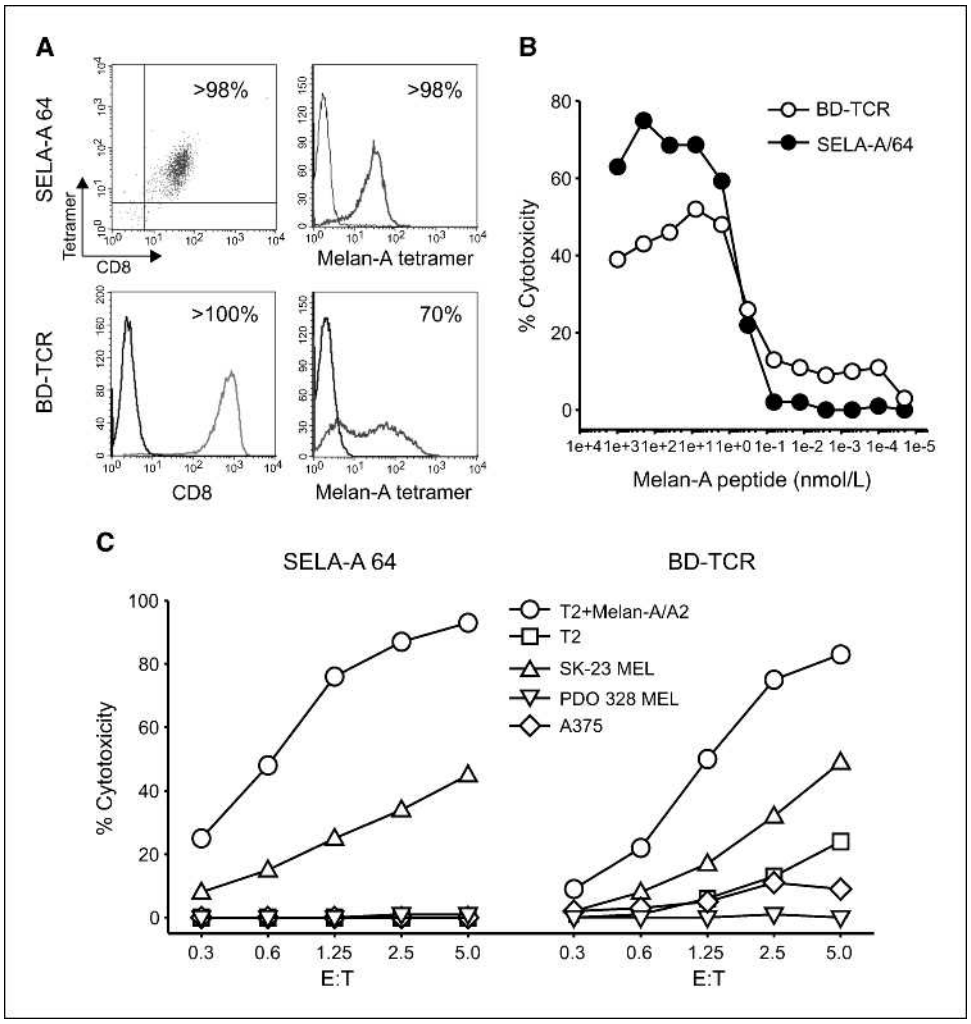

Figure 4. Transgenic TCR reproduces the characteristics of the original CTL clone. *A,* $CD8^+$/$tetramer^+$ or single-positive subsets within the parental SELA-A/64 CTL clone culture and nonsorted T cells from the transduced population. *B,* comparative functional avidity assessment. Effector cells were incubated with ^{51}Cr-labeled T2 cells at an effector:target ratio of 1:1 in the presence of decreasing concentrations of Melan-A peptide. Avidity was defined as the peptide concentration giving 50% of the maximum specific lysis. *C,* comparison of the cytotoxic activities.

recognition capacity and functional properties. Interestingly, a significant fraction of the BD-TCR–transduced population expressed the specific TCR at higher levels than the donor clone. Peptide titration revealed that cultures had comparable avidity values (32 and 60 nmol/L for the original clone and the BD-TCR population, respectively; Fig. 4*B*). Moreover, they exhibited an almost overlapping lytic activity against Melan-A–expressing target cells (Fig. 4*C*). Thus, data indicate that TCR transfer from SELA-A/64 CTL clone to alternative effectors results in the redirection of the antigen specificity of recipient T lymphocytes against the Melan-A antigen, with preservation of the original functional properties (22).

***In vivo* tumor treatment.** To evaluate *in vivo* the antimelanoma therapeutic effect of TCR-transduced T cells, we first carried out a Winn assay (Fig. 5*A*). Whereas untreated control SK-23 MEL tumors underwent progressive growth, no tumor masses formed at the site where melanoma cells and BD-TCR T lymphocytes were coinjected. As a specificity control, treatment of A375 cells led to a nonsignificant delay of tumor growth (Fig. 5*A, inset*). In a subsequent set of experiments, we tested whether engineered CTL could be effective against established tumors. A single dose (10^7) of T cells given 7 days after tumor injection had no apparent effect.[6] Assuming a dose dependence for T-cell therapeutic efficacy, we administered two or three doses of T cells to tumor-bearing animals at days 7 and 12 or at days 7, 12, and 17, respectively. Both schedules determined a significant delay of SK-23 MEL tumor growth, compared with controls ($P < 0.001$; Fig. 5*B*), and led to 25% and 62.5% increase, respectively, in the overall survival rates ($P < 0.05$; Fig. 5*B, inset*). Finally, further experiments were conducted by administering transgenic T cells by tail vein injection at days 5, 10, and 14 after s.c. tumor injection. TCR-transduced cells did not exert any therapeutic effect nor increased survival in treated mice compared with untreated animals (Fig. 5*C*). Moreover, no partial rescue of T-cell activity was observed in mice receiving 30,000 IU rhIL-2 twice daily for 3 days starting from the day of T-cell transfer (Fig. 5*D*).

Tracking engineered T cells *in vivo* by optical imaging reveals their poor homing to the tumor site. Results of *in vivo* experiments showed that locally administered engineered T cells were efficient in mediating an antitumor response even against established tumors, whereas their i.v. injection did not produce detectable effects. This dichotomy prompted us to study the biodistribution of transgenic T cells on i.v. adoptive transfer in tumor-bearing mice to assess their potential homing to the tumor site. Labeling with the fluorescent dye DiR produced a staining persisting for >1 week, although fluorescence intensity declined more rapidly in accordance with the progressive dilution of the dye due to cell division (Supplementary Fig. S3*A*). DiR did not interfere with the expression of transgenic TCR (Supplementary

Fig. S3*B*) nor affected target cell recognition (Supplementary Fig. S3*C*). For tumor-homing studies, nonpigmented Mel3/3 cell line was used as melanoma model, as melanin strongly interferes with DiR signal. SCID mice bearing established Mel3/3 tumors were injected with DiR-labeled engineered T cells and their whole biodistribution was assessed by analyzing a specific region of interest comprising the total body (Fig. 6*A*). Starting from day 1, an increasing specific signal could be detected in inguinal lymph nodes and in tumor, although most cells were retained in liver (Fig. 6*A*), as assessed at day 4 after sacrifice of mice and analysis of single explanted organs.[6] A more detailed analysis of data inside the tumor mass (Fig. 6*B*) showed a progressive increase of a specific signal, indicating that transferred T cells were indeed accumulating to the tumor site. Extrapolation of imaging data indicated that the amount of signal present into the tumor mass accounted for <1% of the total. To ascertain that the visualized signal was not due to dye aspecifically captured by macrophages or other cells, we isolated leukocytes infiltrating the tumor mass at day 4 after transfer. By morphologic gating of the lymphocyte population, a small fraction of double-positive $DiR^+/CD8^+$ cells was identified, thus showing that the signal detected into the tumor could be really ascribed to the labeled population transferred (Fig. 6*C*).

Discussion

Melan-A–specific T cells can be identified from metastatic tumor lesions or tumor-infiltrating lymph nodes and circulating lymphocytes of melanoma patients as well as healthy subjects ($\sim 1/10^3$ of circulating CD8 T cells; ref. 23). Nonetheless, methods to expand the endogenous tumor-specific T-cell component are often extremely laborious and time-consuming, not always successful, and severely limited by interindividual and technical factors. TCR gene transfer approach overcomes such limits by providing both the possibility to select optimal antigen receptors and lymphocyte properties to maximize the effectiveness of ACT

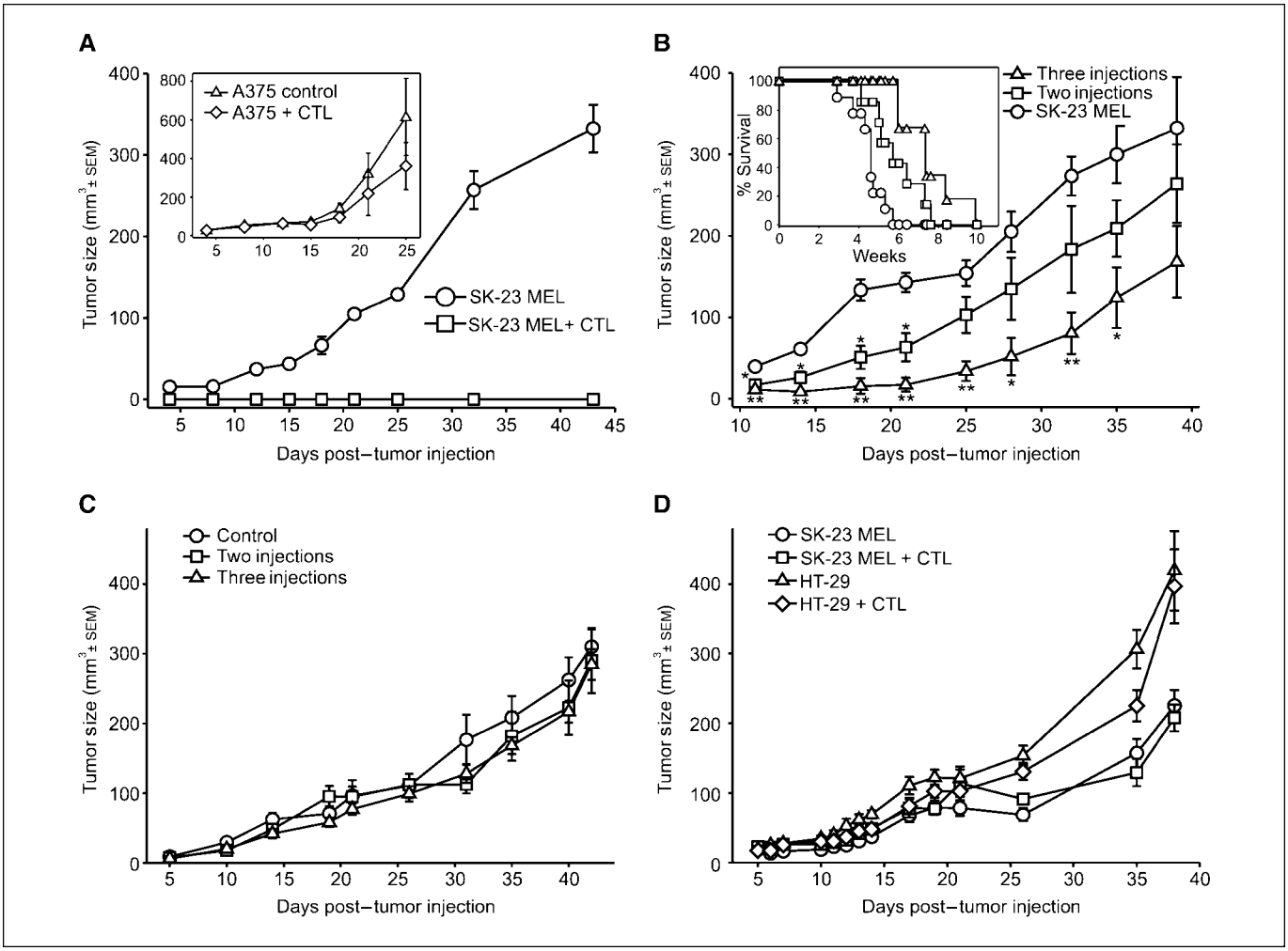

Figure 5. Adoptive transfer. *A,* Winn assay: SK-23 MEL cells were mixed with RPMI or effector T cells and injected s.c. at day 0 in SCID mice (five mice per group). As a specificity control, A375 cells were used (*inset*). *B,* tumor growth and survival analysis (*inset*) in SCID mice inoculated with SK-23 MEL cells and left untreated ($n = 9$) or treated locally with two doses ($n = 7$) or three doses ($n = 6$) of transgenic T cells. Median survivals: control group = 32 d; two-injections group = 40 d; three-injections group = 52 d; control group versus two-injections group, $P < 0.05$; control group versus three-injections group, $P < 0.0002$. *, $P < 0.001$; **, $P < 0.0001$, statistically significant differences. *C,* i.v. administration: mice were left untreated (control, $n = 9$) or received two or three doses of effector cells ($n = 6$ and 11, respectively). *D,* rhIL-2 did not rescue or promote T-cell antitumor efficacy. Mice received SK-23 MEL cells on one flank and HT-29 control cells in the contralateral side at day 0 followed by T cells given i.v. three times (control mice, $n = 5$; treated mice, $n = 5$). Six doses of 30,000 units/dose rhIL-2 were given i.p. after each treatment.

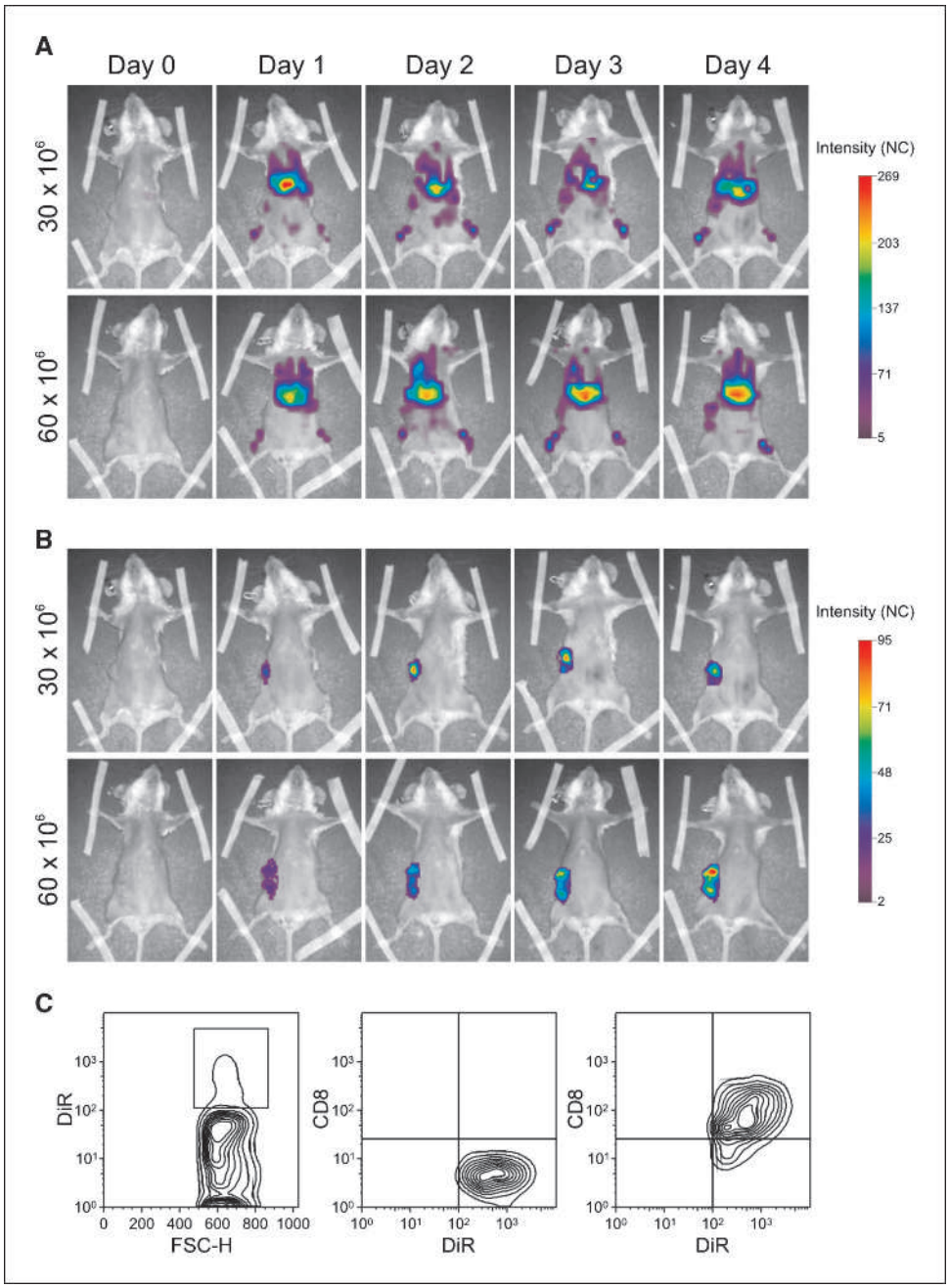

Figure 6. T-cell tracking by optical imaging. *A,* total body analysis of DiR^+ cell biodistribution in tumor-bearing mice. Animals bearing established Mel3/3 tumors were injected i.v. with 30×10^6 (*top*) or 60×10^6 (*bottom*) DiR-labeled transgenic T lymphocytes. Fluorescence imaging was conducted using a 3-mm scan step. *B,* accumulation of DiR^+ cells to the tumor site (scan step at 1 mm). *C,* cytometry analysis of the tumor infiltrate from a representative injected mouse: DiR^+ events were identified morphologically (*left*) and subsequently analyzed for the coexpression of human CD8 molecule [unstained cells (*middle*) versus stained cells (*right*)].

in a reduced time frame compatible with therapeutic application. Clinical use of "redirected" T lymphocytes has moved faster than preclinical research and in the absence of an exhaustive animal analysis. Indeed, in preclinical models (24–29), targeted antigens were mostly highly immunogenic, poorly physiologic tumor-associated antigens, with some major exceptions (30). Another important aspect deals with the viral vectors used for transfer, as recent data relate the loss of transgene expression to the compromised clinical efficacy of gene-modified T cells. The difference in efficacy between clinical studies using TIL (>50% of objective responses; refs. 31, 32) and TCR-modified T cells (13-30% of responses; refs. 2, 33) is accompanied by a decrease in the observed persistence of transferred specific T cells (26 versus 13 months; ref. 2). The relevance of TCR transgene expression is further emphasized by data of a clinical trial using a chimeric antigen receptor (34).

We report here that LV-mediated efficient transfer of a Melan-A–specific human TCR into T lymphocytes, which fully reproduced *in vitro* the characteristics and functionality of the original donor CTL clone, as reported previously (21, 35), exerted relevant therapeutic effects *in vivo* and localized to the tumor mass after systemic administration. In particular, we used a LV carrying a synthetic and optimized strong promoter (14) capable of driving the coordinated and robust expression of both α and β chains of the transgenic TCR. Additionally, LV-BD behaved much more efficiently than RV-BD in mediating TCR transfer, in agreement with recent advancements in the field (20, 36). Notably, RV-BD was developed as self-inactivating vector to reduce the described genotoxicity of

viral long terminal repeat (37). Although further vector refinements may increase performances (38), nonetheless the use of such bidirectional LV and of a rapid and simple expansion protocol likely leads to the establishment of an engineered population characterized by elevated mRNA levels of transgenic TCR chains; this in turn results in a competition ratio favoring the assembly and the surface expression of a completely exogenous receptor. The resulting "functional" shutdown of the endogenous TCR expression can have important rebounds. On one hand, this limits the emergence of potential autoreactive hybrid TCR. On the other hand, it reduces the requirements for several cycles of *in vitro* antigen stimulation to expand the transgenic TCR-expressing population, an aspect that may have important drawbacks on phenotype and functional characteristics of T lymphocytes, limiting their therapeutic outcome *in vivo* on adoptive transfer (39). With respect to this point, *in vivo* imaging revealed that transgenic T lymphocytes progressively accumulated into the tumor mass, although their reduced number was likely responsible of the treatment failure after i.v. administration. The low retention/homing to the tumor is presumably dependent on the experimental model and the poor trafficking of human T lymphocytes in the mouse microenviroment (40), or the lack of specific signals for expansion, whereas their functionality seems to be fully preserved, as shown by therapeutic activity when administered locally. These considerations cannot be disjointed by some comments on the characteristics of TCR selected thus far for transfer studies. CTL and TIL endowed with the highest antigen affinity are currently considered the best choice for ACT protocols (2); however, studies we carried out on the Moloney mouse sarcoma/leukemia virus tumor model (18) disclosed that an increasing antigenic and tumor load triggered a phenomenon of activation-induced cell death in CTL characterized by a very high avidity for the antigen, with the progressive elimination of the effector population.[6] On the other hand, a sufficiently high TCR avidity would likely lead to functional independence from CD4 or CD8 coreceptors, thus allowing transgene expression and functionality in both T-cell subsets (21, 41–45). The TCR used in the present work turned out to be CD8 dependent; accordingly, the $CD4^+$ population, while initially expressing the transgenic TCR, did not expand and was progressively overgrown by $CD8^+$ T cells. Finally, most TCR studied thus far have been derived from CTL clones directed to melanocyte antigens (46). While recognizing the importance for melanoma, a striking advancement in the field will be likely represented by the establishment of TCR-donor T-cell clones specific for universal tumor antigens, such as survivin or telomerase (47–49). This would offer the possibility to set up a bank of TCR restricted for the most common MHC alleles and specific for a limited array of highly relevant tumor-associated antigen, thus leading to the potential targeting of a broad spectrum of histotypically different cancers and fostering the exchange of reagents and comparative analysis of data from multicentric trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

1. Dudley ME, Rosenberg SA. Adoptive-cell-transfer therapy for the treatment of patients with cancer. Nat Rev Cancer 2003;3:666–75.
2. Morgan RA, Dudley ME, Wunderlich JR, et al. Cancer regression in patients after transfer of genetically engineered lymphocytes. Science 2006;314:126–9.
3. Kohn DB, Hershfield MS, Carbonaro D, et al. T lymphocytes with a normal ADA gene accumulate after transplantation of transduced autologous umbilical cord blood $CD34^+$ cells in ADA-deficient SCID neonates. Nat Med 1998;4:775–80.
4. Vigna E, Naldini L. Lentiviral vectors: excellent tools for experimental gene transfer and promising candidates for gene therapy. J Gene Med 2000;2:308–16.
5. Follenzi A, Ailles LE, Bakovic S, Geuna M, Naldini L. Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. Nat Genet 2000;25:217–22.
6. Zufferey R, Donello JE, Trono D, Hope TJ. Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. J Virol 1999;73:2886–92.
7. Pfeifer A, Ikawa M, Dayn Y, Verma IM. Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. Proc Natl Acad Sci U S A 2002;99:2140–5.
8. Naldini L, Blomer U, Gallay P, et al. *In vivo* gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 1996;272:263–7.
9. Huang J, Kerstann KW, Ahmadzadeh M, et al. Modulation by IL-2 of CD70 and CD27 expression on $CD8^+$ T cells: importance for the therapeutic effectiveness of cell transfer immunotherapy. J Immunol 2006;176:7726–35.
10. Klebanoff CA, Gattinoni L, Torabi-Parizi P, et al. Central memory self/tumor-reactive $CD8^+$ T cells confer superior antitumor immunity compared with effector memory T cells. Proc Natl Acad Sci U S A 2005;102:9571–6.
11. Zhou J, Shen X, Huang J, Hodes RJ, Rosenberg SA, Robbins PF. Telomere length of transferred lymphocytes correlates with *in vivo* persistence and tumor regression in melanoma patients receiving cell transfer therapy. J Immunol 2005;175:7046–52.
12. Robbins PF, Dudley ME, Wunderlich J, et al. Cutting edge: persistence of transferred lymphocyte clonotypes correlates with cancer regression in patients receiving cell transfer therapy. J Immunol 2004;173:7125–30.
13. Lanzavecchia A, Sallusto F. Progressive differentiation and selection of the fittest in the immune response. Nat Rev Immunol 2002;2:982–7.
14. Amendola M, Venneri MA, Biffi A, Vigna E, Naldini L. Coordinate dual-gene transgenesis by lentiviral vectors carrying synthetic bidirectional promoters. Nat Biotechnol 2005;23:108–16.
15. Sebestyen Z, Schooten E, Sals T, et al. Human TCR that incorporate CD3ζ induce highly preferred pairing between TCRα and β chains following gene transfer. J Immunol 2008;180:7736–46.
16. Mandruzzato S, Rossi E, Bernardi F, et al. Large and dissimilar repertoire of Melan-A/MART-1-specific CTL in metastatic lesions and blood of a melanoma patient. J Immunol 2002;169:4017–24.
17. Persano L, Moserle L, Esposito G, et al. Interferon-α counteracts the angiogenic switch and reduces tumor cell proliferation in a spontaneous model of prostatic cancer. Carcinogenesis 2009;30:851–60.
18. Rosato A, Milan G, Collavo D, Zanovello P. DNA-based vaccination against tumors expressing the P1A antigen. Methods 1999;19:187–90.
19. Rosato A, Mandruzzato S, Bronte V, et al. Role of anti-LFA-1 and anti-ICAM-1 combined MAb treatment in the rejection of tumors induced by Moloney murine sarcoma virus (M-MSV). Int J Cancer 1995;61:355–62.
20. Indraccolo S, Habeler W, Tisato V, et al. Gene transfer in ovarian cancer cells: a comparison between retroviral and lentiviral vectors. Cancer Res 2002;62:6099–107.
21. Tsuji T, Yasukawa M, Matsuzaki J, et al. Generation of tumor-specific, HLA class I-restricted human Th1 and Tc1 cells by cell engineering with tumor peptide-specific T-cell receptor genes. Blood 2005;106:470–6.
22. Schaft N, Willemsen RA, de Vries J, et al. Peptide fine specificity of anti-glycoprotein 100 CTL is preserved following transfer of engineered TCR α β genes into primary human T lymphocytes. J Immunol 2003;170:2186–94.
23. Zippelius A, Pittet MJ, Batard P, et al. Thymic selection generates a large T cell pool recognizing a self-peptide in humans. J Exp Med 2002;195:485–94.
24. Chamoto K, Tsuji T, Funamoto H, et al. Potentiation of tumor eradication by adoptive immunotherapy with T-cell receptor gene-transduced T-helper type 1 cells. Cancer Res 2004;64:386–90.
25. de Witte MA, Bendle GM, van den Boom MD, et al. TCR gene therapy of spontaneous prostate carcinoma requires *in vivo* T cell activation. J Immunol 2008;181:2563–71.

26. de Witte MA, Coccoris M, Wolkers MC, et al. Targeting self-antigens through allogeneic TCR gene transfer. Blood 2006;108:870–7.

27. Kessels HW, Schepers K, van den Boom MD, Topham DJ, Schumacher TN. Generation of T cell help through a MHC class I-restricted TCR. J Immunol 2006;177:976–82.

28. Kessels HW, Wolkers MC, van den Boom MD, van der Valk MA, Schumacher TN. Immunotherapy through TCR gene transfer. Nat Immunol 2001;2:957–61.

29. Morris EC, Tsallios A, Bendle GM, Xue SA, Stauss HJ. A critical role of T cell antigen receptor-transduced MHC class I-restricted helper T cells in tumor protection. Proc Natl Acad Sci U S A 2005;102:7934–9.

30. Abad JD, Wrzensinski C, Overwijk W, et al. T-cell receptor gene therapy of established tumors in a murine melanoma model. J Immunother 2008;31:1–6.

31. 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;23:2346–57.

32. Rosenberg SA, Dudley ME, Restifo NP. Cancer immunotherapy. N Engl J Med 2008;359:1072.

33. Johnson LA, Morgan RA, Dudley ME, et al. Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. Blood 2009;114:535–46.

34. Lamers CH, Gratama JW, Pouw NM, et al. Parallel detection of transduced T lymphocytes after immunogene therapy of renal cell cancer by flow cytometry and real-time polymerase chain reaction: implications for loss of transgene expression. Hum Gene Ther 2005;16:1452–62.

35. Coccoris M, de Witte MA, Schumacher TN. Prospects and limitations of T cell receptor gene therapy. Curr Gene Ther 2005;5:583–93.

36. Rosenberg SA, Restifo NP, Yang JC, Morgan RA, Dudley ME. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. Nat Rev Cancer 2008;8:299–308.

37. Montini E, Cesana D, Schmidt M, et al. The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a mouse model of HSC gene therapy. J Clin Invest 2009;119:964–75.

38. Frecha C, Costa C, Negre D, et al. Stable transduction of quiescent T cells without induction of cycle progression by a novel lentiviral vector pseudotyped with measles virus glycoproteins. Blood 2008;112:4843–52.

39. Gattinoni L, Klebanoff CA, Palmer DC, et al. Acquisition of full effector function *in vitro* paradoxically impairs the *in vivo* antitumor efficacy of adoptively transferred $CD8^+$ T cells. J Clin Invest 2005;115:1616–26.

40. Pinthus JH, Waks T, Malina V, et al. Adoptive immunotherapy of prostate cancer bone lesions using redirected effector lymphocytes. J Clin Invest 2004;114:1774–81.

41. Cohen CJ, Zheng Z, Bray R, et al. Recognition of fresh human tumor by human peripheral blood lymphocytes transduced with a bicistronic retroviral vector encoding a murine anti-p53 TCR. J Immunol 2005;175:5799–808.

42. Kuball J, Schmitz FW, Voss RH, et al. Cooperation of human tumor-reactive $CD4^+$ and $CD8^+$ T cells after redirection of their specificity by a high-affinity p53A2.1-specific TCR. Immunity 2005;22:117–29.

43. Morgan RA, Dudley ME, Yu YY, et al. High efficiency TCR gene transfer into primary human lymphocytes affords avid recognition of melanoma tumor antigen glycoprotein 100 and does not alter the recognition of autologous melanoma antigens. J Immunol 2003;171:3287–95.

44. Roszkowski JJ, Lyons GE, Kast WM, Yee C, Van Besien K, Nishimura MI. Simultaneous generation of $CD8^+$ and $CD4^+$ melanoma-reactive T cells by retroviral-mediated transfer of a single T-cell receptor. Cancer Res 2005;65:1570–6.

45. Zhao Y, Zheng Z, Robbins PF, Khong HT, Rosenberg SA, Morgan RA. Primary human lymphocytes transduced with NY-ESO-1 antigen-specific TCR genes recognize and kill diverse human tumor cell lines. J Immunol 2005;174:4415–23.

46. Rosenberg SA. A new era of cancer immunotherapy: converting theory to performance. CA Cancer J Clin 1999;49:70–3, 65.

47. Andersen MH, Svane IM, Becker JC, Straten PT. The universal character of the tumor-associated antigen survivin. Clin Cancer Res 2007;13:5991–4.

48. Theobald M, Biggs J, Dittmer D, Levine AJ, Sherman LA. Targeting p53 as a general tumor antigen. Proc Natl Acad Sci U S A 1995;92:11993–7.

49. Vonderheide RH, Hahn WC, Schultze JL, Nadler LM. The telomerase catalytic subunit is a widely expressed tumor-associated antigen recognized by cytotoxic T lymphocytes. Immunity 1999;10:673–9.