Adoptive Transfer of Tumor-Reactive Melan-A-Specific CTL Clones in Melanoma Patients Is Followed by Increased Frequencies of Additional Melan-A-Specific T Cells 1 The Journal of Immunology

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Adoptive Transfer of Tumor-Reactive Melan-A-Specific CTL Clones in Melanoma Patients Is Followed by Increased Frequencies of Additional Melan-A-Specific T Cells[1]

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In this study, we report the adoptive transfer of highly tumor-reactive Melan-A-specific T cell clones to patients with metastatic melanoma, and the follow-up of these injected cells. These clones were generated from HLA-A*0201 patients by in vitro stimulations of total PBMC with the HLA-A*0201-binding Melan-A peptide analog ELAGIGILTV. Ten stage IV melanoma patients were treated by infusion of these CTL clones with IL-2 and IFN-α. The generated T cell clones, of effector/memory phenotype were selected on the basis of their ability to produce IL-2 in response to HLA-A*0201 Melan-A-positive melanoma lines. Infused clones were detected, by quantitative PCR, in the blood of three patients for periods ranging from 7 to 60 days. Six patients showed regression of individual metastases or disease stabilization, and one patient experienced a complete response, but no correlation was found between the detection of the infused clones in PBMC or tumor samples and clinical responses. Nonetheless, frequencies of Melan-A/A2-specific lymphocytes, measured by tetramer labeling, increased after treatment in most patients. In one of these patients, who showed a complete response, this increase corresponded to the expansion of new clonotypes of higher avidity than those detected before treatment. Together, our results suggest that infused CTL clones may have initiated an antitumor response that may have resulted in the expansion of a Melan-A-specific CTL repertoire. *The Journal of Immunology,* **2005, 175: 4797–4805.**

The molecular identification of tumor Ags recognized by tumor-reactive $CD8^+$ lymphocytes (1–3) has enabled the investigation of new approaches to generate tumor-specific lymphocytes that could be used in adoptive immunotherapy. Compared with vaccine strategies, adoptive therapy could overcome the in vivo constraints that influence the magnitude and avidity of the targeted response. T cells of defined specificity and of measurable functions and reactivity toward tumor cells can now be selected and expanded in vitro for infusion to patients.

Among defined melanoma-associated Ags, those from the melanocytic lineage are attractive targets for T cell therapies due to their high level of expression by many of these tumors. The Melan-A/MART-1 (hereafter Melan-A) Ag belongs to this category and has the unique property of being recognized by a large and reactive T cell repertoire. Furthermore, the potential immunogenicity of this Ag is suggested by the frequent enrichment of Melan-A-specific lymphocytes in melanoma-infiltrating lymphocytes (tumor-infiltrating lymphocytes (TIL))[4] (4–6). Due to these properties, Melan-A Ag has been targeted in many immunotherapy studies.

Recently, three recent studies evidenced the efficiency of the adoptive transfer of melanoma-specific lymphocytes in melanoma treatment. In these studies, infused lymphocytes were either oligoclonal TIL populations enriched in CD8 tumor-reactive T cells (7), clonal (8) or polyclonal (9) Melan-A-specific CTL. These three studies established for the first time that transferred T cells could survive for long periods in vivo. These transfers were followed in several cases by the decrease of Melan-A Ag expression in melanoma tumors, suggesting that Melan-A-specific T cells have trafficked to tumor sites and contributed to eliminate the original Melan-A-positive tumor cells. Furthermore, tumor regressions or stabilizations observed in these studies (7, 9, 10) strongly support the carrying out of similar adoptive therapies.

Generation of a high number of T cells of defined Ag specificity with adequate functional and survival properties is the major difficulty for adoptive therapy. Methods used so far to generate these cells consisted of stimulating patient PBMC with peptide-loaded dendritic cells (DC) (11, 12) and then enriching specific T cells by MHC/peptide tetramer sorting (9) and/or cloning (8). A major limit of this approach is that it remains logistically difficult to generate a sufficient number of autologous DC from each patient enrolled in

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[4] Abbreviations used in this paper: TIL, tumor-infiltrating lymphocyte; DC, dendritic cell; GMP, good manufactory practice; MU, million unit.

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Table I. *Number of infused CTL clones to melanoma patients*[a]

Patient ID	CTL ID	Number of Infusions	Number of Infused CTL (10^6)
Mela01	CTL01	1	200
Mela02	CTL02	1	140
Mela03[b]	CTL03.1	2	1400, 5000
	CTL03.2	1	900
Mela04[b]	CTL04	2	692, 513
Mela05[b]	CTL05	2	164, 36
Mela06	CTL06	1	2000
Mela10	CTL10	1	187
Mela13	CTL13	1	1500
Mela15	CTL15	1	322
Mela16	CTL16	1	2000

[a]Amount of infused Melan-A specific CTL clones (10^6) to melanoma patients. Melanoma patients received a single injection of Melan-A-specific CTL clones and three of them received two injections[b]. Following each T cell clone injection (day 0), patients received 9 MU of IL-2 from days 1–5, and from days 8–12, and IFN-α 9 MU for 1 mo three times a week.

a clinical study. In our study we used, as APCs, an allogeneic irradiated melanoma cell line, produced in good manufactory practice (GMP) conditions, and pulsed with the Melan-A26-35L analog peptide, according to a stimulation method that we described previously (13). With this method, PBL containing between 20 and 60% of Melan-A-specific cells are systematically obtained, so that a simple limiting dilution culture always provides Melan-A-specific clones highly reactive against melanoma cells. Therefore, we aimed at testing the absence of toxicity and antitumor potential of these cells in a phase I/II clinical assay in 16 metastatic (stage IV) melanoma patients.

In this study, we describe the properties of the clones infused to the first 10 patients included in this trial, the clinical responses observed, the results of immunological and molecular studies concerning the tracking of infused clones, and the evolution of Melan-A-specific T cell response in patient blood.

Materials and Methods

Patients and treatment schedule

Treated patients were HLA-A2 melanoma patients with locoregional relapse or evaluable cutaneous metastasis expressing Melan-A/MART-1 Ag, detected by RT-PCR. The trial was designed and conducted in accordance with the Declaration of Helsinki. The therapy protocol was approved by the Institutional Ethics Committee and registered with the regulatory state authority. All patients gave written informed consent before enrolling in the study. After blood collection (80 ml), Melan-A-specific clones were generated from PBMC and injected 90–120 days later. The first 10 patients received between 1.64×10^8 and 2×10^9 autologous Melan-A-specific T cell clones through a first injection. Three patients received between 3.6×10^7 and 5×10^9 CTL clones through a second injection. Following T cell clone injection (day 0), patients received 9 million units (MU) of IL-2 from days 1 to 5, and from days 8 to 12, and IFN-α 9 MU for 1 mo, three times a week. Several PBMC samples were collected for immunomonitoring at days 0, 7, 30, and 60.

Cell lines

Melanoma cell lines (M) were established in our laboratory from metastatic tumor fragments as previously described (14). The human mutant cell line CEM $\times$ 721 T2 (T2) used as a presenting cell was a gift from T. Boon (Ludwig Institute for Cancer Research, Brussels, Belgium). All cell lines were cultured in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FCS (Eurobio) and L-Glutamine (2 mM; Invitrogen Life Technologies).

Synthetic peptides

The Melan-A peptides $AAGIGILTV_{27-35}$ and $EAAGIGILTV_{26-35}$ and the decamer analog ELAGIGILTV were purchased from Epytop for in vitro assays and from Clinalfa for clinical grade peptide. Purity (>70%) was controlled by reversed-phase HPLC. Lyophilized peptides were dissolved in DMSO at 10 mg/ml and stored at −80°C.

Generation of Melan-A-specific CTL clones from PBMC

Melan-A-specific T cell clones were produced according to the GMP conditions in the Unit of Cellular and Gene Therapy (Centre Hospitalier Régional Universitaire, Nantes, France). To isolate and expand Melan-A-specific T cell clones from melanoma patients, we used a stimulation method previously described (13). Briefly, total PBMC (10^5) from HLA-A*0201 melanoma patients were stimulated in 96-well culture plates with a 2×10^4 irradiated HLA-A*0201 melanoma line pulsed with the Melan-A peptide analog (ELAGIGILTV), in RPMI 1640 medium containing 8% human serum supplemented with 5 ng/ml IL-6 (Endogen) during the first stimulation and 10 U/ml IL-2 (Chiron, clinical grade) during the second and third stimulations. PBMC from microcultures containing a significant fraction of Melan-A_{27-35} peptide-reactive cells were cloned in U-bottom 96-well plates (Falcon) by limiting dilution. Growth was induced using PHA (15 μg/ml) and irradiated allogeneic feeder cells: EBV-transformed LAZ cells (10^4/well) and allogeneic PBMC (10^5/well). A clinically agreed-upon master cell bank was established for the LAZ cell line and allogeneic PBMC were controlled according to the recommendations of the French security agency (AFSSAPS, Paris, France). Microcultures showing >95% probability of monoclonality, according to the single-hit Poisson law, were transferred into new plates with freshly irradiated feeder cells and PHA. Melan-A-specific CTL clones were selected on the basis of their capacity to produce IL-2 in response to HLA-A*0201 Melan-A-expressing melanoma cell lines. Selected T cell clones were then expanded according to the method described previously (15).

^{51}Chromium microcytotoxicity assay

Cytotoxic activity was measured in a standard 4-h assay against ^{51}Cr-labeled cells. Briefly, target cells (peptide-pulsed T2 or melanoma cells) were incubated with 100 μCi $Na_2{}^{51}CrO_4$ (Oris Industrie) and incubated with effector cells at 37°C for 4 h, at a 10:1 effector/target ratio for T2 cells and 20:1 for melanoma cells. For peptide recognition assays, T2 cells were preincubated with a range of Melan-A peptide concentrations for 1 h at 37°C and then washed before addition of effector cells. After the 4 h coculture, 25 μl of supernatant were mixed with 100 μl of scintillation mixture (Optiphase Supermix) for measurement of radioactive content.

Table II. *TCR α and β sequences expressed by Melan-A-specific T cell clones*

Patient	T Cell Clone	Vα[a]	TCR α-Chain CDR3α	Vβ[a]	TCR β-Chain CDR3β
Mela01	CTL01	**Vα2S1**	CAV GLRDM RFG	Vβ3	CAS SFGGLGTEA FFG
Mela02	CTL02	**Vα2S1**	CAG QAGTAL IFG	Vβ14	CAS SLSSLIVDTQ YFG
Mela03	CTL03.1	**Vα2S1**	CAV NLEGNNRL AFG	Vβ7	CAS SPGTLSDTQ YFG
	CTL03.2	**Vα2S1**	CAA SQGFQKL VFG	Vβ7	CAS SQDRGGAETQ YFG
Mela04	CTL04	**Vα2S1**	CAV SNARL MFG	Vβ17	CAS RPGPLAGYGY TFG
Mela05	CTL05	**Vα2S1**	CAV NKDNFNKF YFG	Vβ14	CAS SYGPTQ YFG
Mela06	CTL06	**Vα2S1**	CAV NFDQTGANNLF FFG	**Vβ2**	CSA RDGLGEL FFG
Mela10	CTL10	ND	ND	**Vβ2**	CSA RAGVGGDTQ YFG
Mela13	CTL13	Vα16S1	CAV RPYNNNDM RFG	Vβ6	CAS SDGLAVTQ YFG
Mela15	CTL15	**Vα2S1**	CAV GGSGGGADGL TFG	**Vβ2**	CSA RDGLGEL FFG
Mela16	CTL16	Vα21S1	CAA SDENGGATNKL IFG	**Vβ2**	CSA PRAGVGQPQ HFG

[a] Shown in bold are the most frequently used Vα or Vβ.

Table III. *Frequency of Melan-A-specific infused CTL clones in patients' PBMC*[a]

Patient ID		Frequency of Infused CTL in CD8$^+$ T Cells[a]							
		Days after the first injection				Days after the second injection			
		0	7	30	60	0	7	30	60
Mela02	CTL02	$<10^{-6}$	10^{-5}	ND	3×10^{-6}				
Mela03	CTL03.1	$<10^{-6}$	2×10^{-3}	2×10^{-4}	nd	Pos	10^{-3}	2×10^{-4}	nd
	CTL03.2	$<10^{-6}$	5×10^{-4}	5×10^{-4}	nd				
Mela13	CTL13	$<10^{-6}$	Pos	Pos	nd				

[a] The frequency of the infused T cell clone within blood CD8 T cells at indicated time points following their infusion was determined by clonotypic PCR with CDR3-specific primers. $<10^{-6}$, Undetectable, under the detection limit; nd, not done; Pos, positive, with too low a frequency to be calculated. CTL clones 01, 04, 05, 06, 10, 15, and 16 from corresponding patients Mela 01, 04, 05, 06, 10, 15, and 16 were nonpersisting, undetectable in the blood by clonotypic PCR at days 0, 7, 30, and 60 after the first injection, and the second injection for CTL04 and CTL05.

mAbs and flow cytometric analysis

Surface marker analysis of T cell clones was performed using the following mAbs: CD8-FITC, CD3-PE, CD27-FITC, CD28-FITC, CD69-FITC, CD45-RO-PE, CD45 RA-FITC, CCR7-FITC, CD62 ligand-FITC, NKG2D, CD94, NKG2A, ILT-2, and NKRP-1 (all from BD Biosciences). The production of IFN-γ and IL-2 by Melan-A-specific CTL clones, in response to target cells, was assessed by intracellular cytokine labeling using a method described by Jung et al. (16). PE-conjugated MQ1-17H12 (anti-IL-2) and FITC-conjugated 4S.B3 (anti-IFN-γ) purified Abs were obtained from Pharmingen SA. T cell clones were stimulated by Ag-expressing melanoma cells at the 1:2 ratio in RPMI 1640 10% FCS in the presence of Brefeldin A (10 μg/ml; Sigma-Aldrich). After 6 h, cells were fixed for 10 min at room temperature in a solution of PBS 4% paraformaldehyde. A total of 5×10^4 T cells were stained with anti-cytokine mAbs, at a concentration of 5 μg/ml, for 30 min, at room temperature. Reagent dilutions and washes were performed with PBS containing 0.1% BSA (A-9647; Sigma-Aldrich) and 0.1% saponin (S-2149; Sigma-Aldrich). After staining, cells were resuspended in PBS and 5000 events were analyzed on a FACScan (BD Biosciences).

*Construction of HLA-A*0201/peptide tetramers*

HLA-A0201/peptide α3-mutated monomers were generated as previously described (17). Recombinant proteins were produced as inclusion bodies in *Escherichia coli* XA90F'Lac^{Q1}, dissolved in 8 M urea, and refolded with 50 μg/ml Melan-A peptide (ELAGIGILTV). Tetramerization was performed as previously described (17). Briefly, HLA monomers were biotinylated for 4 h at 30°C with 6 μg/ml BirA (Immunotech), purified on a monoQ column (Pharmacia) and tetramerized with PE-labeled streptavidin (Sigma-Aldrich) at a molar ratio of 4:0.8.

Tetramer staining

To minimize nonspecific staining, HLA-A2-Melan-A tetramer was titered and used at the lowest concentration that showed a clearly distinguishable positive population in Melan-A-specific CTL generated as described above. PBMC were coincubated for 1 h at 4°C in the dark with Melan-A tetramer (10 μg/ml) and CD8-FITC mAb (5 μg/ml). After washing in PBS-0.1%BSA, cells were resuspended in PBS and analyzed on a FACScan. Compensation was checked before each acquisition. Events (10^5 among CD8-positive T cells) were collected using a FACScan set on a maximal flow rate of 1000 events/s. For analysis, tetramer-positive events were evaluated within a Boolean gate including cells within an extended lymphoid light scatter gate and a CD8 gate. The frequency of circulating Melan-A-specific CTL is presented as a percentage of Melan-A-tetramer-positive T cells among CD8 lymphocytes.

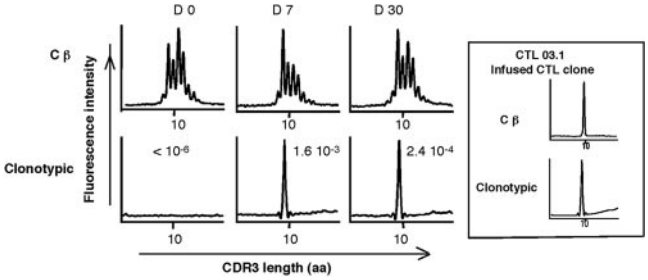

FIGURE 1. Example of the in vivo follow-up of infused Melan-A-specific T cell clones by quantitative clonotypic PCR. Sorted CD8 T cells, here from an Mela03 patient, were analyzed at days 0, 7, and 30 following the first injection of CTL03.1 clone. Immunoscope profiles of the fluorescent Vβ-Cβ runoff products are shown on the *top panel* and CTL03.1 clonotypic profile is shown on the *bottom panel*. The frequency of the CTL03.1 clone among CD8 T cells is indicated for each sample. The *x*-axis indicates CDR3 length.

Immunomagnetic cell sorting and expansion of T cell-sorted populations

HLA-A*0201/Melan-A monomers (20 μg/ml) were incubated for 1 h at room temperature with 6.7×10^6 streptavidin-coated beads (Dynabeads M-280 streptavidin; DYNAL) and washed in PBS-0.1% BSA. A total of 5×10^6 PBMC were rotated for 4 h at 4°C with monomer-coated beads. After 10 washes, bead-coated cells were expanded using a polyclonal T cell stimulation protocol described previously (18). Briefly, 2000 bead-coated T cells/well were distributed in 96-well plates mixed with irradiated feeder cells (LAZ EBV-B cells (2×10^4/well) and allogeneic PBMC (10^5/well)), in 200 μl of culture medium supplemented with IL-2 (150 U/ml) and PHA (15 μg/ml).

TCRVβ quantitative repertoire

Total RNA was extracted using the RNeasy Mini kit (Qiagen) as recommended by the manufacturer. A total of 50 μl of RNA was reverse-transcribed with oligo(dT)$_{17}$ and 400 U of SuperScript II RNase H-reverse transcriptase (Invitrogen Life Technologies). An aliquot of cDNA synthesis reaction was amplified with each of the 24 TCRVβ family-specific primers, together with a TCRCβ primer and a fluorochrome-labeled nested oligonucleotide TaqMan probe for TCRCβ. Real-time PCR was conducted in an ABI7300 system (Applied Biosystems). All quantitative PCRs were conducted in a total volume of 25 μl with 1× TaqMan buffer (Applied Biosystems), 25 mU of AmpliTaq, 5 mM $MgCl_2$, 0.2 mM dNTPs, 400 nM of each primer, 200 nM of probe. The conditions used were 95°C for 10 min followed by 95°C for 15s, 60°C for 1 min and 72°C for 30s for 40 cycles. The relative usage of each TCRVβ segment was computed as previously described (19). Two microliters from each amplification reaction were used as template in a run-off reaction initiated by a nested TCRCβ fluorescent primer in a total volume of 10 μl with 1× polymerase buffer (Promega), 25 mU of *Taq*DNA polymerase, 3 mM MgCl2, 0.2 mM dNTP, 0.1 mM nested TCRCβ fluorescent primer. The fluorescent products were then separated on a denaturing 6% polyacrylamide gel, run on an automated 373 DNA sequencer (Applied Biosystems) and analyzed with Immunoscope software (20) to profile the TCRVβ repertoire.

Quantitative clonotypic analysis

Real-time quantitative PCR was performed using the 5′ nuclease assay (TaqMan; Applied Biosystems) as previously described. For each clonotypic analysis, a clonotypic primer specific for the CDR3 region of the injected clone's TCR β-chain was designed, together with a nested oligonucleotide TaqMan probe specific for its rearranged Vβ gene segment. To determine the frequency of the clonotypic transcripts among transcripts sharing the same BV segment within a given sample, two series of quantitative amplifications were performed in parallel with the Vβ family-specific primer and the TaqMan probe, and either the clonotypic primer or the Cβ-specific primer. The frequency was determined as previously described

Table IV. *Phenotypic characteristics of infused CTL clones*[a]

	Effector/Memory Markers								NKRs				
	CD45RO	CD45RA	CD69	CD27	CD28	CCR7	CD62L	Perforin	NKG2D	CD94	NKG2A	ILT-2	NKRP-1
Clones detected in blood after transfer													
CTL02	**32**[b] (100%)	**1** (100%)	**6** (100%)	Neg	Neg	Neg	Neg	**10** (28%)	**25** (100%)	Neg	Neg	**12** (11%)	**22**[c] (86%)
CTL03.1	**283** (100%)	**9** (100%)	**5.3** (100%)	**9.3** (59%)	Neg	Neg	Neg	**5.5** (86%)	**189** (100%)	Neg	Neg	Neg	**22.4** (76%)
CTL03.2	**274** (100%)	**27** (100%)	**4.3** (100%)	Neg	Neg	Neg	Neg	**3** (78%)	**114** (100%)	Neg	Neg	Neg	**22** (21%)
CTL13	**233** (100%)	**33** (100%)	**3.5** (100%)	Neg	Neg	Neg	Neg	**7** (40%)	**64.5** (100%)	Neg	Neg	**59** (24%)	Neg
Clones not detected in blood after transfer													
CTL01	**612** (100%)	**3** (100%)	**12** (100%)	Neg	Neg	Neg	Neg	**17** (10%)	**130** (100%)	**26** (19%)	**37** (15%)	**42** (24%)	Neg
CTL04	**454** (100%)	**2.4** (100%)	**23** (100%)	Neg	Neg	Neg	Neg	**7.5** (100%)	**130** (100%)	**76** (29%)	**93** (27%)	**48** (38%)	Neg
CTL05	**86** (100%)	**39** (100%)	**32** (100%)	Neg	Neg	Neg	Neg	**6** (95%)	**15** (100%)	Neg	Neg	**35** (23%)	Neg
CTL06	**394** (100%)	**10** (100%)	**10** (100%)	Neg	Neg	Neg	Neg	**5** (87%)	**74** (100%)	**91** (33%)	**134** (29%)	**142** (60%)	Neg
CTL10	**44** (100%)	**1** (100%)	**3** (100%)	Neg	Neg	Neg	Neg	**5** (80%)	**5** (100%)	Neg	Neg	Neg	Neg
CTL15	**351** (100%)	**3.2** (100%)	**4.4** (100%)	**5.6** (78%)	Neg	Neg	Neg	**6.6** (98%)	**107** (100%)	Neg	Neg	**29** (9%)	Neg
CTL16	**428** (100%)	**7.3** (100%)	**14** (100%)	Neg	Neg	Neg	Neg	**5** (93%)	**79** (100%)	Neg	Neg	Neg	Neg

[a] Phenotypic analysis of T cell clones was performed by flow cytometry using FITC or PE-labeled mAbs.
[b] Ratio fluorescence intensity (RFI) are indicated in bold and have been calculated as follows: mean fluorescence intensity with the indicated mAb/mean fluorescence intensity with the isotype-matched control mAb.
[c] Underlined values emphasize the heterogeneous expression of the considered marker.

(19). The run-off reactions, electrophoresis of the single-stranded fluorescent DNA fragments, as well as computer analyses, were conducted as described above.

Results

Generation of Melan-A-specific T cell clones and injection to melanoma patients

Patient PBMC were stimulated three times with a melanoma cell line loaded with the 26-35L Melan-A peptide analog (21), and then cloned by limiting dilution as previously described (13), yielding several Melan-A/A2-specific clones from all the patients. One or two clones selected on the basis of their reactivity against Melan-A-expressing HLA-A2 melanoma cell lines (highest lytic activity and IL-2 secretion capacity), were expanded by a stimulation protocol previously used to grow TIL populations (15). Each patient received between 1.64×10^8 and 2×10^9 Melan-A-specific CTL in a single injection (Table I). One patient (Mela03) received a mixture of two distinct Melan-A-specific clones (CTL03.1 and CTL 03.2). Three patients received a second clone injection (between 3.5×10^7 and 5×10^9 cells), 6 mo later.

To follow the fate of infused T cells, we sequenced α and β CDR3 chains of all infused T cell clones to develop clonotype-specific probes. In accordance with previous results on Melan-A-specific repertoire (22–28), the Vα2.1 chain was frequently used by these clones (8 of 10 tested clones) and there was no preferential Vβ usage, although Vβ2 was shared by 4 of 11 clones (Table II). Unexpectedly, we found a shared CDR3β region for two clones obtained from two distinct patients (CTL06 and CTL15). From the CDR3β sequence of each clone, a PCR probe was designed and used to monitor the fate of infused cells in patient PBMC.

Follow-up of infused Melan-A-specific T cells clones in patient blood

Quantitative clonotypic PCR was performed on sorted $CD8^+$ T cells at days 0, 7, 30, and 60 following each injection. For all the patients, the clonotypic PCR performed before infusion was negative in $CD8^+$ T cells. After immunotherapy, the infused T cell clones were detected in three patients (Mela02, 03, and 13) and their frequencies could be estimated for two of these clones (Table III and Fig. 1). These frequencies were the highest 7 days after the injection, ranging from 10^{-5} and 2×10^{-3} $CD8^+$ T cells. In the Mela02 patient, the infused clone was still detected at day 60, with a frequency of 3×10^{-6} $CD8^+$ T cells. In the Mela03 patient, the two infused clones were present at day 30 with frequencies of 2×10^{-4} and 5×10^{-4} CD8 T cells, and one of these clones was still present at day 165, just before the second injection, at a very low frequency. Its frequency increased again after the second injection up to 10^{-3} at day 7, and then decreased to 2×10^{-4} at day 30. Finally, the clone to Mela13 patient could be detected at days 7, 30, and 60, but its frequency could not be determined due to a low representation of its Vβ family. Overall, these results show that infused Melan-A-specific T cell clones survived in blood for >2 mo in three patients, whereas they were undetectable in the other patients as soon as 7 days after the infusion. Therefore, we asked whether phenotypic or functional characteristics of infused T cell clones could be predictive of their variable persistence in the peripheral blood.

Phenotypic and functional characterization of Melan-A-specific T cell clones

Phenotypic analysis revealed that all the clones expressed CD45RO, CD69, and perforin and lacked expression of CCR7,

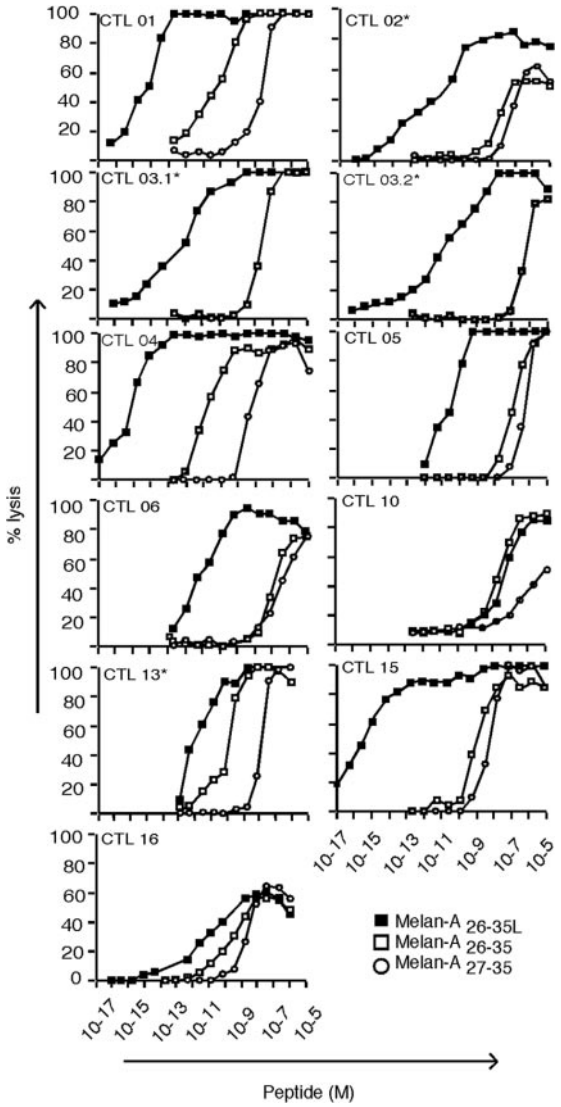

FIGURE 2. Titration analysis of Melan-A peptides recognized by the Melan-A-specific CTL clones. Target cell was a TAP-deficient cell line T2 expressing the HLA-A*0201 loaded with either the natural peptides Melan-A_{26-35} (□) or Melan-A_{27-35},(○) or the modified decapeptide $_{26-35L}$ (■) at various concentrations. Lytic activity was measured, at an E:T ratio of 10:1 with the classical 4-h ^{51}Cr release assay. *, These clones persisted in blood CD8 T cells following their transfer.

CD62L, and CD28 (Table IV). Some of them expressed low levels of CD45 RA or CD27. Therefore, all the clones have a similar phenotype corresponding to effector/memory cells (29), most of them lacking CD27 expression. With regard to the expression of NK receptors (NKRs), all the clones expressed the activating NKG2D receptor but the expression of other NKRs (CD94/NKG2A, ILT-2, NKRP-1) was more heterogeneous. The CD94/NKG2A heterodimer was expressed only by three nonpersisting CTL clones (CTL01, 04, and 06) and the IL-T2 receptor by seven clones, among which were five nonpersisting ones. Finally, NKRP-1 was expressed only by three persisting clones (CTL02, 03.1, and 03.2) (Table IV). Therefore, there is no clear phenotypic profile associated with the persistence in blood of infused Melan-A-specific T cell clones. Nonetheless, the inhibitory receptor CD94/NKG2A appears to be preferentially expressed by nonpersistent clones, whereas the potentially activating NKRP-1 receptor is expressed only by persistent ones.

The relative avidity of Melan-A-specific CTL clones for specific peptides was then evaluated. All the clones were highly reactive against the analog peptide, with EC_{50} ranging from 7×10^{-16} to 5×10^{-8} M (Fig. 2 and Table V). As expected from their selection based on tumor cell recognition, CTL clones also recognized the natural Melan-A peptides, usually with an advantage for the natural decapeptide. EC_{50} ranged respectively from 2×10^{-11} to 10^{-7} M and from 8×10^{-9} to up to 10^{-5} M for the natural deca- and nonapeptide.

All the clones were highly lytic against $A2^+$/Melan-A^+ melanoma cell lines and they efficiently produced IFN-γ upon stimulation with these cells, as illustrated by the high fraction of IFN-γ-positive cells (Fig. 3). A strong correlation was found between the percentage of IFN-γ-positive cells and the percentage of lysis, in agreement with previous observations (30) (Fig. 3*A*). The ability of clones to produce IL-2 was more heterogeneous. Four clones, among which three were nonpersisting in blood, had a limited capacity to produce this cytokine (as shown by a lower fraction of clone cells producing this cytokine) (Fig. 3*B*). Therefore, this analysis did not reveal any clear association between either T cell clone avidity or functional activity (lytic activity and cytokine synthesis) and in vivo persistence in the blood.

Clinical evolution of the patients

All the patients enrolled in this study had, at the time of the first T cell transfer, progressive metastatic disease, refractory to conventional therapy. Adoptive T cell therapy was followed in one patient (Mela01) by a complete response lasting 27 mo, in three patients

Table V. *Relative avidity of Melan-A-specific CTL clones on Melan-A peptides*[a]

T Cell Clone	EC_{50} Melan-A_{26-35L}	EC_{50} Melan-A_{26-35}	EC_{50} Melan-A_{27-35}
Clones detected in blood after transfer			
CTL02	4×10^{-11} M	10^{-8} M	8×10^{-8} M
CTL03.1	9×10^{-13} M	5×10^{-8} M	3×10^{-8} M
CTL03.2	2×10^{-11} M	8×10^{-7} M	8×10^{-7} M
CTL13	10^{-11} M	10^{-9} M	2×10^{-7} M
Clones not detected in blood after transfer			
CTL01	10^{-14} M	2×10^{-11} M	3×10^{-8} M
CTL04	10^{-15} M	8×10^{-11} M	8×10^{-9} M
CTL05	5×10^{-11} M	10^{-7} M	8×10^{-7} M
CTL06	10^{-11} M	10^{-7} M	8×10^{-7} M
CTL10	5×10^{-8} M	2×10^{-8} M	$>10^{-5}$ M
CTL15	7×10^{-16} M	10^{-9} M	8×10^{-9} M
CTL16	8×10^{-11} M	5×10^{-9} M	3×10^{-8} M

[a] Titration analysis was performed with the two natural Melan-A peptides (26–35) and (27–35) and the modified decamer (26–35L) recognized by the HLA-A2-restricted CTL clones. Target cells were a TAP-deficient cell line T2 loaded with the synthetic peptides at various concentrations. Lytic activity was measured at an E:T ratio of 10:1, by the classical 4-h ^{51}Cr release assay. EC_{50} represents the peptide concentration inducing 50% of maximal lysis.

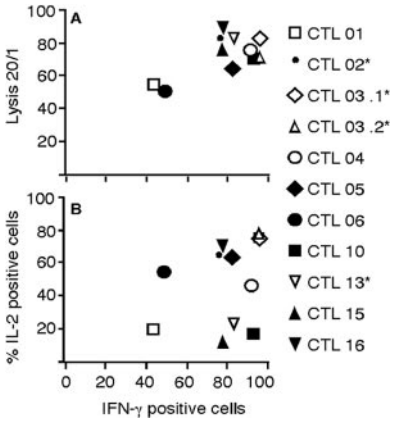

FIGURE 3. *A*, Lysis and IFN-γ production by Melan-A-specific CTL clones in response to an HLA-A2 Melan-A-positive melanoma cell line. For the lysis experiment, effector and target cells were incubated at a 20:1 ratio. For IFN-γ production, effector and target cells were incubated at a 1:2 ratio in the presence of Brefeldin A before being fixed, permeabilized with saponin, stained with anti-IFN-γ Ab and analyzed on a FACScan (5×10^3 cells). *B*, IFN-γ and IL-2 production by Melan-A-specific CTL clones in response to an HLA-A2 Melan-A-positive melanoma cell line. Effector and target cells were incubated at a 1:2 ratio in the presence of Brefeldin A before being fixed, permeabilized with saponin, stained with both anti-IFN-γ and IL-2 mAbs and analyzed on a FACScan (5×10^3 cells). ∗, Persisting CTL clones after in vivo transfer.

(Mela06, 13, and 16) by disease stabilization lasting, respectively, 26, 13, and 8 mo, and, in an additional three patients (Mela05, 10, and 15), by partial or mixed responses followed by disease progression. The three other patients progressed after treatment (Mela02, 03, and 04). The seven patients who experienced a clinical response are hereafter named "responder" and the other three patients "progressor." Surprisingly, injected clones did not persist in the blood of responding patients except for Mela13 (Table III), while they survive for long periods in the blood of two nonresponding patients (Mela02 and 03). Therefore, we wondered whether the failure to detect clones in blood from patients who experienced responses could be due to their selective trapping in tumor lesions.

Tracking of transferred T cell clones into tumor sites

We could monitor the presence of infused CTL clones in tumor samples from five treated patients. For two of them (Mela10 and Mela13), we detected the presence of the injected clone inside tumor lesions, before and after treatment (Table VI). For the Mela10 patient, the frequency of the clone appeared to be lower in the postimmune tumor sample (2×10^{-4}) compared with the preimmune one (7×10^{-3}) (Table VI). For the other three patients, we could not detect the presence of the injected clone after treatment, in the analyzed tumor lesions. Together, these data do not prove the migration or the absence of migration of transferred T cells into tumor sites.

Frequency of Melan-A/A2 tetramer$^+$ CD8 T cells in the blood of patients infused with Melan-A/A2-specific clones

Because clinical responses suggest that infused T cells migrate into the tumors, we wondered whether this migration could have resulted in the expansion of additional *Melan-A/A2*-specific CTL. To check this hypothesis, we measured the frequency of total circulating Melan-A/A2 tetramer$^+$ T cells, during the course of immunotherapy treatment (Fig. 4). Before T cell transfer, the frequencies of tetramer-positive cells ranged from 0.03 to 0.26% of CD8$^+$ T cells (Fig. 4*B*). After infusion, these frequencies increased significantly in 9 of the 10 treated patients (Fig. 4*B*), this increase is illustrated by a representative example on Fig. 4*A*. All the patients who experienced a complete response or stable disease showed a marked increase in tetramer-positive cells around day 30 (*upper panel* on Fig. 4*B*). The Mela15 patient, who experienced a partial response, also showed an increase in tetramer-positive cells at day 30, and one of the two patients with mixed responses showed such a response at day 60. The three progressor patients (*lower panel* on Fig. 4*B*) also showed this type of response in blood, at day 30. Because, in six patients (Mela01, 04, 06, 10, 15, and 16), the injected Melan-A-specific clone was undetectable in blood at any time postinfusion, the tetramer$^+$ T cell population detected in these patients did correspond to other Melan-A-specific CTL. For the other three patients (Mela02, 03, and 13), comparison of frequencies obtained by PCR and tetramer labeling indicated that the increase in tetramer$^+$ cells was only partially due to the presence of the infused clone in blood. Thus, for 9 of 10 patients, this increase likely results from an in vivo expansion of Melan-A-specific cells, following the treatment.

Table VI. *Detection of infused Melan-A-specific CTL clones in tumors*[a]

Patient ID	T Cell Clone	Frequency of Infused CTL in CD8$^+$ TIL	
		Before infusion	After infusion (day)
Mela04	CTL04	nd	$<10^{-6}$ (30)
Mela05	CTL05	nd	$<10^{-6}$ (30)
Mela10	CTL10	7×10^{-3}	2×10^{-4} (180)
Mela13	CTL13	Pos	Pos (30)
Mela16	CTL16	nd	$<10^{-6}$ (7)

[a] The frequency of infused T cell clone within tumor-derived CD8 T cells before or after immunotherapy was determined by clonotypic PCR with CDR3-specific primers. $<10^{-6}$, Undetectable, under the detection limit; nd, not done; Pos, positive, with too low a frequency to be calculated.

Characterization of Melan-A-specific lymphocytes in the blood of Mela01 patient

To characterize more accurately these expanded Melan-A-specific cells, we performed an in-depth analysis of their avidity and repertoire in the Mela01 patient who showed a complete response. To this end, Melan-A-specific lymphocytes were sorted from pre- and postimmune PBMC, using MHC/peptide multimers (Fig. 5*A*).

The attempt to detect the infused Melan-A CTL clone by clonotypic PCR within the Melan-A-tetramer-sorted population confirmed its absence in the blood at day 30. Surprisingly, the Vβ repertoire of these populations, analyzed by Immunoscope, was different before and after adoptive therapy. Lymphocytes expressing the Vβ2 TCR chain were dominant before treatment (83%), while after treatment these cells were hardly detectable, whereas Vβ3- and Vβ21-expressing lymphocytes became the dominant clonotypes, with, respectively, 45 and 31% (data not shown). Therefore, the composition of the Melan-A-specific repertoire in the blood was altered following adoptive cell therapy.

The relative avidity of tetramer-sorted cells was increased at day 30 vs day 0, EC_{50} on the natural deca- and nonapeptide being, respectively, 4×10^{-10} M and 5×10^{-9} M at day 30 vs 7×10^{-9} M and 2×10^{-8} M at day 0 (Fig. 5*B*). In accordance with these results, the lytic activity of Melan-A-specific CTL against two Melan-A-expressing melanoma cell lines was higher at day 30 compared with day 0 (Fig. 5*C*). These functional differences reflect the changes observed at the Vβ level.

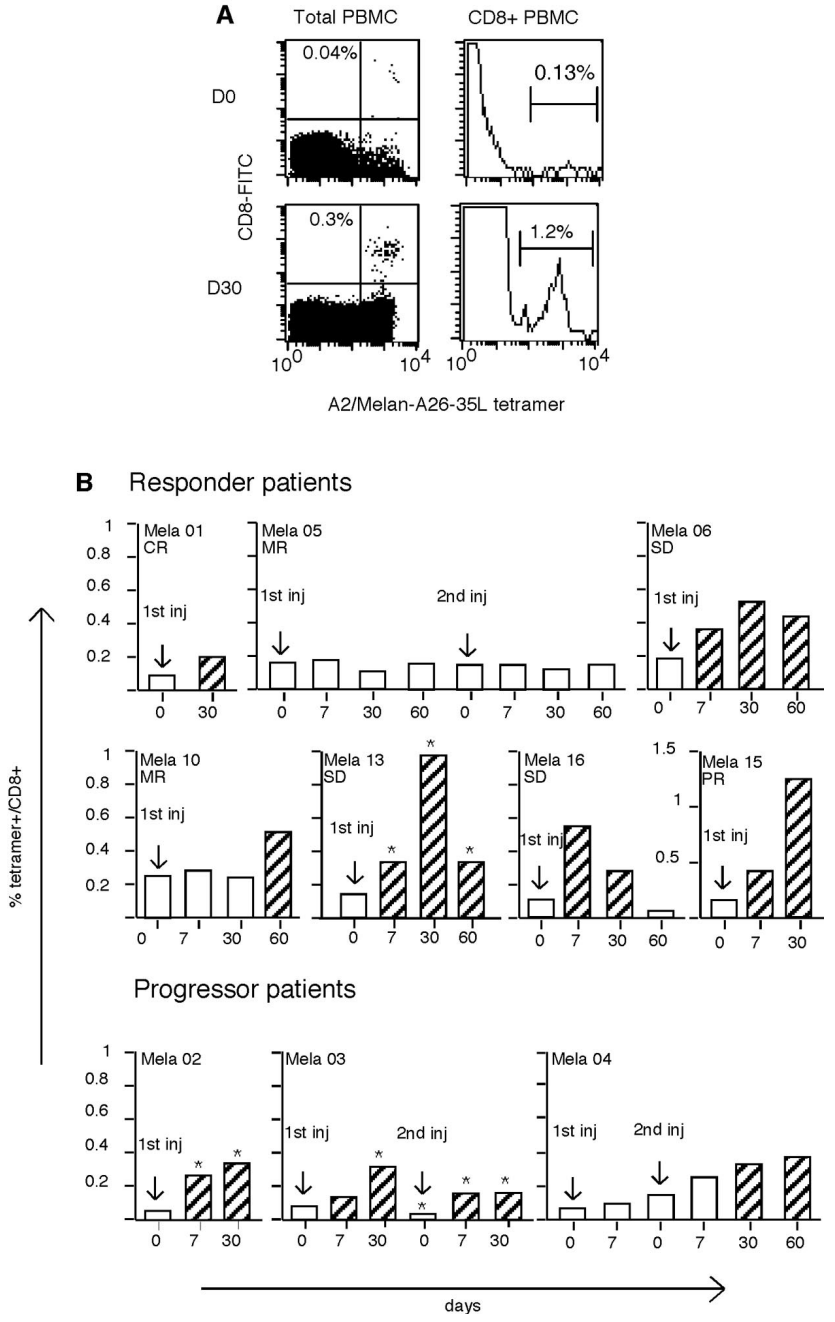

FIGURE 4. *A*, Example of tetramer-CD8 labeling performed on Mela15 PBMC before and 30 days after clone infusion. On the *left panel*, values indicate the fraction of tetramer-positive cells among total PBMC. On the *right panel*, values indicate the fraction of tetramer-positive cells among CD8-positive PBMC. *B*, Frequencies of Melan-A-specific T cells among blood CD8 T cells after infusion of Melan-A-specific CTL clones. Peripheral blood samples were collected from patients before T cell transfer (day 0), and at days 7, 30, and 60 after CTL clone infusion, and double-stained with HLA-A2-Melan-A-tetramer and CD8-specific mAbs. Histograms represent the percentages of tetramer-positive cells among CD8 T cells. ▨, An increase (≥2-fold) in the frequency of tetramer-positive cells at the indicated time point compared with the baseline frequency (day 0). Bars with an asterisk represent blood samples for which we detected the presence of the infused CTL clone by clonotypic PCR. The clinical status of responder patients are indicated as follows: CR, complete response; SD, stable disease; MR, mixed response; and PR, partial response.

Discussion

In this study, we generated Melan-A-specific CTL clones from 10 melanoma patients by stimulating PBL with the Melan-A_{26-35L} peptide analog. Infused T cell clones were selected on the basis of their ability to produce IL-2 in response to Melan-A-positive melanoma cell lines, which reflects a high reactivity toward tumor cells (13). Indeed, we assume that the avidity for the Ag expressed on tumor cells is one of the main parameters that could determine the efficiency of adoptive transfer.

The fate of the transferred T cells in the patient is a crucial question for the success of adoptive therapy. Recent studies have shown that infused tumor-specific T cells survive in the circulation and suggested that these cells migrate into the tumor (8, 9, 31). In these studies, transferred T cells could be detected during >4 mo, when patients were pretreated with a lymphodepleting chemotherapy regimen (31, 32) whereas, in the absence of previous lymphodepletion, persistence of infused cells in peripheral blood did not exceed 2 wk (8, 33). In our study, although patients were not lymphodepleted, we observed the persistence in blood of infused clones in three patients, for periods ranging from 1 to 2 mo (Table III). This is, to our knowledge, the longest survival period ever reported for T cell clones transferred to patients, without previous lymphodepletion. The persistence in the blood of those CTL clones compared with those undetectable as soon as day 7 was not correlated with the number of transferred cells, as shown in Table I.

We then investigated whether the presence in peripheral blood of infused T cell clones was correlated with specific functional properties or phenotype. Results failed to support this hypothesis since all the clones exhibited a similar reactivity toward melanoma cells and no significant differences were found between clones regarding phenotype or other functional properties such as the apoptosis induced by CD3 or tetramer stimulation (Table V, Fig. 3, and data not shown).

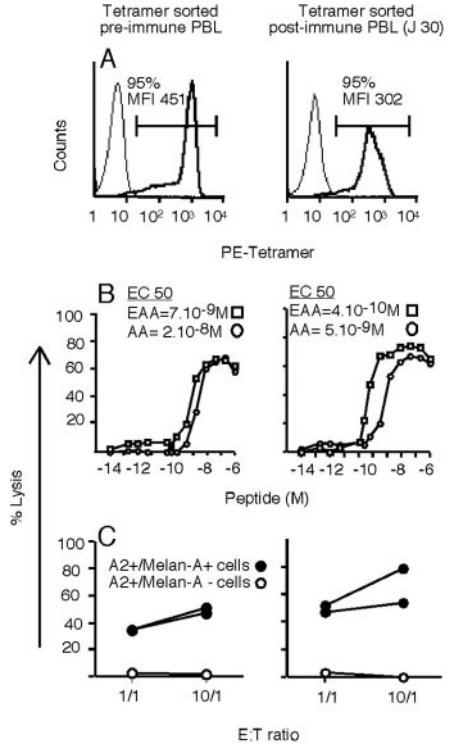

FIGURE 5. *A*, Tetramer labeling of Melan-A-specific lymphocytes from a Mela01 patient. HLA-A*0201/Melan-A monomers coated on magnetic beads were used to isolate Melan-A-specific T cells from PBMC at days 0 and 30. PE-conjugated tetramers were used to assess the Ag specificity of the sorted populations. Values indicate the geometric means of fluorescence compared with an irrelevant peptide folded in HLA-A*0201. *B*, Titration analysis of Melan-A peptides recognized by tetramer-positive lymphocytes. Target cell was a TAP-deficient cell line T2 expressing the HLA-A*0201 loaded with the natural peptides Melan-A_{27-35} (○) and $_{26-35}$ (□) at various concentrations. Lytic activity was measured at an E:T ratio of 10:1 with the classical 4-h ^{51}Cr release assay. *C*, Lytic activity of sorted population on HLA-A2 melanoma lines expressing (●) or not expressing (○) Melan-A/MART-1.

Only, the NKR expression profile appeared heterogeneous (Table IV). None of the blood-persisting clones expressed CD94/NKG2A whereas three of four expressed NKRP-1. Conversely, the heterodimer CD94/NKG2A was expressed by three of seven nonpersisting clones. Association of this inhibitory receptor with its ligand, HLA-E, expressed on melanoma cells, can lead to the inhibition of TCR-mediated cytotoxicity and TNF production by human melanoma specific T cell clones (34, 35). Therefore, the expression of this inhibitory receptor could limit both the activation and the survival of injected clones. The role of NKRP-1 in T cell activation is more controversial. Its expression on activated memory T cells could stimulate their activation and proliferation (36). Thus, its expression by infused T cell clones could be involved in increased proliferation and persistence in vivo. However, at this stage, the potential incidence of NKR expression on blood persistence of transferred cells remains elusive and requires a more extensive study on additional T cell clones.

The rapid disappearance of transferred cells from the blood of seven patients could be due either to their premature death or to their specific trapping in other tissues, especially in tumors lesions. In fact, we detected the infused T cells inside tumor lesions of two of five tested patients (Mela10 and Mela13) (Table VI). Unfortunately, in these two cases, we could not conclude about the migration of transferred cells into tumor sites, because we also detected these same clonotypes in tumor lesions before treatment. Conversely, the absence of transferred cells in tumor infiltrates from the three other patients does not prove an absence of migration, considering that only one of multiple tumor sites was checked for the presence of infused cells. Indeed, they could have been trapped in other nontested sites. Overall, these results underlie the difficulty to ascribe qualitative or quantitative variations in tumor tissue infiltrates, to an immunotherapy treatment.

Nonetheless, seven patients experienced a clinical response (Mela01, 05, 06, 10, 13, 15, and 16), suggesting that transferred cells did initiate an antitumor response. Because in six of them, infused clones were not detected in the peripheral blood, we hypothesized that they might have migrated in tumor sites where, by killing tumor cells, they could have recruited other tumor-specific lymphocytes, especially other Melan-A-specific ones. To test this hypothesis, we analyzed the evolution of Melan-A-specific peripheral response, using A2/Melan-A tetramer staining. In fact, we observed a significant increase in the frequency of tetramer-positive cells in the blood of nine patients after treatment, among which were six responder patients (Fig. 4). For most of them the highest frequencies of tetramer-positive cells were detected 30 days after treatment and still persisted until day 60 for some patients. This suggests that adoptive therapy may help to recruit other Melan-A-specific T cell clonotypes, and may be CTL specific for other tumor Ags, not checked in this study.

Finally, we focused our interest on the Mela01 patient, who experienced the best clinical response and for whom the infused clone was not detected in the blood at any time, although we observed an increase in tetramer-positive cells at day 30. The immunoscope analysis of sorted Melan-A-specific T cells confirmed the absence of the infused clone either before or after adoptive therapy, and revealed the presence of two dominant clonotypes (Vβ3, Vβ21) in Melan-A-specific cells posttransfer, which were hardly detectable before treatment (data not shown). Although we cannot formally exclude that these two clonotypes could have been preferentially amplified during the course of in vitro expansion, we favor the hypothesis that this new Melan-A-specific repertoire appeared in PBMC following T cell therapy. Interestingly, this postimmune repertoire displayed a higher reactivity against Melan-A peptides and melanoma cells (Fig. 5, *B* and *C*).

Overall, this suggests that qualitative or quantitative variations in the Melan-A-specific repertoire could be indicative of an antitumor response, induced by immunotherapy treatment. The efficiency of this antitumor response will then probably depend on the extent of the disease at the time of immunotherapy in these stage IV patients.

It should be noted that this repertoire expansion had not been previously reported in studies of T cell transfer in melanoma patients. This could be due either to the high tumor reactivity of the CTL clones used in our study, or to methodological variations concerning the follow-up of infused cells. Indeed, in other clinical trials using Melan-A-specific T cell clones, these clones were selected on the basis of IFN-γ secretion upon stimulation with a Melan-A-derived peptide (9) or on specific lysis of Ag-positive tumor target (8), but not on IL-2 secretion which characterizes clones with high avidity and high tumor reactivity. Another possible explanation is the method used to follow-up infused cells. Indeed, in our study, we coupled an Immunoscope analysis with clonotypic-specific PCR, to prevent the detection of false-positive results with CDR3β regions differing by only few base pairs from the CDR3β of the original CTL clone (Fig. 1). This approach prevented an overestimation of CTL clone survival and was critical to detect Melan-A-specific T cell repertoire spreading following adoptive therapy.

A recent vaccination assay using MAGE-A3 Ag in a melanoma patient, which resulted in a nearly complete tumor regression, supports the hypothesis of a repertoire/epitope spreading induced by

immunotherapy: the authors reported the development in blood and metastases of new clonotypes recognizing MAGE-C2 and gp100 Ags without increased frequencies of anti-vaccine T cells in the blood (37, 38).

In conclusion, our study suggests that 1) high avidity Melan-A-specific clones can survive for long periods in melanoma patients, 2) the persistence in the blood of transferred cells is not correlated with clinical responses, and 3) an antitumor response initiated by transferred cells can be evidenced by measuring quantitative or qualitative variations in the Melan-A-specific repertoire.

Disclosures

The authors have no financial conflict of interest.

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