Naturally Occurring Human Lymphocyte Antigen-A2 Restricted CD8+ T-Cell Response to the Cancer Testis Antigen NY-ESO-1 in Melanoma Patients[1]

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ABSTRACT

Cancer testis (CT) antigens are particularly interesting candidates for cancer vaccines. However, T-cell reactivity to CT antigens has been detected only occasionally in cancer patients, even after vaccination. A new group of CT antigens has been recently identified using the SEREX technique based on immunoscreening of tumor cDNA expression libraries with autologous sera. We have used fluorescent HLA-A2/peptide tetramers containing an optimized antigenic peptide to directly identify HLA-A2-restricted CD8+ T cells specific for the SEREX-defined CT antigen NY-ESO-1 in melanoma patients. High frequencies of NY-ESO-1-specific CD8+ T cells were readily detected in peptide-stimulated peripheral blood mononuclear cells as well as in lymphocytes infiltrating melanoma lesions from patients with measurable antibody responses to NY-ESO-1. NY-ESO-1-specific CD8+ T cells were also detectable in peptide-stimulated peripheral blood mononuclear cells from some seronegative patients. Whereas the frequencies of NY-ESO-1-specific CD8+ T cells in circulating lymphocytes were usually below the limit of detection by tetramer staining, the presence of NY-ESO-1 CD8+ T cells displaying a memory phenotype was clearly detectable *ex vivo* in blood from a seropositive patient over an extended period of time. These results indicate that sustained CD8+ T-cell responses to CT antigens can naturally occur both locally and systemically in melanoma patients.

INTRODUCTION

The molecular identification of human melanoma-associated antigens recognized by CTLs has recently prompted the implementation of clinical trials aimed at inducing CTL responses against defined melanoma antigens. These studies are mainly focused on the use of antigenic peptides derived from the two major groups of melanoma-associated antigens, namely melanocyte differentiation antigens (*i.e.,* Melan-A[3], tyrosinase, and gp100) and CT antigens (*i.e.,* MAGE, BAGE, and GAGE). In contrast to melanocyte differentiation antigens, which are expressed in both melanoma cells and normal melanocytes, CT antigens are usually expressed only in the testis and in several cancer types (hence, their designation) but not in other normal tissues. Thus, vaccination with CT antigen-derived peptides has the advantage of being potentially applicable to multiple cancer types.

Six CT antigen families have been identified thus far, either by using tumor-reactive CTL clones from melanoma patients (1) or by immunoscreening of cDNA expression libraries from tumors [serological analysis of recombinant cDNA expression libraries (SEREX)] with autologous sera from cancer patients (2–4). Whereas CD8+ T cells directed against melanocyte differentiation antigens are readily detectable in both peripheral blood lymphocytes and TILs from melanoma patients, T-cell responses to "MAGE-related" CT antigens have been detected only rarely in cancer patients, even after vaccination (5). T-cell reactivity to "SEREX-defined antigens" has only recently been addressed (6, 7). In particular, three peptides corresponding to overlapping sequences spanning the 155–167 region of the CT antigen NY-ESO-1 have been shown to be recognized by a tumor-reactive CTL line from a melanoma patient in an HLA-A2-restricted fashion (6).

Here, we report a detailed study of CD8+ T-cell responses to NY-ESO-1 in HLA-A2 melanoma patients, which includes identification of an optimal antigenic peptide sequence(s), assessment of tumor reactivity of specific CTLs, generation of HLA-A2/NY-ESO-1 peptide tetramers, and-tetramer based identification of NY-ESO-1-specific CD8+ T cells in peripheral blood and melanoma lesions.

MATERIALS AND METHODS

Patients, Tissues, and Cells. Ten HLA-A2 melanoma patients (listed in Table 1) were selected for this study. Melanoma cell lines Me 275 and Me 242 were established in our laboratory from melanoma metastases surgically excised from patients LAU 50 and LAU 92, respectively. The melanoma cell line NA8-MEL was kindly provided by Dr. F. Jotereau (U211; Institut National de la Santé et de la Recherche Médicale, Nantes, France). NY-ESO-1-specific serum antibody titers were determined by ELISA, as described previously, with minor modifications (8). Two patients showed detectable levels of NY-ESO-1-specific antibodies: LAU 50 (antibody titer 1:1′400) and LAU 156 (antibody titer > 1:10′000). No detectable levels of NY-ESO-1-specific antibodies were observed in patients LAU 42, LAU 97, LAU 165, LAU 198, LAU 212, LAU 233, and LAU 253. Patient LAU 203 was not tested.

Surgically resected lymph node or metastatic lesions were finely minced with needles in sterile RPMI 1640 supplemented with 10% FCS. Cell suspensions were placed in 24-well tissue culture plates (Costar, Cambridge, MA) in 2 ml of Iscove's Dulbecco medium (Life Technologies, Inc., Basel, Switzerland) supplemented with 0.24 mM asparaginine, 0.55 mM arginine, 1.5 mM glutamine, 8% pooled human A+ serum (CTL medium), 100 units/ml hrIL-2, and 10 ng/ml hrIL-7. Cells were cultured 2–3 weeks before fluorescence-activated cell-sorting or ELISPOT analysis. NY-ESO-1-specific CTL clones were derived from TILNs of melanoma patient LAU 156 from limiting dilution cultures in the presence of irradiated allogeneic PBMCs, PHA, and human recombinant IL-2, as described elsewhere (9).

For peptide stimulation experiments, CD8+ lymphocytes were positively selected by magnetic cell sorting from PBMCs of HLA-A2 melanoma patients using a miniMACS device (Miltenyi Biotec GmBH, Sunnyvale, CA). Cells from the CD8− fraction were irradiated (3.000 rads) and used as autologous APCs. CD8+ highly enriched lymphocytes (1 × 10⁶/well) were stimulated with peptide (1 μM) and irradiated autologous APCs in 2 ml of CTL medium (10) containing hrIL-2 [10 units/ml; Glaxo, Geneva, Switzerland; kindly provided by Dr. M. Nabholz (Institut Suisse de Recherches Experimentales sur le Cancer, Epalinges, Switzerland)] and hrIL-7 (10 ng/ml; R&D System

Table 1 *IFN-γ ELISPOT analysis of NY-ESO-1-specific T cells in peptide-stimulated CD8^{+} lymphocytes from HLA-A2 melanoma patients*

Days after stimulation:	Day 7				Day 14			
Stimulated with peptide:	SLLMWITQC		SLLMWITQA		SLLMWITQC		SLLMWITQA	
Peptide SLLMWITQC[a]	−	+	−	+	−	+	−	+
Patient code								
LAU 42	7[b]	5	10	16	20	34	34	44
LAU 50	11	7	16	**87**	6	**152**	13	**140**
LAU 97	17	27	18	7	25	36	48	37
LAU 156	12	**45**	17	**70**	16	**>200**	11	**88**
LAU 165	11	20	10	7	12	17	24	14
LAU 198	22	21	27	18	25	28	24	**82**
LAU 203	10	16	10	17	21	23	51	33
LAU 212	39	25	12	7	25	37	25	18
LAU 233	8	13	10	7	24	15	42	40
LAU 253	15	2	6	12	18	41	28	34

[a] The assay was performed as detailed in "Materials and Methods." Results are reported for 10 HLA-A*0201 melanoma patients using CD8^{+}-enriched PBMCs stimulated *in vitro* with either NY-ESO-1 parental peptide 157–165 or the peptide analogue C165A for the indicated time period.

[b] Numbers represent the mean of IFN-γ spot numbers measured in duplicate cultures containing 5×10^4 APCs (T2) and 5×10^4 peptide-stimulated CD8^{+}-enriched T cells. Figures in *bold* represent significant numbers (three times over background values) obtained in the presence of NY-ESO-1 parental peptide 157–165 in comparison with values obtained in the presence of T2 cells alone.

Europe, Oxon, United Kingdom). Cells were cultured for the indicated period of time before ELISPOT or A2/NY-ESO-1 tetramer analysis.

HLA-A2 Binding and Antigen Recognition Assays. Peptide binding to HLA-A2 was assessed in a functional competition assay based on inhibition of recognition of the antigenic peptide Tyrosinase 368-376 (YMDGTMSQV) by the HLA-A2-restricted CTL clone LAU132/2 (10). Briefly, T2 cells were ^{51}Cr-labeled in the presence of the anticlass I mAb W6/32. Various concentrations of competitor peptides ranging from $1–10^4$ nM (50 μl) were incubated with ^{51}Cr-labeled T2 cells (50 μl; 1000 cells/well) for 15 min at room temperature. A suboptimal dose (1 nM) of the antigenic peptide (50 μl) was then added together with specific CTL (5000 cells/well; 50 μl). Chromium release was measured after a 4-h incubation at 37°C. The concentration of each competitor peptide required to achieve 50% inhibition of target cell lysis was then determined as [nM] 50%. To facilitate comparison, the relative competitor activity of each peptide was calculated as the [nM] 50% of the reference influenza A matrix peptide 58-66 divided by the [nM] 50% of the competitor peptide. Antigen recognition was assessed using standard ^{51}Cr release assays. Target cells were labeled with ^{51}Cr for 1 h at 37°C and washed two times. Labeled target cells (1000 cells in 50 μl) were incubated in the presence of various concentrations of peptide (50 μl) for 15 min at room temperature before the addition of effector cells (50 μl). In the case of TILNs, the effector cells were preincubated for at least 20 min at 37°C with unlabeled K562 cells (50,000/well) to eliminate nonspecific lysis due to natural killer-like effectors. Chromium release was measured in supernatant (100 μl) harvested after a 4-h incubation at 37°C. The percentage of specific lysis was calculated as: 100 × [(experimental − spontaneous release)/(total − spontaneous release)].

The relative antigenic activity of each peptide was calculated as the [nM] 50% of the parental NY-ESO-1 nonapeptide 157–165 SLLMWITQC divided by the [nM] 50% of the competitor peptide.

Tetramers, mAbs, and Flow Cytometry Immunofluorescence Analysis. HLA-A2/peptide tetramers were synthesized as described (11, 12). Antigenic peptides included influenza A matrix peptide 58-66 (GILGFVFTL), HIV-derived peptide pol 476-484 (ILKEPVHGV), and NY-ESO-1 parental peptide 157-165 (SLLMWITQC) or peptide analogue C165A (SLLMWITQA). The cells were first incubated with tetramers in 20 μl of PBS containing 2% BSA and 0.2% azide during 1 h at room temperature, then 20 μl of anti-CD8[FITC] or of a mixture containing anti-CD8[FITC] and anti-CD45-RA[Cyc] or anti-CD8[PerCP] and anti-CD62L[FITC] were added, and the incubation was continued for another 20 min at 4°C. All of the mAbs used were purchased from Becton Dickinson (Basel, Switzerland). Cells were washed once in the same buffer and analyzed by flow cytometry. Data analysis was performed using Cell Quest software.

IFN-γ ELISPOT Assay. IFN-γ ELISPOT assay (13) was performed in nitrocellulose-lined 96-well microplates (Millipore MAHA S45; Millipore, Bedford, MA) using a IFN-γ ELISPOT kit (Mabtech, Stockholm, Sweden) according to the manufacturer's instructions, with minor modifications. Plates were coated overnight with antibody to human IFN-γ and washed six times. T2 cells (5×10^4/well) were then added together with the indicated number of responder T cells and peptide (1 μM where indicated). After incubation for 20 h at 37°C, cells were removed and plates were developed with a second (bio-

tinylated) antibody to human IFN-γ and streptavidin-alkaline phosphatase. Spots were counted using a stereomicroscope with a magnification of ×15.

RESULTS

Detection of a High Proportion of NY-ESO-1-specific CD8^{+} T Cells in TILs of HLA-A2^{+} Melanoma Patient LAU 156 and Generation of Specific CTL Clones. Melanoma patient LAU 156 presented a slowly progressive disease with recurrent metastases all localized at a single paravertebral site. RT-PCR of RNA from this lesion showed that the tumor cells expressed NY-ESO-1. In addition, high titers of anti-NY-ESO-1 antibodies were detected in the patient's serum. In a first attempt to identify NY-ESO-1-specific CD8^{+} T cells among TILs derived from this lesion, short-term cultured TILs were tested for NY-ESO-1 peptide-induced IFN-γ production by ELISPOT (13). To this end, we used three different peptides corresponding to overlapping sequences spanning the NY-ESO-1 155-167 region (peptide 157-167 SLLMWITQCFL, peptide 157-165 SLLMWITQC, and peptide 155-163 QLSLLMWIT) that have been previously shown to be recognized by a NY-ESO-1-reactive CTL line (6). As shown in Fig. 1, high frequencies of IFN-γ producing cells were readily observed with any of the three NY-ESO-1 peptides. From the ELISPOT data we estimated that about 20% of TILs were NY-ESO-1 specific.

IFN-γ-producing cells were also detected in the same TIL population after stimulation with two other HLA-A2-restricted CTL anti-
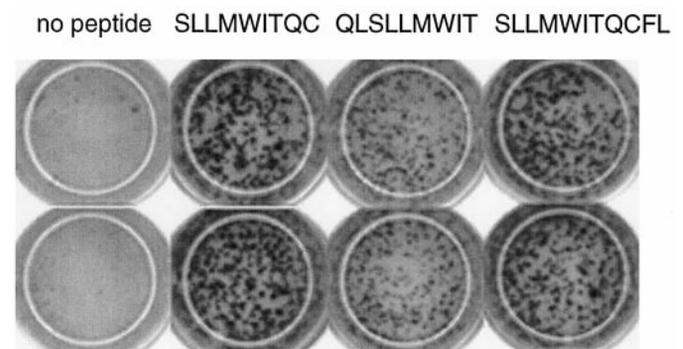
no peptide   SLLMWITQC   QLSLLMWIT   SLLMWITQCFL

Fig. 1. IFN-γ ELISPOT analysis of TIL Me 336. Short-term cultured TIL Me 336 from patient LAU 156 (2×10^3 cells/well) were tested by IFN-γ ELISPOT assay in the presence of APCs alone (T2, 5×10^4 cells/well) or APCs plus the indicated peptide (1 μM) in duplicate cultures. Nineteen spots were counted in cultures with T2 cells alone, 424 spots in cultures with T2 cells plus NY-ESO-1 peptide 157-165, 358 spots in cultures with T2 cells plus NY-ESO-1 peptide 155-163, and 396 spots in cultures with T2 cells plus NY-ESO-1 peptide 157-167.
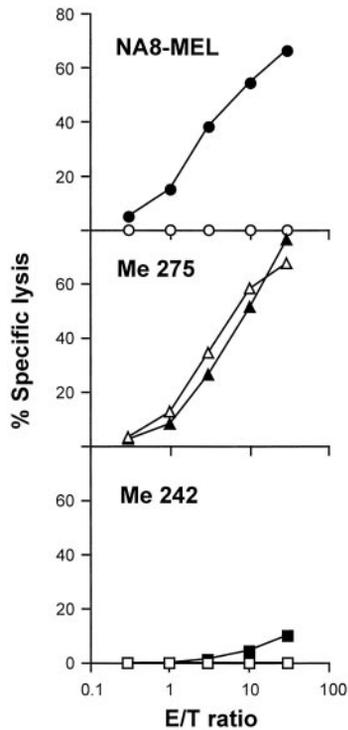
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Fig. 2. Cytolytic activity of CTL clone ESO 5 against NY-ESO-1+ and NY-ESO-1− melanoma cell lines. Specific lysis of NY-ESO-1-positive or -negative melanoma cell lines by clone ESO 5 was assessed in a 4-h chromium release assay as detailed in "Materials and Methods." Melanoma cell lines NA8-MEL (*circles*, A2+ NY-ESO-1−), Me 275 (*triangles*, A2+ NY-ESO-1+), and Me 242 (*squares*, A2− NY-ESO-1+) were tested either in the absence (*open symbols*) or in the presence (*filled symbols*) of exogenously added NY-ESO-1 peptide 157-165 (1 μM).

genic peptides, Melan-A peptide 26-35 (EAAGIGILTV) and Tyrosinase peptide 368-376 (YMDGTMSQV; data not shown). No reactivity was detected against other known HLA-A2-restricted CTL antigenic peptides, including gp100 peptides 154-162 (KTWGQYWQV), 209-217 (ITDQVPFSV), 280–288 (YLEPGPVTA), 457–466 (LLDGTATLRL), and 476-485 (VLYRYGSFSV; Ref. 14); 619-628 (RLMKQDFSV) and 639-647 (RLPRIFCSC; Ref. 15); Tyrosinase peptide 1-9 (MLLAVLYCL; Ref. 16); and the recently defined CAMEL peptide 1-11 (MLMAQEALAFL; Ref. 17; data not shown).

Monoclonal NY-ESO-1-specific CD8+ T-cell populations were generated by limiting dilution cloning of the TIL in the presence of PHA and allogeneic feeder cells, as described previously (9). In agreement with the ELISPOT data, 5 of 24 (21%) CTL clones were found to specifically recognize NY-ESO-1-derived peptides presented in association with HLA-A2. Fig. 2 shows the cytolytic activity of a representative clone (ESO 5) that was able to specifically lyse melanoma line Na8-MEL (A2+, NY-ESO-1−) in the presence, but not in the absence, of exogenously added NY-ESO-1 157-165 nonapeptide. In addition, this CTL clone efficiently lysed melanoma line Me 275 (A2+, NY-ESO-1+) in the absence of added peptide. In contrast, no lysis of melanoma line Me 242 (A2−, NY-ESO-1+) was observed, even in the presence of added peptide. Furthermore, other A2+, but not A2−, NY-ESO-1-expressing melanoma lines generated in our laboratory were efficiently lysed by CTL clone ESO 5 (data not shown).

Identification of the Optimal NY-ESO-1 Antigenic Peptide and Generation of Peptide Analogues with Enhanced Antigenic Activity. To determine whether one of the three tested peptides was better recognized by NY-ESO-1-specific CTLs, we assayed each one for both binding to HLA-A2 and recognition by cloned CTLs. Binding to HLA-A2 was measured in a functional competition assay as described previously (18). A peptide from influenza matrix protein (Flu-MA 58-66), which is a high-affinity HLA-A2 binder, was used as the reference competitor peptide. As shown in Fig. 3A, NY-ESO-1 nonapeptide 155-163 was 100-fold less efficient than Flu-MA 58-66. NY-ESO-1 nonapeptide 157-165 was even less active (data not shown). Surprisingly, NY-ESO-1 peptide 157-167 displayed a competitor activity higher than that of either nonapeptide (only 25-fold less efficient than Flu-MA 58-66; data not shown). These results led us to speculate that the poor binding of NY-ESO-1 nonapeptide 157-165 could be due to the presence of a cysteine (C) residue at the carboxyl terminus of the peptide. To test this hypothesis, we produced single amino acid-substituted nonapeptides containing either A or L at position 165 (NY-ESO-1 C165A and NY-ESO-1 C165 L). As shown in Fig. 3A, both substitutions dramatically enhanced peptide binding to HLA-A2 (100-fold as compared with the parental sequence). Recognition of both parental and analogue peptides by CTL clone ESO 5 is shown in Fig. 3B. Each of the three parental peptides was specifically recognized at high peptide concentrations. However, the nonapeptide 157-165 was recognized more efficiently (50% maximal lysis at 0.6 nM) than the other parental peptides (50% maximal lysis at
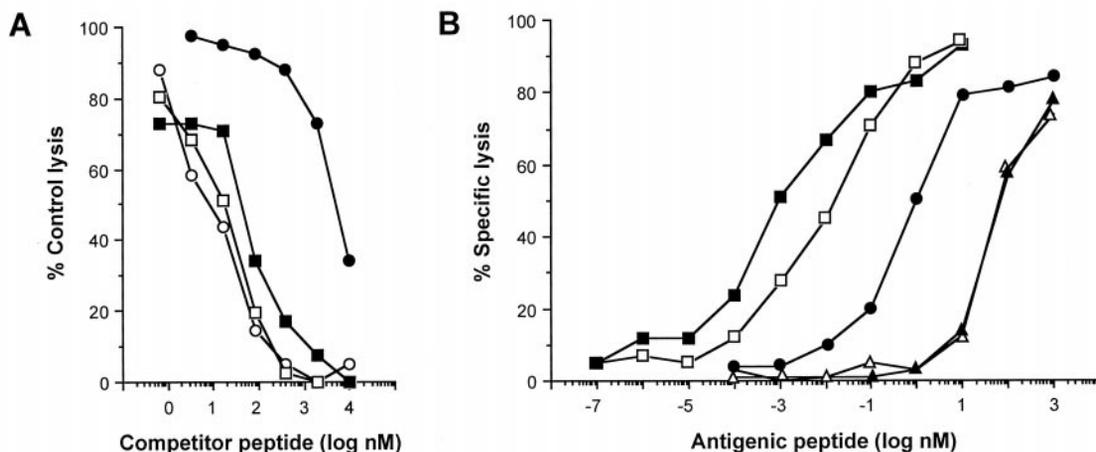

Fig. 3. Identification of NY-ESO-1 peptide analogues with enhanced antigenic activity. *A*, NY-ESO-1 parental or analogue peptides were tested for binding to HLA-A2 in a functional peptide competition assay, as reported previously (9). Relative competitor activity was calculated as described in "Materials and Methods." ○, influenza A matrix peptide 58-66; ●, NY-ESO-1 parental peptide 157-165 SLLMWITQC; □, NY-ESO-1 peptide analogue SLLMWITQL; ■, NY-ESO-1 peptide analogue SLLMWITQA. *B*, NY-ESO-1 parental or analogue peptides were tested for antigen recognition by CTL clone ESO 5 in a 4-h chromium release assay as detailed in "Materials and Methods." △, SLLMWITQCFL; ▲, QLSLLMWIT; ●, SLLMWITQC; ■, SLLMWITQA; □, SLLMWITQL.

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50 nM). In addition, both NY-ESO-1 C165 L and NY-ESO-1 C165A nonapeptide analogues were recognized more efficiently than the parental nonapeptide 157-165 (50% maximal lysis at 10 pM and 0.5 pM, respectively).

Assessment of NY-ESO-1 Peptide-reactive CD8$^+$ T Cells in Blood from Melanoma Patients. To assess the proportion of HLA-A2 melanoma patients with NY-ESO-1-specific CTL precursors, highly enriched CD8$^+$ T cells from blood samples collected in 10 A2$^+$ patients were stimulated *in vitro* with autologous APCs in the presence of the parental peptide NY-ESO-1 157-165 or the peptide analogue NY-ESO-1 C165A and then tested by ELISPOT at different time points after stimulation (Table 1). At day 7, high proportions of NY-ESO-1-specific IFN-γ-producing cells were detected in CD8$^+$ T-cell cultures from patient LAU 156 stimulated with either of the two peptides. Similar results were obtained with cultures from patient LAU 50, albeit only after stimulation with NY-ESO-1 peptide C165A. Analysis of cultures at day 14 after stimulation confirmed the results obtained at day 7. In addition, specific IFN-γ-producing cells were now detected among CD8$^+$ T cells from patient LAU 50 stimulated with the parental nonapeptide. Remarkably, patients LAU 156 and LAU 50 were the only two patients among the nine tested (patient LAU 203 was not tested) with both detectable serum levels of anti NY-ESO-1 antibodies and NY-ESO-1-expressing tumors as assessed by RT-PCR and Western blot analysis (data not shown). Specific IFN-γ-producing cells were detected at day 14 in CD8$^+$ T-cell cultures from an additional patient, LAU 198, after stimulation with peptide NY-ESO-1 C165A.

Enumeration of NY-ESO-1-specific CD8$^+$ T Cells in Peptide-stimulated Blood Lymphocytes from Melanoma Patients. To directly enumerate NY-ESO-1-specific CD8$^+$ T cells in the cultures described above, we prepared fluorescent A2/NY-ESO-1 peptide tetramers (11). Because high-affinity peptide binding to HLA-A2 greatly facilitates the generation of stable tetramer preparations, the peptide analogue NY-ESO-1 C165A was chosen for tetramer assembly. As shown in Fig. 4, A2/NY-ESO-1 C165A tetramers (A2/NY-ESO-1 tetramers thereafter) specifically stained CTL clone ESO 5. No detectable staining over background values was observed for the Flu-MA 58–66-specific CTL clone FLU 17 (Fig. 4A). Conversely, A2/Flu-MA 58–66 tetramers stained specifically the latter but not

CTL clone ESO 5 (Fig. 4B). Remarkably, the efficacy (both in terms of mean fluorescence intensity and tetramer dose response) of A2/NY-ESO-1 tetramer staining of clone ESO 5 was comparable with that of A2/Flu-MA 58–66 tetramer staining of clone FLU 17 (Fig. 4C). Specific staining of CTL clone ESO 5 was also obtained with tetramers containing the parental peptide NY-ESO-1 157-165, although with lower staining efficiency compared with tetramers containing the peptide analogue. In addition, both NY-ESO-1 tetramers detected identical proportions of CD8$^+$ T cells in polyclonal PBMC cultures stimulated with NY-ESO-1 peptide 157-165 (data not shown).

Enumeration of NY-ESO-1 tetramer$^+$ CD8$^+$ T cells in peptide-stimulated cultures from the experiment illustrated in Table 1 was performed at day 14 after stimulation (Fig. 5). In agreement with the ELISPOT results (Table 1), relatively high proportions of A2/NY-ESO-1 tetramer$^+$ lymphocytes were detected in cultured CD8$^+$ T cells from patients LAU 50 and LAU 156. In the case of patient LAU 50, the majority of tetramer$^+$ lymphocytes displayed a bright fluorescence signal, whereas a tetramer$^+$ dull population was also clearly detectable in the cultures from patient LAU 156. Moreover, high proportions of A2/NY-ESO-1 tetramer$^+$ lymphocytes with intermediate fluorescence intensity were detected in cultures from patient LAU 198. Interestingly, significant numbers of A2/NY-ESO-1 tetramer$^+$ cells were also detected in cultures from patients LAU 97 and LAU 42, although these cultures scored negatively in the ELISPOT assay (Table 1). The positive staining of these cultures with tetramers was confirmed after a second round of *in vitro* stimulation. No A2/NY-ESO-1 tetramer$^+$ lymphocytes were detected in cultures from the remaining patients. Altogether, these results indicated that 5 of the 10 patients tested exhibited significant numbers of circulating NY-ESO-1 peptide-reactive CD8$^+$ T cells that could be expanded *in vitro*.

Tetramer-guided Isolation of NY-ESO-1-specific CTLs. To further validate the use of the A2/NY-ESO-1 tetramers for the detection of NY-ESO-1-specific CD8$^+$ T cells, A2/NY-ESO-1 tetramer$^+$ and tetramer$^-$ populations from cultures of patient LAU 50 (Fig. 4) were isolated by tetramer-guided cell sorting and immediately tested for IFN-γ production. In addition, antigen-specific cytolytic activities of both subpopulations were assessed after mitogen-driven expansion. As shown in Fig. 6, specific IFN-γ-producing cells were detected only
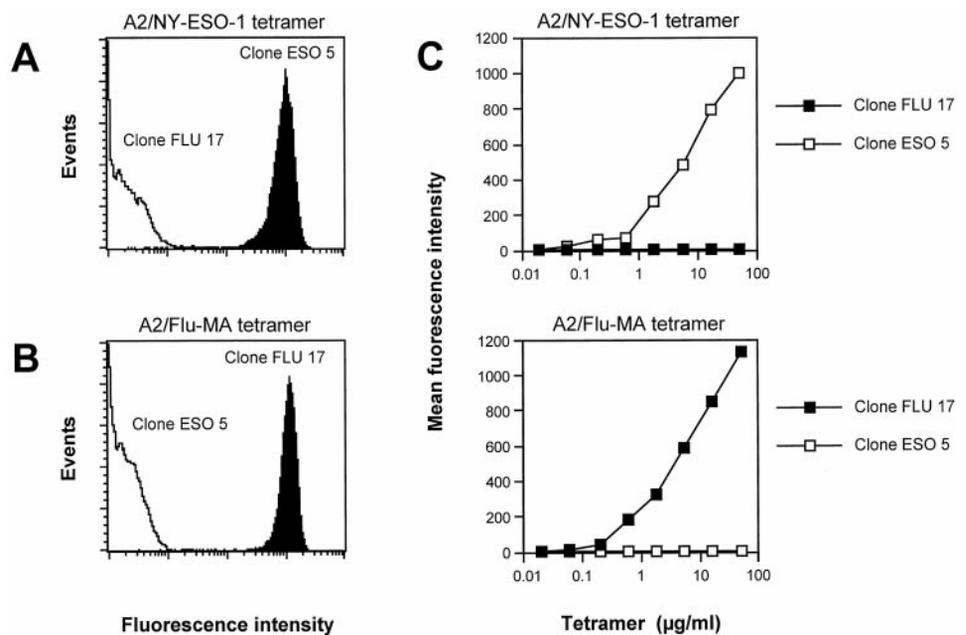

Fig. 4. Characterization of fluorescent A2/NY-ESO-1 tetramers. *A*, cloned CTL specific for the influenza A matrix 58-66 peptide (clone FLU 17) or NY-ESO-1 (clone ESO 5) were stained with phycoerythrin-labeled A2/NY-ESO-1 tetramers (50 μg/ml) and analyzed by flow cytometry. Data analysis was performed using Cell Quest software. *B*, staining was performed as described in *A* with phycoerythrin A2/influenza A matrix peptide 58-66 tetramers. *C*, cloned CTL FLU 17 (■) or ESO 5 (□) were stained with the indicated tetramer concentrations.
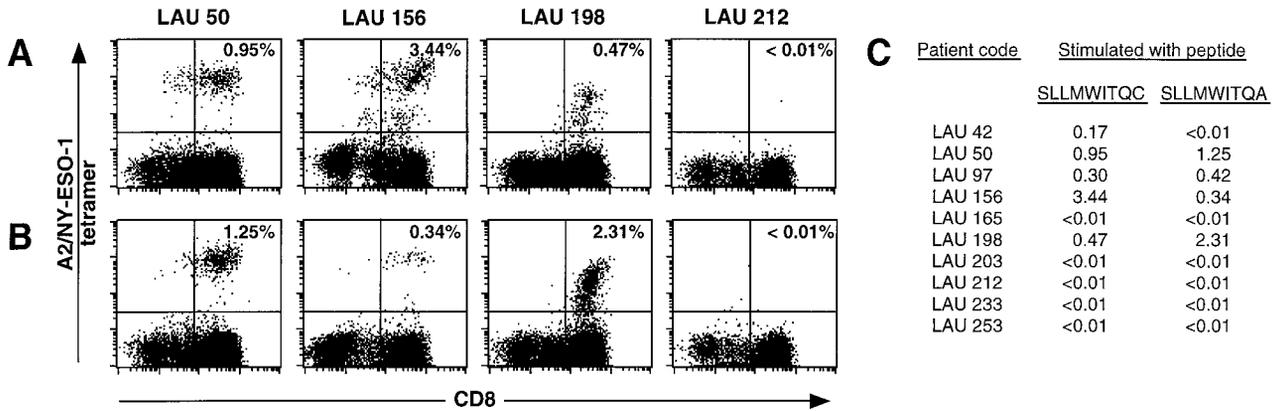
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Fig. 5. Tetramer-guided analysis of NY-ESO-1-specific CTL responses in peptide-stimulated CD8$^+$ lymphocytes. CD8$^+$-enriched PBMCs from melanoma patients LAU 50, LAU 156, LAU 198, and LAU 212 were stained with A2/NY-ESO-1 tetramers together with anti-CD8^{FITC} mAb on day 14 after *in vitro* stimulation either with NY-ESO-1 parental peptide 157-165 (*A*) or NY-ESO-1 peptide analogue C165A (*B*). *Numbers in the top right quadrant* indicate the percentage of A2/NY-ESO-1 tetramer$^+$ cells within CD8$^+$ lymphocytes. *C*, results are reported for 10 HLA-A2 melanoma patients on day 14 after stimulation with the indicated peptide. *Numbers* represent percentages of A2/NY-ESO-1 tetramer$^+$ cells within CD8$^+$ lymphocytes.

in CD8$^+$ tetramer$^+$, but not in CD8$^+$ tetramer$^-$ subpopulations derived from cultures stimulated with either NY-ESO-1 peptide 157-165 (Fig. 6*A*) or NY-ESO-1 peptide analogue C165A (Fig. 6*B*). Moreover, CD8$^+$ tetramer$^+$, but not CD8$^+$ tetramer$^-$, subpopulations were able to kill peptide-pulsed target cells as well as autologous tumor cells (Fig. 6*C*).

NY-ESO-1-specific CD8$^+$ T Cells in TIL(N)s. Using tetramers containing a Melan-A immunodominant peptide, we have previously reported that metastatic lesions of HLA-A2 melanoma patients often contain high numbers of Melan-A-specific CD8$^+$ T cells (12). To assess whether a similar accumulation of NY-ESO-1-specific CD8$^+$ T cells at the tumor site may occur, TIL and TILN samples available from the group of melanoma patients analyzed in this study were stained with A2/NY-ESO-1 peptide tetramers. As shown in Fig. 7,

high numbers of NY-ESO-1 tetramer$^+$ CD8$^+$ T cells were found in TILNs from patient LAU 50 and, in agreement with the ELISPOT and cloning data mentioned above, in TILs from patient LAU 156. However, no NY-ESO-1 tetramer$^+$ CD8$^+$ T cells were detectable in fresh or short-term cultured TIL or TILN samples from the eight other patients analyzed.

Circulating NY-ESO-1-specific CD8$^+$ T cells in Melanoma Patients. Finally, in an attempt to directly assess the frequency and phenotype of NY-ESO-1-circulating CD8$^+$ T cells *ex vivo*, uncultured PBMCs from the 10 melanoma patients included in this study were analyzed by A2/NY-ESO-1 tetramer staining. NY-ESO-1 tetramer$^+$ CD8$^+$ T cells were not detectable in blood samples from 9 of the 10 patients analyzed, including patients LAU 42, LAU 97, LAU 156, and LAU 198, who exhibited detectable NY-ESO-1-specific CD8$^+$ T-cell
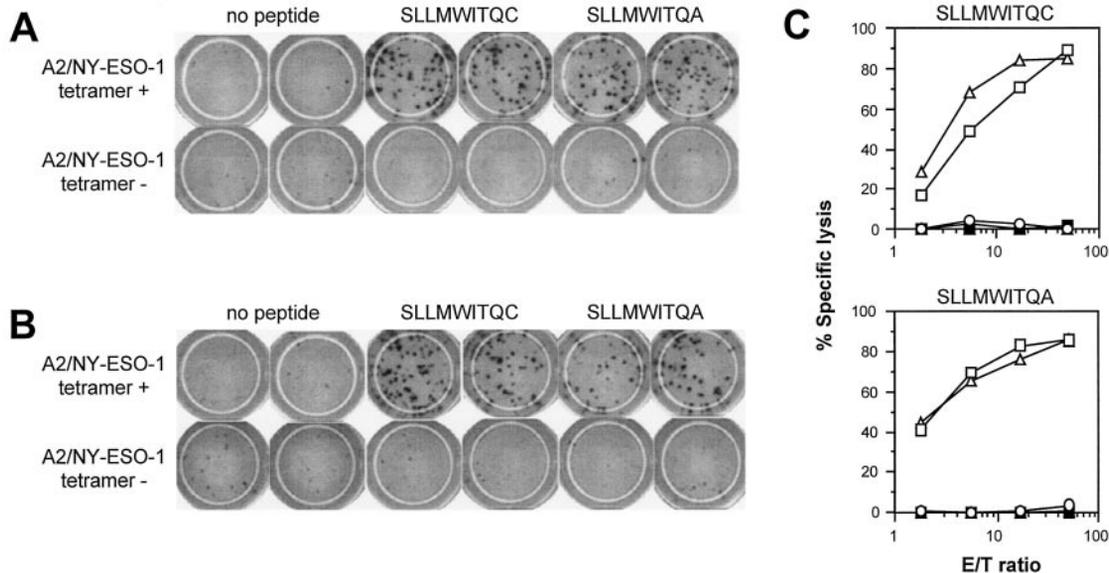

Fig. 6. Validation of the use of A2/NY-ESO-1 tetramers for the detection and isolation of NY-ESO-1-specific CD8$^+$ T cells. CD8$^+$-enriched PBMCs from patient LAU 50 stimulated with NY-ESO-1 parental peptide 157-165 (*A*) or NY-ESO-1 peptide analogue C165A (*B*) were stained with A2/NY-ESO-1 tetramers on day 14 after stimulation. CD8$^+$ A2/NY-ESO-1 tetramer$^+$, and CD8$^+$ A2/NY-ESO-1 tetramer$^-$ fractions were then purified by tetramer-guided cell sorting and the isolated populations (100 cells/well) immediately tested by IFN-γ ELISPOT assay. Cultures stimulated with peptide SLLMWITQC: four spots with T2 alone, 60 spots with peptide SLLMWITQC, and 63 spots with peptide SLLMWITQA for the A2/NY-ESO-1 tetramer$^+$ fraction; five spots with T2 alone, two spots with peptide SLLMWITQC, and eight spots with peptide SLLMWITQA for the A2/NY-ESO-1 tetramer$^-$ fraction. Cultures stimulated with peptide SLLMWITQA: eight spots with T2 alone, 66 spots with peptide SLLMWITQC, and 27 spots with peptide SLLMWITQA for the A2/NY-ESO-1 tetramer$^+$ fraction; seven spots with T2 alone, seven spots with peptide SLLMWITQC, and eight spots with peptide SLLMWITQA for the A2/NY-ESO-1 tetramer$^-$ fraction. *C*, lytic activities displayed by CD8$^+$ A2/NY-ESO-1 tetramer$^+$ (*open symbols*) and CD8$^+$ A2/NY-ESO-1 tetramer$^-$ (*filled symbols*) populations 2 weeks after expansion in the presence of PHA were determined using ^{51}Cr-labeled autologous melanoma cell line Me 275 (*triangles*) or T2 target cells in the presence (*squares*) or in the absence (*circles*) of NY-ESO-1 parental peptide 157-165 (1 μM) at the indicated E:T ratios.
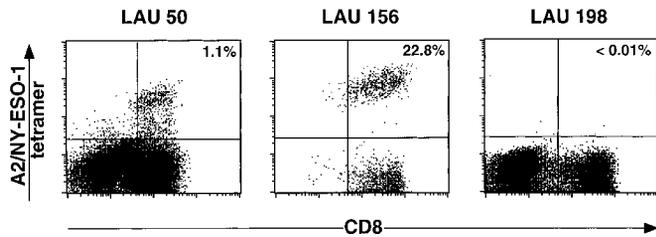
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Fig. 7. NY-ESO-1-specific CTL responses in TIL(N)s. TILs from patient LAU 156 and TILNs from patients LAU 50 and LAU 198 were cultured in complete medium supplemented with hrIL-2 and hrIL-7 for 14 days and stained with A2/NY-ESO-1 tetramers together with anti-CD8[FITC] mAb. *Numbers in the top right quadrant* indicate the percentage of A2/NY-ESO-1 tetramer+ cells within CD8+ lymphocytes.

responses after *in vitro* stimulation of PBMC with NY-ESO-1 peptide. In contrast, circulating A2/NY-ESO-1 tetramer+ CD8+ T cells were detected repeatedly in patient LAU 50 (0.04%; Fig. 8). This value was clearly above background values obtained with the unrelated A2/HIV tetramer in eight HLA-A2 normal donors (<0.01%; data not shown). This patient presented a primary skin melanoma of the lower limb in October 1992. A few months later, numerous cutaneous metastases appeared in the same limb. He was subjected to isolated limb perfusion with melphalan and adjuvant IFN-α therapy. A complete response ensued, lasting until 1995 when an inguinal lymph node dissection revealed that 1 of 14 nodes was infiltrated by melanoma cells. Since then, the patient remained tumor free until his last control in April 1999. A2/NY-ESO-1 tetramer staining of blood samples collected at different time points of his clinical history is shown in Fig. 8. A2/NY-ESO-1 tetramer+ CD8+ T cells were clearly detectable in three consecutive blood samples collected between 1997 and 1999 but were below the detection limit at an earlier time point. Of note, A2/NY-ESO-1 tetramer+ cells displayed a typical memory phenotype at all time points analyzed (CD45RA−, CD28+; Fig. 8 and data not shown). Moreover, staining with anti-CD62L mAb subdivided NY-ESO-1-specific CD8+ T cells in two subpopulations of comparable size. Thus, although the frequency of circulating NY-ESO-1-specific CD8+ T cells is usually below the limit of detection by tetramer staining, the data obtained with patient LAU 50 clearly indicate that CD8+ T-cell memory to NY-ESO-1 can persist for several years in clinically tumor-free melanoma patients.

DISCUSSION

In the present study, we investigated the HLA-A2-restricted CD8+ T-cell response against the NY-ESO-1 CT antigen in melanoma patients. We report two main findings: (*a*) we identified NY-ESO-1 peptide analogues with enhanced antigenicity; and (*b*) we demonstrated the existence of natural CD8+ T-cell responses against this CT antigen in a significant proportion of melanoma patients. In one patient, high numbers of NY-ESO-1-specific CD8+ T cells were detectable both in blood and at the tumor site concomitantly with high titers of NY-ESO-1-specific antibodies in serum.

The CT antigens include presently the products of at least six gene families: *MAGE*, *GAGE*, *BAGE* (1), *SSX-2*, *SCP1* (2, 3), and *NY-ESO-1* (4). Because of their tumor specificity, CT antigens are good candidates for the design of cancer vaccines aimed at eliciting tumor-reactive CTL responses. Indeed, one of these antigens, the MAGE-3.A1 peptide, has been already used in a Phase I-II clinical trial (5). Although a relatively high tumor response rate was observed among a group of 25 HLA-A1 patients, there was no evidence of specific CTL induction after peptide immunization. Moreover, naturally occurring CD8+ T-cell responses to CT antigens have been reported only occasionally possibly due to the very low frequency of specific

precursors (19). Together, these observations raise questions on the immunogenicity of CT antigens and, hence, on the induction of specific CTL responses by appropriate peptide immunization.

The recently identified CT antigen NY-ESO-1 has been shown to be involved in both cellular and humoral immune responses. High titers of IgG antibodies were first demonstrated in a patient with a squamous cell carcinoma of the esophagus (4). Circulating antibodies to NY-ESO-1 have been found in about 10% of melanoma patients and in lower proportions of patients with other tumor types, such as ovarian, lung, and breast adenocarcinomas (8). In this study, we focused on the assessment of naturally occurring CD8+ T-cell responses to the HLA-A2-restricted NY-ESO-1 antigenic peptides previously described by Jäger *et al.* (6). Using a NY-ESO-1-specific CTL clone isolated from a TIL population, we confirmed previous observations made with a polyclonal CTL line indicating that three overlapping NY-ESO-1 peptides could be efficiently recognized at saturating peptide concentrations. Our study established that among these three peptides NY-ESO-1 peptide 157-165 (SLLMWITQC) was the most efficiently recognized in antigenic peptide titration assays.

The cysteine residue at the carboxyl terminus of NY-ESO-1 peptide 157-165 is not only suboptimal for binding to HLA-A2 (20) but may also be disadvantageous due to its propensity to form cystine dimers on oxidation. As expected, we found that NY-ESO-1 peptide analogues carrying major HLA-A2 anchor residues (leucine or valine) at the COOH terminus exhibited enhanced binding to HLA-A2. Surprisingly, the peptide analogue containing a COOH-terminal alanine, an amino acid that has not been identified as a major anchor residue, also bound to HLA-A2 more efficiently than the parental nonapeptide.
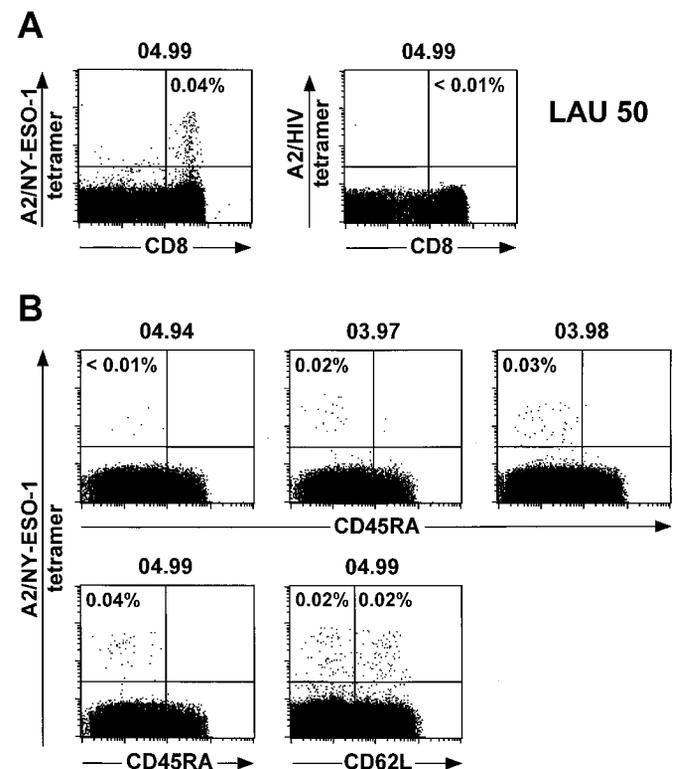

Fig. 8. Circulating A2/NY-ESO-1 tetramer+ CD8+ T cells. Cryopreserved PBMC samples from melanoma patient LAU 50 were cultured overnight in complete medium and then stained with the indicated mAbs. *A*, PBMCs were stained with A2/NY-ESO-1 tetramers or with irrelevant A2/peptide tetramers containing the HIV peptide pol 476–484 (ILKEPVHGV), together with anti-CD8[PerCP]. *B*, PBMCs obtained at the dates indicated were stained with A2/NY-ESO-1 tetramers and anti-CD45RA[FITC] mAbs or anti-CD62L[FITC] mAbs. A2/NY-ESO-1 tetramer *versus* CD45RA or CD62L dot plots are shown for gated CD8+ live lymphocytes, and the percentage of cells staining positively with the tetramers is indicated.

Moreover, this peptide analogue was more efficiently recognized by cloned CTL. Importantly, the CTL generated after *in vitro* stimulation with this peptide analogue were able to efficiently cross-recognize the naturally processed NY-ESO-1 peptide. As reported recently for another CTL-defined antigenic peptide analogue (21), additional studies using HLA-A2-transgenic mice should establish whether the NY-ESO-1 analogue described here displays enhanced immunogenicity *in vivo*.

The availability of a potent NY-ESO-1 peptide analogue provided the opportunity to generate stable fluorescent tetramers for the direct visualization of NY-ESO-1-specific CD8^{+} T cells. We have previously shown that an analogue of the HLA-A2-restricted Melan-A/MART-1 antigenic peptide was superior to the parental peptide in terms of tetramer staining efficiency (12). We made similar observations in this study when comparing HLA-A2 tetramers assembled with either the NY-ESO-1 parental peptide or its analogue. It is noteworthy, however, that the two tetramers exhibited virtually identical staining properties when used at saturating concentrations.

Fluorescent tetramers were used to monitor naturally occurring CD8^{+} T-cell responses to NY-ESO-1 in HLA-A2 melanoma patients. Five of the 10 patients analyzed had circulating NY-ESO-1-specific CD8^{+} T cells that were readily detectable with tetramers after a single round of *in vitro* peptide stimulation. Although we could not directly determine the frequency of circulating NY-ESO-1-specific CD8^{+} T cells in the majority of the patients, our results suggest that these cells are much more frequent than CD8^{+} T cells directed against the CT MAGE-3-derived antigenic peptide EVDPIGHLY (19). NY-ESO-1 seropositive patients exhibited relatively strong NY-ESO-1 CD8^{+} T-cell responses *in vitro*. The melanoma lesions of these patients were found to express NY-ESO-1 by RT-PCR as well as by Western blot. Surprisingly, low but significant NY-ESO-1 CD8^{+} T-cell responses were also detected in three seronegative patients. It is noteworthy that the melanoma lesions from these latter patients did not express NY-ESO-1 mRNA.[4] It is conceivable that these patients may have been primed by NY-ESO-1 antigen expressed in melanoma lesions that were not accessible for RT-PCR analysis. If so, the NY-ESO-1-specific CD8^{+} T cells should express an antigen-experienced phenotype. Moreover, their existence would implicate that CD8^{+} T-cell responses to this antigen may take place in the absence of specific humoral responses. Alternatively, the circulating NY-ESO-1-specific lymphocytes may express a naive phenotype. Indeed, we have recently reported that both melanoma patients and normal individuals display high numbers of circulating Melan-A-specific CD8^{+} T cells displaying a naive phenotype (22). Additional analyses of the naive/memory phenotype of circulating NY-ESO-1-specific CD8^{+} T cells in NY-ESO-1 seronegative patients should clarify these issues.

In line with our previous findings with Tyrosinase- and Melan-A-tetramer^{+} lymphocytes (10, 23), NY-ESO-1-tetramer^{+} CD8^{+} T cells could be isolated by flow cytometry sorting and expanded *in vitro* in cultures containing allogeneic PBMCs, PHA, and hrIL-2. Moreover, the cultured tetramer^{+} cells were able to kill melanoma cells either sensitized with NY-ESO-1 peptide or expressing the naturally processed antigenic peptide. It, thus, seems that NY-ESO-1-specific CD8^{+} T cells are not necessarily in an anergic state, in contrast to the recently reported tyrosinase-specific CD8^{+} T cells derived from a melanoma patient (24). Although there is no direct evidence that the NY-ESO-1-specific CD8^{+} T cells identified in this study by tetramer staining can exert lytic functions *in vivo*, their ability to proliferate and display potent effector functions *in vitro* suggest that appropriate stimulation and/or addition of growth factors may easily overcome

any putative anergy *in vivo*. NY-ESO-1-specific CD8^{+} T cells among TILs from patients LAU 50 and LAU 156 reveal the existence of tumoricidal NY-ESO-1-specific responses *in vivo* at tumor sites.

Direct *ex vivo* enumeration of NY-ESO-1-specific CD8^{+} T cells in the five patients with detectable *in vitro* responses indicated that the frequencies of these cells in blood were below the tetramer detection limit (0.01%), with one exception. Indeed, the frequency of NY-ESO-1 tetramer^{+} cells in patient LAU 50 was $\sim 1/2'500-5'000$ circulating CD8^{+} lymphocytes. Remarkably, this high frequency was stable over a prolonged period of time. The NY-ESO-1-specific CD8^{+} T cells exhibited an activated/memory phenotype and could be divided into two distinct subpopulations by staining with anti-CD62L mAb. These subpopulations may correspond, respectively, to the resting memory (CD62L^{+}) and effector memory (CD62L^{−}) CD8^{+} T cells that have been described recently (25, 26). It is unclear whether the coexistence of these two phenotypically distinct memory cells in the circulating lymphocyte pool may reflect an ongoing CD8^{+} T-cell response that is sustained over time. Conceivably, resting memory cells could become activated by tumor antigen-presenting dendritic cells after homing into lymph nodes via CD62L. Following activation (and CD62L down-regulation), these cells could recirculate for some time before migrating into tumor lesions. In a previous study, we showed selective accumulation of Melan-A-specific CD8^{+} T cells with an antigen-experienced phenotype in metastatic lymph nodes (12). In contrast, the Melan-A-specific CD8^{+} T-cell populations found in the blood were a mixture of naive and memory lymphocytes at least in some patients (22). Unfortunately, the relatively large numbers of cells required for flow cytometry analysis has thus far hampered an extensive *ex vivo* assessment of the phenotype of antigen-specific CD8^{+} T cells present at tumor sites. Future studies using tetramers for *in situ* detection of antigen-specific CD8^{+} T cells and laser scanning confocal microscopy might provide information on the functional state of tumor antigen-specific T cells that are located in tumor lesions and in tumor-invaded lymph nodes.

ACKNOWLEDGMENTS

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Naturally Occurring Human Lymphocyte Antigen-A2 Restricted CD8 + T-Cell Response to the Cancer Testis Antigen NY-ESO-1 in Melanoma Patients

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