[CANCER RESEARCH 63, 5601–5606, September 1, 2003]

Tumor-reactive, SSX-2-specific CD8$^+$ T Cells Are Selectively Expanded during Immune Responses to Antigen-expressing Tumors in Melanoma Patients[1]

Maha Ayyoub,[2,3] Donata Rimoldi, Philippe Guillaume, Pedro Romero, Jean-Charles Cerottini, Danila Valmori,[3,4] and Daniel Speiser[4]

Division of Clinical Onco-Immunology, Ludwig Institute for Cancer Research, University Hospital, Lausanne [M. A., P. R., D. V., D. S.], and Ludwig Institute for Cancer Research, Lausanne Branch, University of Lausanne, Epalinges [D. R., P. G., J-C. C.], Switzerland

ABSTRACT

The SSX-2 gene encodes a tumor-specific antigen expressed in neoplasms of various histological types. By analyzing a tumor-infiltrated lymph node of a melanoma patient bearing an SSX-2-expressing tumor, we have recently identified the first SSX-2-derived CD8$^+$ T-cell epitope, that corresponds to peptide SSX-2$_{41–49}$, and is recognized by specific CTL in an HLA-A2 restricted fashion. Here, we have used fluorescent HLA-A2/SSX-2$_{41–49}$ peptide multimeric complexes to analyze the response to SSX-2$_{41–49}$ in melanoma patients and healthy donors. Multimer$^+$ CD8$^+$ T cells were readily detected in the majority of patients bearing SSX-2-expressing tumors and, at lower proportions, in patients with nonexpressing tumors and healthy donors. Importantly, isolated A2/SSX-2$_{41–49}$ multimer$^+$ CD8$^+$ T cells exhibited a large functional heterogeneity in terms of antigen recognition and tumor reactivity. SSX-2-specific CTLs isolated from tumor-infiltrated lymph node of antigen-expressing patients as well as from the corresponding peripheral blood mononuclear cells exhibited high functional avidity of antigen recognition and efficiently recognized antigen-expressing tumors. In contrast, SSX-2-specific CTLs isolated from patients with undetectable responses in the tumor-infiltrated lymph node, as well as from healthy donors, recognized the antigen with decreased functional avidity and were not tumor reactive. Together, these data indicate that CD8$^+$ T-cell responses to SSX-2$_{41–49}$ frequently occur in SSX-2-expressing melanoma patients and suggest that SSX-2$_{41–49}$-specific CTLs of high avidity and tumor reactivity are selectively expanded during immune responses to SSX-2-expressing tumors *in vivo*.

INTRODUCTION

The group of CT[5] antigens includes nonmutated self-proteins whose expression is limited to germ-line cells and tumors of different histological types, but it is not found in most normal tissues (1–3). As germ-line cells do not express MHC-class I molecules and can therefore not be targeted by CTL, immune responses against CT antigens are strictly tumor specific. Therefore, CT antigens are among the most attractive molecules to be used for generic immunotherapy of cancer. It has been shown that expression of CT antigens in tumors can give rise to antibody as well as T cell-mediated immune responses (4–6). Because it is currently admitted that tumor-specific CD8$^+$ T cells constitute the main antitumor effector arm of the immune response, their elicitation is the aim of most antitumor immunotherapy clinical trials currently carried out worldwide. CT antigens are encoded by a number of genes and gene families (7–9). Numerous CD8$^+$ T-cell epitopes derived from these antigens have been identified (4, 10–12). The potential of these epitopes and of the corresponding synthetic peptides to be used as generic cancer vaccines depends, among other parameters, on the frequency of peptide-specific tumor-reactive CD8$^+$ T cells in the T-cell repertoire. Although the frequency of tumor antigen-specific T-cell precursors in the naive repertoire is generally low and difficult to evaluate, the analysis of tumor antigen-specific T-cell responses in patients bearing antigen-expressing tumors can provide important information. Interestingly, the occurrence of specific CD8$^+$ T-cell responses to CT antigen-derived epitopes identified thus far has been found to be highly variable depending on the epitope, being rare for some of them and rather frequent for others (13, 14). In addition to tumor-reactive CD8$^+$ T cells, peptide-specific CD8$^+$ T cells that recognize the antigen with decreased functional avidity and are nontumor reactive can also be found in the repertoire (15). The evaluation of the antigen-specific immune response to newly identified epitopes is therefore a prerequisite for the implementation of immunotherapy clinical trials using these epitopes.

SSX-2 was initially described for being one of the two partner genes involved in a recurrent chromosomal translocation found in synovial sarcoma (16, 17). The gene was later shown to be identical to that encoding HOM-MEL-40, a CT antigen identified by SEREX analysis of serum from a melanoma patient, which is expressed in a wide variety of tumors. Significant levels of specific antibodies to SSX-2 were found in 10% of melanoma patients (18), indicating that spontaneous immune responses directed against the SSX-2 antigen can occur. By analyzing CD8$^+$ T lymphocytes from a TILN of an SSX-2-expressing melanoma patient, we have recently identified an HLA-A2 restricted SSX-2-derived CD8$^+$ T-cell epitope corresponding to peptide SSX-2$_{41–49}$. SSX-2$_{41–49}$-specific CD8$^+$ T cells isolated from the TILN exhibited high functional avidity of antigen recognition and specifically recognized SSX-2-expressing tumor cells (19, 20). To implement the development of cancer vaccination trials with peptide SSX-2$_{41–49}$, we analyzed the relative frequency at which SSX-2$_{41–49}$-specific CD8$^+$ T-cell responses occur in melanoma patients bearing SSX-2 expressing or nonexpressing tumors as well as in healthy donors. Using fluorescent HLA-A2/SSX-2$_{41–49}$ multimeric complexes (multimers thereafter), we could clearly detect SSX-2$_{41–49}$-specific CD8$^+$ T cells both among TILN and peptide-stimulated PBMCs from patients bearing SSX-2-expressing tumors and, to a lesser extent, among peptide-stimulated PBMCs from patients with SSX-2 nonexpressing tumors and healthy donors. CD8$^+$ HLA-A2/SSX-2$_{41–49}$ multimer$^+$ T-cell populations displayed a wide range of functional avidity of antigen recognition and tumor reactivity. Interestingly, SSX-2$_{41–49}$-specific CD8$^+$ T cells exhibiting high functional avidity of antigen recognition and high tumor reactivity were consistently isolated from both TILN and peptide-stimulated PBMCs from patients bearing SSX-2-expressing tumors, indicating that high-avidity tumor-reactive CTLs are selectively expanded during immune responses to SSX-2-expressing tumors.

Received 2/26/03; revised 4/23/03; accepted 6/11/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported in part by a grant from the Cancer Antigen Discovery Collaborative (Cancer Research Institute) to M. A. and D. S. M. A. was the recipient of an NCCR Molecular Oncology postdoctoral fellowship.

[2] To whom requests for reprints should be addressed, at Ludwig Institute Clinical Trial Center, at Division of Medical Oncology, Department of Medicine, Columbia University College of Physicians and Surgeons, New York, NY. Phone: (212) 305-3923; Fax: (212) 305-7348; E-mail: ayyoub@cancercenter.columbia.edu.

[3] Present address: Ludwig Institute Clinical Trial Center, at Division of Medical Oncology, Department of Medicine, Columbia University College of Physicians and Surgeons, New York, NY.

[4] D. V. and D. S. share senior contribution.

[5] The abbreviations used are: CT, cancer testis; TILN, tumor-infiltrated lymph node; mAb, monoclonal antibody; APC, antigen presenting cells; TIL, tumor-infiltrating lymphocyte; PE, phycoerythrin; PBMC, peripheral blood mononuclear cell.

MATERIALS AND METHODS

Patients, Tumors, and Cells. Frozen tumor samples from stage II-IV HLA-$A2^+$ melanoma patients were tested for the expression of SSX-2. To prepare TIL or TILN cultures, surgically resected metastatic tumor lesions from melanoma patients were finely minced in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD). Cell suspensions were either directly cryopreserved or placed in 2 ml of Iscove's modified Dulbecco's medium (Life Technologies, Inc., Basel, Switzerland) supplemented with 8% heat inactivated pooled human serum (CTL medium), 100 units/ml human recombinant rIL-2 (Glaxo, Geneva, Switzerland), and 10 ng/ml rIL-7 (Biosource International, Camarillo, CA) for 2–3 weeks. Melanoma cell lines Me 275, T343B, and T567A were established at the Ludwig Institute for Cancer Research, Lausanne Branch, from surgically excised melanoma lesions from patients LAU 50, LAU 343, and LAU 567, respectively. The cells were characterized for surface expression of total HLA class I or HLA-A2 molecules by fluorescence-activated cell sorter analysis using W6/32 and BB7.2 mAbs, respectively. All lines expressed the melanoma marker HMW-MAA and the adhesion molecule ICAM-1/CD54. SK-MEL-37 and SK-MEL-23 cells were kindly provided by Dr. Y. T. Chen (Ludwig Institute for Cancer Research, New York Branch, New York, NY). Tumor cell lines and the HLA-$A2^+$ human mutant cell line CEMx721.T2 (T2 thereafter) were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS.

PCR Analysis. RNA extraction from frozen tissue samples and cell lines was performed with guanidinium thiocyanate/CsCl gradient method (21) and TRIzol reagent (Life Technologies, Inc., Basel, Switzerland), respectively. cDNA synthesis was performed as described previously (21), and aliquots (equivalent to 100 ng of RNA) were used for different PCR using a Qiagen HotStar Taq polymerase Master Kit (Basel, Switzerland). SSX-2 was amplified as described (22). Actin was amplified on each cDNA sample to assess the quality and quantity of input RNA.

Peptide Stimulation and Isolation of SSX-2_{41-49}-specific $CD8^+$ T Cells. For peptide stimulation experiments, $CD8^+$ lymphocytes were positively selected by magnetic cell sorting from PBMCs of HLA-A2 melanoma patients or healthy individuals using a miniMACS device (Miltenyi Biotec, Sunnyvale, CA). Cells from the $CD8^-$ fraction were irradiated (3000 rad) and used as APC. $CD8^+$ lymphocytes ($0.5–1 \times 10^6$/well) were stimulated with peptide (1 μM) in the presence of irradiated autologous APC in 2 ml of CTL medium containing 10 units/ml rIL-2 and 10 ng/ml rIL-7. Cells were restimulated at day 10 with T2 cells that were preincubated with peptide (1 μM), washed three times, and irradiated (10,000 rad). SSX-2-specific $CD8^+$ T cells were isolated by multimer-guided cell sorting from short-term cultured TILN or peptide-stimulated PBMCs and cultured either directly or after limiting dilution in the presence of irradiated allogeneic PBMC, 1 μg/ml phytohemagglutinin, and 150 units/ml rIL-2 to obtain polyclonal monospecific CTL lines or CTL clones, respectively. Clones were derived from wells with a probability of clonality $> 90\%$ according to single-hit Poisson distribution.

Tetramer Staining and Flow Cytometry Immunofluorescence Analysis. A2/SSX-2_{41-49} multimeric complexes were synthesized as described (23). Cells were stained with PE-labeled multimers in 20 μl of PBS, 5% FCS during 20 min at 25°C, and then 20 μl of a 1/25 dilution of the following mAbs (anti-CD8, -CD45RA, -CCR7, -CD27, or -CD28; BD PharMingen) were added where indicated and incubated for an additional 30 min at 4°C. Where indicated, antiperforin mAb (BD PharMingen) was added after cells were fixed and permeabilized using saponine (Sigma). Cells were washed once, analyzed, and/or sorted by flow cytometry (FACScan or FACSVantage SE; BD Biosciences, San Jose, CA). Data analysis was performed using Cell Quest software.

Chromium Release Assay. Antigen recognition was assessed using target cells labeled with ^{51}Cr for 1 h at 37°C and washed three times. Labeled target cells (1000 cells in 50 μl) were then added to effector cells (100 μl) at the indicated effector:target cell ratios in V-bottomed microwell plates in the presence or absence of antigenic peptide (50 μl) at the indicated concentrations. Chromium release was measured in supernatant harvested after 4-h incubation at 37°C. The percentage of specific lysis was calculated as: $100 \times$ [(experimental − spontaneous release)/(total − spontaneous release)].

Stimulation and Detection of Cytokine Production. PBMCs were stained with PE-labeled multimers as described above, washed, and then stimulated during 4 h with T2 cells in the absence or presence of 10 μg/ml peptide SSX-2_{41-49}. Brefeldin A (Sigma Chemical Co., Steinheim, Germany; 20 μg/ml final) was added 1 h after the beginning of the stimulation to inhibit cytokine secretion. At the end of the incubation, cells were stained with cell surface mAbs for 20 min at 4°C, washed once, and fixed. Cells were then permeabilized (using saponine, 0.1%), stained by incubation with anti-IFN-γ FITC for 30 min at 4°C, washed once, fixed, and analyzed by flow cytometry.

RESULTS

Assessment of SSX-2_{41-49}-specific $CD8^+$ T-Cell Responses in HLA-A2 Melanoma Patients and Healthy Donors. To evaluate the frequency at which SSX-2_{41-49}-specific responses are found among HLA-$A2^+$ individuals, we selected a group of 7 HLA-$A2^+$ melanoma patients that expressed SSX-2 (as assessed by reverse transcription-PCR) in their tumor lesions and/or in melanoma cell lines obtained from some of these lesions (Table 1). The expression of SSX-2 in the tumor samples generally correlated with the expression observed in tumor cell lines derived from the same lesion, with the exception of patient LAU 233, for whom SSX-2 expression was detectable in the melanoma cell line but not in the tumor sample. It is noteworthy that SSX-2 expression levels detected in the cell lines were higher than in the corresponding tumor samples, possibly because of the presence, in tumor samples, of variable proportions of other cell types (most tumor samples were from tumor invaded lymph nodes) and/or because of the variable expression of SSX-2 within the tumor (24). In addition to SSX-2-expressing patients, a series of 10 melanoma patients bearing SSX-2 nonexpressing lesions and 6 healthy donors (all HLA-$A2^+$) were included in the analysis of the SSX-2_{41-49}-specific $CD8^+$ T-cell response.

Fluorescent HLA-A2/SSX-2_{41-49} multimers (19) were used to assess the presence of SSX-2_{41-49}-specific $CD8^+$ T cells in TILs or TILN, cultured during 2 weeks in the presence of cytokines but without the addition of peptide SSX-2_{41-49}, as well as among peptide-stimulated PBMCs. Representative dot plots are shown in Fig. 1, *A* and *B*. Results obtained for all patients and healthy donors are summarized in Table 1. $Multimer^+$ $CD8^+$ T cells were readily detectable in 5 of 7 PBMC samples from SSX-2-expressing patients but only 2 of 10 SSX-2-negative patients and 2 of 6 healthy donors. Importantly, $multimer^+$ cells were also detected among $CD8^+$ T lymphocytes from tumor lesions of 3 of 5 SSX-2-expressing patients.

Isolation and Functional Characterization of SSX-2_{41-49} $Multimer^+$ $CD8^+$ T-Cell Populations. $Multimer^+$ $CD8^+$ T cells were isolated from positive cultures by multimer-guided flow cytometry cell sorting. The sorted populations were analyzed by multimer staining after *in vitro* mitogen-driven stimulation. Representative dot plots for a specific $CD8^+$ T-cell clone derived from TILN and three $CD8^+$ T-cell lines derived from peptide-stimulated PBMCs are shown in Fig. 1, *C* and *D*. Lines used in functional assays contained >75% $multimer^+$ $CD8^+$ T cells.

For each population, the functional avidity of antigen recognition was determined in a standard chromium release assay by assessing the ability of the CTL to specifically lyse HLA-$A2^+$ T2 targets in the presence of serial dilutions of peptide SSX-2_{41-49}. Results obtained for representative populations are shown in Fig. 2*A*. The peptide concentration giving half-maximal lysis (EC_{50}) for all populations tested is given in Table 2. The EC_{50} was variable among different CTL populations ranging from 2 nM to 10 pM. It is noteworthy that, although CTL populations derived from PBMCs exhibited variable functional avidity of antigen recognition, all CTL clones derived from TILN recognized peptide SSX-2_{41-49} with very high functional avidity ($EC_{50} < 200$ pM). The ability of each SSX-2-specific CTL population to specifically recognize antigen-expressing tumor cells was similarly assessed in a chromium release assay using as target cells HLA-$A2^+$ melanoma cell lines Me 275 (SSX-2^+, Table 1; Ref. 19) and SK-MEL-23 (SSX-2^-, data not shown and Ref. 19). As shown in Fig. 2, *B* and *C*, high avidity CTL (*e.g.*, CTL clone LAU 672/B3.4,

Table 1 *Analysis of SSX-2 expression in tumors from melanoma patients and assessment of CD8$^+$ T-cell response against peptide SSX-2_{41-49} in TIL(N) and PBMCs*

	SSX-2 expression[a]		%SSX-2_{41-49} multimer$^+$ CD8$^+$ T cells[b]		
Patient	Sample	RT-PCR	Sample	IVC	*Ex vivo*
LAU 50	Sub. cut. 93	+/−	PBMC	0.01	<0.01
	Me 275*	+++	TILN, 95	0.64	
LAU 156	Paravert., 97	++	PBMC	0.18	<0.01
	Paravert., 99	+/−	TIL	<0.01	
LAU 233	LN, 96	−	PBMC	<0.01	<0.01
	Me 203*	+			
	Me 317.M2*	−			
LAU 321	LN, 98	+	PBMC	0.03	<0.01
			TILN, 98	<0.01	
LAU 343	LN2, 99	++	PBMC	<0.01	<0.01
	LN3, 99	+			
	T343B*	+++			
LAU 567	LN, 01	+/−	PBMC	2.73	1.3
	T567A	++	TILN, 01	0.6	0.56
LAU 672	LN, 02	+	PBMC	0.1	<0.01
			TILN, 02	0.26	0.82
LAU 4	Cut., 94	−	PBMC	<0.01	<0.01
	Cut, 95	−			
LAU 27	Cut, 93	−	PBMC	<0.01	<0.01
LAU 42	LN, 98	−	PBMC	<0.01	<0.01
	T42B*	−			
	Me 331*	−			
LAU 97	LN, 96	−	PBMC	<0.01	<0.01
LAU 142	Primary, 95	−	PBMC	0.01	<0.01
LAU 155	Liver met., 95	−	PBMC	<0.01	<0.01
LAU 165	LN, 96	−	PBMC	<0.01	<0.01
	Me 280.M2*	−			
LAU 169	Sub. cut., 95	−	PBMC	<0.01	<0.01
LAU 270	Cut., 99	−	PBMC	0.01	<0.01
	LN, 99	−			
LAU 465	T465A*	−	PBMC	<0.01	<0.01
			TIL, 00		<0.01
HD 1			PBMC	<0.01	<0.01
HD 2			PBMC	<0.01	<0.01
HD 3			PBMC	<0.01	<0.01
HD 4			PBMC	<0.01	<0.01
HD 5			PBMC	0.01	<0.01
HD 6			PBMC	0.54	<0.01

[a] SSX-2 expression was assessed in fresh tumor samples or tumor cell lines. Semiquantitative assessment of mRNA expression was performed using SK-Mel-37 melanoma cell line as reference. Expression levels were scored as follows: +++, 50–200%; ++, 10–50%; and +, 1–10% of the levels found in the reference cell line. Sub. cut., s.c. metastasis; paravert., paravertebral metastasis; cut., cutaneous metastasis; LN, lymph node. Two digit numbers correspond to the year of surgical resection. * Tumor cell line. All lymph nodes tested are tumor invaded as assessed by RT-PCR expression of melanoma differentiation antigen Melan-A.

[b] SSX-2_{41-49} multimer$^+$ CD8$^+$ T cells were assessed in TIL(N) either *ex vivo* or after short-term (2–4 weeks) culture with cytokines only. PBMCs were analyzed similarly either *ex vivo* or after peptide stimulation using multimers and anti-CD8 mAb. Results correspond to the percentage of multimer$^+$ cells among CD8$^+$ cells. HD, healthy donors. IVC, *in vitro* culture.

CTL line LAU 50) efficiently lysed Me 275 cells both in the presence and absence of exogenously added peptide SSX-2_{41-49} but lysed SK-MEL-23 cells only in the presence of peptide. Similar results were obtained for all high avidity CTL populations (Table 2). In contrast, CTLs exhibiting lower avidity of antigen recognition (EC_{50} in the nanomolar range) were unable to significantly lyse Me 275 or SK-MEL-23 cells in the absence of exogenously added SSX-2_{41-49} (*e.g.*, CTL lines from LAU 321 and HD 6). To further assess tumor recognition by SSX-2-specific CTLs exhibiting different functional avidity of antigen recognition, we tested the capacity of these different

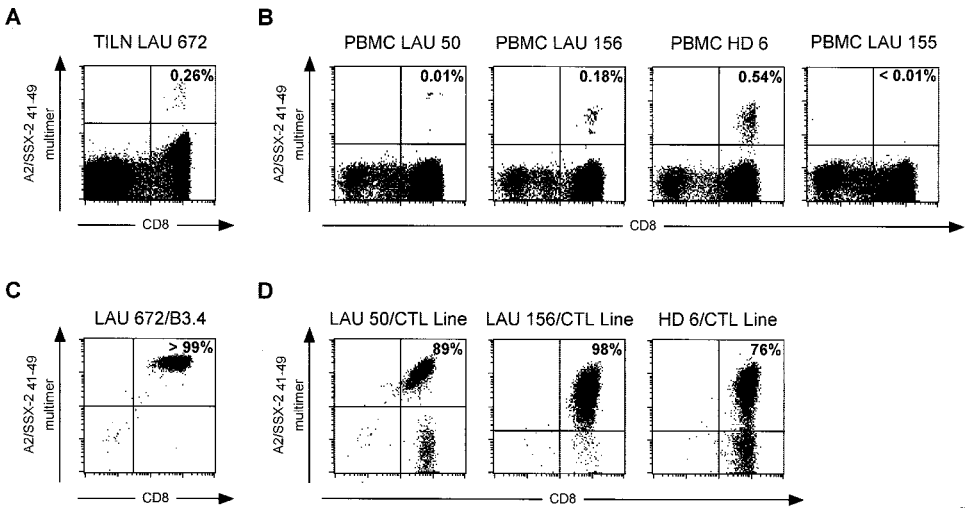

Fig. 1. Enumeration of SSX-2_{41-49}-specific CD8$^+$ T cells and isolation of specific polyclonal monospecific and clonal populations. Cells (short term cultured TILN, peptide-stimulated PBMC, CTL lines, and clones) were stained with PE-labeled SSX-2_{41-49} multimers and anti-CD8^{FITC} and analyzed by flow cytometry. Figures in the *top right quadrants* correspond to the percentage of multimer$^+$ cells among CD8$^+$ cells. *A*, short term cultured TILN from patient LAU 672 expressing SSX-2 in the corresponding lesion (Table 1). *B*, peptide-stimulated PBMC from 2 patients bearing SSX-2-expressing lesions (LAU 50 and LAU 156), one healthy individual (HD 6), and a patient having no SSX-2 expression (LAU 155). *C*, SSX-2_{41-49}-specific clone B3.4 from patient LAU 672. *D*, SSX-2_{41-49}-specific polyclonal CTL lines derived from peptide-stimulated PBMC.

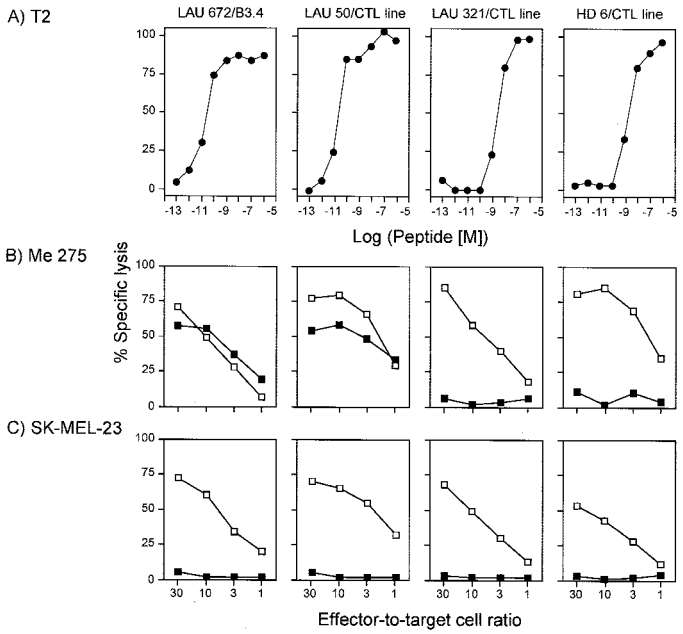

Fig. 2. Functional activity of SSX-2_{41-49}-specific CTL clones and lines obtained from melanoma patients and healthy individuals. Functional avidity of antigen recognition (*A*) and lysis of tumor cell lines (SSX-2^+ $A2^+$ Me 275 in *B* and SSX-2^- $A2^+$ SK-MEL-23 in *C*) were determined in a standard ^{51}Cr release assay. Tumor lysis was determined in the absence (*closed symbols*) or presence (*open symbols*) of peptide SSX-2_{41-49} (0.1 μM). As summarized in Table 2, the CTL clone LAU 672/B3.4 was derived from TILN, whereas the LAU 50, LAU 321, and HD 6 CTL lines were derived from peptide-stimulated PBMC.

CTL populations to recognize three additional SSX-2^+ $A2^+$ melanoma cell lines. Representative results from these experiments are shown in Fig. 3. Consistent with the results obtained using Me 275, only the CTL clone able to recognize peptide SSX-2_{41-49} with high functional avidity was able to efficiently lyse SSX-2-expressing (Table 1 and Ref. 19) HLA-$A2^+$ melanoma cell lines (SK-MEL-37, T343B, and T567A).

Assessment of CD8$^+$ SSX-2_{41-49} Multimer$^+$ T Cells *ex Vivo*. The high frequency of $CD8^+$ SSX-2_{41-49} multimer$^+$ T cells detected among short-term cultured TILN from SSX-2-expressing melanoma patients prompted us to assess the frequency and phenotype of $CD8^+$ SSX-2_{41-49} multimer$^+$ T cells *ex vivo*. TILN samples cryopreserved on the day of surgery were thawed and rested overnight in cell culture medium in the absence of exogenously added cytokines before being stained using HLA-A2/SSX-2_{41-49} multimers in combination with anti-CD8 and anti-CD45RA mAbs. TILN samples from patients LAU 567 and LAU 672, corresponding to the samples assessed previously after *in vitro* culture (Table 1), were tested *ex vivo*. In both cases, $CD8^+$ SSX-2_{41-49} multimer$^+$ T cells exhibiting an antigen experienced phenotype ($CD45RA^-$) were clearly detectable *ex vivo* and at relatively high frequencies among TILN (0.56 and 0.82% of $CD8^+$ T cells for patients LAU 567 and LAU 672, respectively; Table 1). In contrast, these cells were undetectable among TILN of the SSX-2-nonexpressing patient LAU 465. The *ex vivo* frequency of $CD8^+$ SSX-2_{41-49} multimer$^+$ T cells was also assessed in circulating $CD8^+$ lymphocytes from patients and donors (Table 1 and Fig. 4). Interestingly, with the exception of a single case, $CD8^+$ SSX-2_{41-49} multimer$^+$ T cells were below multimer detection limits *ex vivo*. However, in the case of patient LAU 567, $CD45RA^-$ $CD8^+$ SSX-2_{41-49} multimer$^+$ T cells were detectable at high frequency among circulating lymphocytes analyzed at three different time points spanning a time period of 20 months (Fig. 4*A*). The first time point corresponds to the date analyzed previously after *in vitro* stimulation (Table 1) and was derived from a blood sample obtained at the time of surgical removal of the corresponding TILN. The frequency of $CD8^+$ SSX-2_{41-49} multimer$^+$ T cells was increased (~6-fold) at the next time point analyzed and remained stable afterward. At the last time point analyzed, the patient had progressed and presented a bulky duodenal metastasis. After radiological embolization of the local vessels, however, the tumor mass did not progress further. The patient is presently stable and receiving chemotherapy. Interestingly, circulating SSX-2_{41-49}-specific $CD8^+$ T cells from this patient displayed a $CD45RA^-$, $CCR7^-$, $CD27^-$, and $CD28^-$ phenotype; contained high intracellular levels of perforin; and were able to efficiently secrete IFN-γ on stimulation with peptide SSX-2_{41-49} *ex vivo* (Fig. 4, *B* and *C*).

DISCUSSION

SSX-2 belongs to the SSX gene family, including 9 genes (SSX-1 to -9; Refs. 22 and 25) described thus far, 7 of which can be expressed in tumor cells, albeit to very different extents. Among SSX-family members, SSX-1, -2, -4, and -5 are more frequently expressed in tumors of different histological types, although SSX-3, -6, and -7 are expressed only rarely. Importantly, antibodies directed against SSX-1, -2, -3, and -4 gene products have been found in sera of melanoma, colon, and breast cancer patients (25). The tumor-specific expression profile of SSX genes, together with the spontaneous immunogenicity of the corresponding gene products, encourage the use of the latter for generic anticancer immunotherapy. In a recent study, we have found a spontaneous $CD8^+$ T-cell response specific for SSX-2 in a TILN from a melanoma patient bearing a SSX-2-expressing tumor and identified the first SSX-derived $CD8^+$ T-cell epitope corresponding to peptide SSX-2_{41-49} (19, 20). The peptide was recognized by specific CTL in the context of the HLA-A2 allele, one of the HLA-class I alleles most frequently expressed.

In this study, we have analyzed the $CD8^+$ T-cell response against this recently defined epitope in HLA-$A2^+$ melanoma patients and healthy donors. Our results show that SSX-2_{41-49}-specific CTLs can be found relatively frequently in both melanoma patients and healthy donors. These results are similar to those found previously for other highly immunogenic CT antigen-derived epitopes described recently (NY-ESO-1 and Mage-A10; Refs. 13 and 14). The repertoire of $CD8^+$ T cells specific for peptide SSX-2_{41-49} included populations displaying diverse functional avidity of antigen recognition that directly correlated with the capacity of CTL to recognize or not the antigen endogenously expressed by tumor cells (15, 26, 27). High avidity of tumor antigen recognition has been shown to be important for the

Table 2 *Functional activity of CD8$^+$ HLA-A2/SSX-2_{41-49} multimer$^+$ T-cell clones or polyclonal lines obtained from melanoma patients or healthy individuals*

Donor	Source[a]	CTL[b]	EC_{50} (M)[c]	Tumor lysis[d]
LAU 50	PBMC	Line	2×10^{-11}	+
	TILN, 95	Clone D2.5	1×10^{-11}	+
		Clone E3.7	2×10^{-10}	+
LAU 567	TILN, 01	Clone F3.2	4×10^{-11}	+
		Clone B4.6	7×10^{-11}	+
LAU 672	PBMC	Clone 1B7	5×10^{-11}	n.d.
	TILN, 02	Clone E2.4	2×10^{-11}	+
		Clone B3.4	2×10^{-11}	+
LAU 156	PBMC	Line	4×10^{-9}	−
LAU 321	PBMC	Line	3×10^{-9}	−
HD 6	PBMC	Line	2×10^{-9}	−

[a] $CD8^+$ multimer$^+$ T-cell populations were derived either from short-term cultured TILN or peptide-stimulated PBMCs.
[b] $CD8^+$ multimer$^+$ CTLs were grown either as polyclonal lines or clonal populations.
[c] Functional avidity of antigen recognition was measured by peptide titration in a standard ^{51}Cr release assay. EC_{50} corresponds to the peptide concentration leading to half-maximal lysis.
[d] Tumor lysis was considered positive when, at an effector: target cell ratio of 30:1, >50% specific lysis was obtained on the Me 275 SSX-2^+ $A2^+$ melanoma cell line and <10% lysis on the SSX-2^- $A2^+$ SK-MEL-23 melanoma cell line. n.d., not done.

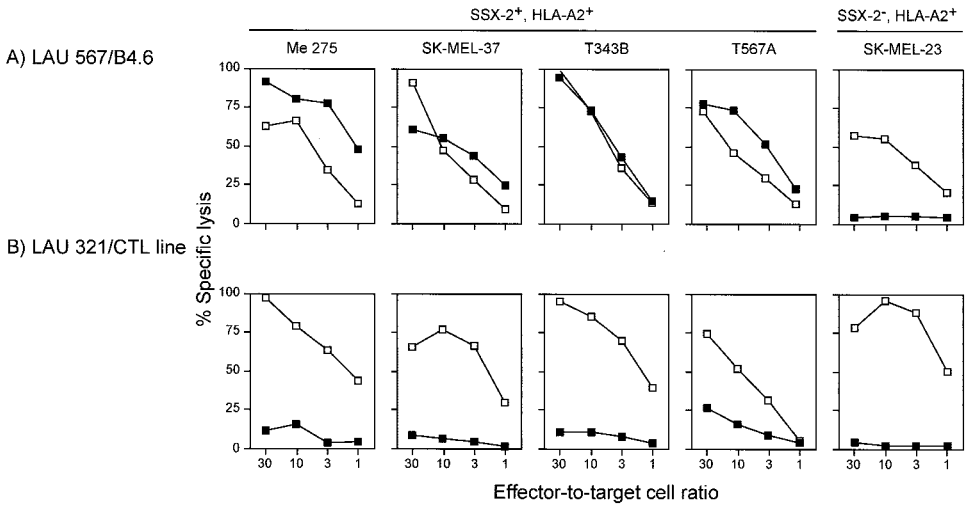

Fig. 3. Tumor lysis by high and low functional avidity SSX-2_{41-49}-specific CTL. Tumor lysis was determined in a standard ^{51}Cr release assay in the absence (*closed symbols*) or presence (*open symbols*) of peptide SSX-2_{41-49} (0.1 μM).

efficacy of antitumor responses *in vivo* (27). Although findings similar to those described here for SSX-2_{41-49} have been documented previously for other epitopes derived from tumor antigens, it is noteworthy that the functional avidity of antigen recognition (defined as the dose of antigenic peptide required to obtain half-maximal lysis in the CTL assay, EC_{50}) necessary to observe efficient recognition of antigen-expressing tumor cells can considerably vary depending on the antigenic system, *e.g.*, although in the case of CTLs specific for the differentiation antigen Melan-A and recognizing Melan-A peptides with a EC_{50} of 1–10 nM exhibited efficient tumor recognition (28), only MAGE-A10-specific CTLs recognizing peptide MAGE-$A10_{254-262}$ with an EC_{50} of $\leq$100 pM were clearly tumor reactive (15). Although these discrepancies could be dependent on possible differences between the synthetic peptide used in the CTL assay and natural antigen expressed by tumor cells, they could also be explained by different levels of antigen present on the surface of tumor cells as the result of different antigen expression levels or different efficiency of antigen processing. In the case of SSX-2_{41-49}, an increase in the EC_{50} of 20-fold resulted in lack of tumor recognition by specific CTL. This observation underlines the importance of designing vaccination protocols to selectively expand CTL of very high functional avidity and possibly directed against several different epitopes to minimize the risk of tumor escape by down-regulation of antigen expression.

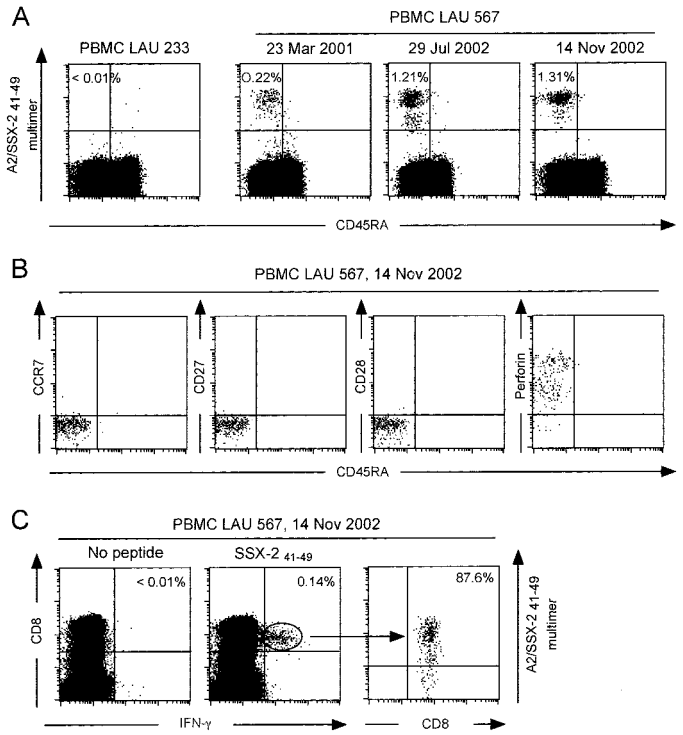

Fig. 4. Assessment of the frequency, phenotype, and functional activity of $CD8^+$ SSX-2_{41-49} multimer$^+$ T cells *ex vivo*. PBMCs were thawed, rested overnight, stained, and analyzed by flow cytometry. In *A*, cells were stained with PE-labeled SSX-2_{41-49} multimers, anti-CD8, and anti-CD45RA mAbs. Dot plots are shown on gated $CD8^+$ cells. Numbers in the *top left quadrants* correspond to the percentage of multimer$^+$ cells among $CD8^+$ cells. In *B*, cells were stained as in *A* and in addition with either mAbs against CCR7, CD27, CD28, or perforin. Dot plots are shown on gated $CD8^+$ multimer$^+$ cells. *C*, PBMCs were stained with multimers, stimulated with T2 cells in the absence or presence of peptide SSX-2_{41-49} for 4 h, and stained with anti-IFN-γ and anti-CD8 mAbs as detailed in "Materials and Methods." Figures in the *top right quadrants* correspond to the percentage of IFN-γ^+ cells among $CD8^+$ cells in the first two panels and to multimer$^+$ cells among IFN-γ^+ cells in the last panel.

An interesting finding of this study was that, although both high and low avidity SSX-2_{41-49} T cells were isolated from circulating lymphocytes from HLA-$A2^+$ individuals and therefore coexist in the T-cell repertoire, we isolated only high avidity T cells from lymphocytes present at the tumor site of patients bearing SSX-2-expressing lesions. This result clearly indicates that high avidity tumor reactive T cells specific for tumor antigens are selectively expanded *in situ* during spontaneous responses to the autologous tumor. Importantly, SSX-2_{41-49}-specific CTLs exhibiting an antigen-experienced phenotype were readily detected *ex vivo* at relatively high frequency ($\sim$1 of 100 $CD8^+$ T cells) in the TILN of two SSX-2-expressing patients. The frequency of HLA-A2/SSX-2_{41-49} multimer$^+$ cells among circulating lymphocytes from the majority of the patients remained below detection limits, as was the case in healthy donors (data not shown). However, in one patient (LAU 567), HLA-A2/SSX-2_{41-49} multimer$^+$ $CD8^+$ cells were clearly detected among circulating lymphocytes. Interestingly, in this patient, the frequency of SSX-2-specific cells increased with disease progression, a finding similar to what described previously in the case of tumor antigen-specific humoral responses (29). It is possible that the high quantity of tumor antigen present in progressing patients may be crucial for optimal cross-priming by professional APCs as recently suggested by Spiotto *et al.* (30).

It is important to underline that, in contrast with the concept suggested previously, that tumor antigen-specific T cells in tumor-bearing patients are anergic (31), and consistent with our previous findings in the case of $CD8^+$ T cells specific for other tumor antigens

(32, 33), SSX-2_{41-49}-specific CD8$^+$ T cells were functional. They exhibited a CD45RA-, CCR7-, CD27-, CD28-, phenotype; contained high intracellular levels of perforin; and produced IFN-γ on brief stimulation with antigen *ex vivo*. It is not completely obvious why, in the presence of a high and functional tumor antigen-specific CD8$^+$ T-cell response, patient LAU 567 presented a rapid tumor progression. Albeit the development of metastasis that have lost antigen expression may be a possible explanation (34), a more likely hypothesis is that, in general, spontaneous tumor antigen-specific CD8$^+$ T-cell responses of high magnitude develop only in the presence of a high tumor load, late in the progression of the disease. It is possible that vaccination of patients bearing tumor antigen-expressing lesions with tumor antigen-derived peptides, such as SSX-2_{41-49}, at earlier stages of the disease, could stimulate a timely response that could favorably impact on the control of their disease by the immune system.

Together, the results of this study encourage the onset of SSX-2_{41-49}-based immunotherapy protocols in cancer patients. In addition, identification of additional SSX-2-derived CD8$^+$ T-cell epitopes recognized by tumor reactive CTL restricted by other HLA-class I molecule is currently being attempted in our laboratory to enlarge the proportion of patients eligible for SSX-2-based cancer immunotherapy.

ACKNOWLEDGMENTS

We thank melanoma patients for their generous participation in this study. We also thank Renate Milesi and Geneviève Metthez for excellent technical assistance. We would like to dedicate this work to Dr. Pascal Batard who performed the flow cytometry cell sorting experiments and very recently tragically disappeared, leaving all of us in deep sorrow.

REFERENCES

1. Old, L. J., and Chen, Y. T. New paths in human cancer serology. J. Exp. Med., *187:* 1163–1167, 1998.
2. Van den Eynde, B. J., and van der Bruggen, P. T cell defined tumor antigens. Curr. Opin. Immunol., *9:* 684–693, 1997.
3. Scanlan, M. J., Gure, A. O., Jungbluth, A. A., Old, L. J., and Chen, Y. T. Cancer/testis antigens: an expanding family of targets for cancer immunotherapy. Immunol. Rev., *188:* 22–32, 2002.
4. Jäger, E., Chen, Y. T., Drijfhout, J. W., Karbach, J., Ringhoffer, M., Jäger, D., Arand, M., Wada, H., Noguchi, Y., Stockert, E., Old, L. J., and Knuth, A. Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. J. Exp. Med., *187:* 265–270, 1998.
5. Wang, R. F. The role of MHC class II-restricted tumor antigens and CD4+ T cells in antitumor immunity. Trends Immunol., *22:* 269–276, 2001.
6. Zeng, G., Touloukian, C. E., Wang, X., Restifo, N. P., Rosenberg, S. A., and Wang, R. F. Identification of CD4+ T cell epitopes from NY-ESO-1 presented by HLA- DR molecules. J. Immunol., *165:* 1153–1159, 2000.
7. Sahin, U., Tureci, O., and Pfreundschuh, M. Serological identification of human tumor antigens. Curr. Opin. Immunol., *9:* 709–716, 1997.
8. Van den Eynde, B. J., and Boon, T. Tumor antigens recognized by T lymphocytes. Int. J. Clin. Lab. Res., *27:* 81–86, 1997.
9. Gure, A. O., Stockert, E., Arden, K. C., Boyer, A. D., Viars, C. S., Scanlan, M. J., Old, L. J., and Chen, Y. T. CT10: a new cancer-testis (CT) antigen homologous to CT7 and the MAGE family, identified by representational-difference analysis. Int. J. Cancer, *85:* 726–732, 2000.
10. Aarnoudse, C. A., van den Doel, P. B., Heemskerk, B., and Schrier, P. I. Interleukin-2-induced, melanoma-specific T cells recognize CAMEL, an unexpected translation product of LAGE-1. Int. J. Cancer, *82:* 442–448, 1999.
11. Huang, L. Q., Brasseur, F., Serrano, A., De Plaen, E., van der Bruggen, P., Boon, T., and Van Pel, A. Cytolytic T lymphocytes recognize an antigen encoded by MAGE-A10 on a human melanoma. J. Immunol., *162:* 6849–6854, 1999.
12. Traversari, C., van der Bruggen, P., Luescher, I. F., Lurquin, C., Chomez, P., Van Pel, A., De Plaen, E., Amar-Costesec, A., and Boon, T. A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E. J. Exp. Med., *176:* 1453–1457, 1992.
13. Valmori, D., Dutoit, V., Liénard, D., Rimoldi, D., Pittet, M., Champagne, P., Ellefsen, U., Sahin, U., Speiser, D., Lejeune, F., Cerottini, J-C., and Romero, P. Naturally occurring HLA-A2 restricted CD8+ T cell response to the cancer testis antigen NY-ESO-1 in melanoma patients. Cancer Res., *60:* 4499–4506, 2000.
14. Valmori, D., Dutoit, V., Rubio-Godoy, V., Chambaz, C., Liénard, D., Guillaume, P., Romero, P., Cerottini, J-C., and Rimoldi, D. Frequent cytolytic T-cell responses to peptide MAGE-$10_{254-262}$ in melanoma. Cancer Res., *61:* 501–512, 2001.
15. Dutoit, V., Rubio-Godoy, V., Dietrich, P. Y., Quiqueres, A. L., Schnuriger, V., Rimoldi, D., Lienard, D., Speiser, D., Guillaume, P., Batard, P., Cerottini, J. C., Romero, P., and Valmori, D. Heterogeneous T-cell response to MAGE-A10(254–262): high avidity- specific cytolytic T lymphocytes show superior antitumor activity. Cancer Res., *61:* 5850–5856, 2001.
16. Clark, J., Rocques, P. J., Crew, A. J., Gill, S., Shipley, J., Chan, A. M., Gusterson, B. A., and Cooper, C. S. Identification of novel genes, SYT and SSX, involved in the t(X;18)(p11.2;q11.2) translocation found in human synovial sarcoma. Nat. Genet., *7:* 502–508, 1994.
17. Crew, A. J., Clark, J., Fisher, C., Gill, S., Grimer, R., Chand, A., Shipley, J., Gusterson, B. A., and Cooper, C. S. Fusion of SYT to two genes, SSX1 and SSX2, encoding proteins with homology to the Kruppel-associated box in human synovial sarcoma. EMBO J., *14:* 2333–2340, 1995.
18. Tureci, O., Sahin, U., Schobert, I., Koslowski, M., Scmitt, H., Schild, H. J., Stenner, F., Seitz, G., Rammensee, H. G., and Pfreundschuh, M. The SSX-2 gene, which is involved in the t(X;18) translocation of synovial sarcomas, codes for the human tumor antigen HOM-MEL-40. Cancer Res., *56:* 4766–4772, 1996.
19. Ayyoub, M., Stevanovic, S., Sahin, U., Guillaume, P., Servis, C., Rimoldi, D., Valmori, D., Romero, P., Cerottini, J. C., Rammensee, H. G., Pfreundschuh, M., Speiser, D., and Levy, F. Proteasome-assisted identification of a SSX-2-derived epitope recognized by tumor-reactive CTL infiltrating metastatic melanoma. J. Immunol., *168:* 1717–1722, 2002.
20. Rubio-Godoy, V., Ayyoub, M., Dutoit, V., Servis, C., Schink, A., Rimoldi, D., Romero, P., Cerottini, J. C., Simon, R., Zhao, Y., Houghten, R. A., Pinilla, C., and Valmori, D. Combinatorial peptide library-based identification of peptide ligands for tumor-reactive cytolytic T lymphocytes of unknown specificity. Eur. J. Immunol., *32:* 2292–2299, 2002.
21. Brasseur, F., Rimoldi, D., Lienard, D., Lethe, B., Carrel, S., Arienti, F., Suter, L., Vanwijck, R., Bourlond, A., Humblet, Y., Vacca, A., Conese, M., Lahaye, T., Degiovanni, G., Deraemaecker, R., Beauduin, M., Sastre, X., Salamon, E., Dreno, B., Jäger, E., Knuth, A., Chevreau, C., Suciu, S., Lachapelle, J. M., Pouillart, P., Parmiani, G., Lejeune, F., Cerottini, J. C., Boon, T., and Marchand, M. Expression of MAGE genes in primary and metastatic cutaneous melanoma. Int. J. Cancer, *63:* 375–380, 1995.
22. Gure, A. O., Tureci, O., Sahin, U., Tsang, S., Scanlan, M. J., Jäger, E., Knuth, A., Pfreundschuh, M., Old, L. J., and Chen, Y. T. SSX: a multigene family with several members transcribed in normal testis and human cancer. Int. J. Cancer, *72:* 965–971, 1997.
23. Altman, J. D., Moss, P. A. H., Goulder, P. J. R., Barouch, D. H., McHeyzer-Williams, M. G., Bell, J. I., McMichael, A. J., and Davis, M. M. Phenotypic analysis of antigen-specific T lymphocytes. Science (Wash. DC), *274:* 94–96, 1996.
24. dos Santos, N. R., Torensma, R., de Vries, T. J., Schreurs, M. W., de Bruijn, D. R., Kater-Baats, E., Ruiter, D. J., Adema, G. J., van Muijen, G. N., van Kessel, A. G., Wang, H. Y., Zhou, J., Zhu, K., Riker, A. I., Marincola, F. M., and Wang, R. F. Heterogeneous expression of the SSX cancer/testis antigens in human melanoma lesions and cell lines. Cancer Res., *60:* 1654–1662, 2000.
25. Gure, A. O., Wei, I. J., Old, L. J., and Chen, Y. T. The SSX gene family: characterization of 9 complete genes. Int. J. Cancer, *101:* 448–453, 2002.
26. Rimoldi, D., Rubio-Godoy, V., Dutoit, V., Lienard, D., Salvi, S., Guillaume, P., Speiser, D., Stockert, E., Spagnoli, G., Servis, C., Cerottini, J. C., Lejeune, F., Romero, P., and Valmori, D. Efficient simultaneous presentation of NY-ESO-1/LAGE-1 primary and nonprimary open reading frame-derived CTL epitopes in melanoma. J. Immunol., *165:* 7253–7261, 2000.
27. Zeh, H. J., III, Perry-Lalley, D., Dudley, M. E., Rosenberg, S. A., and Yang, J. C. High avidity CTLs for two self-antigens demonstrate superior in vitro and in vivo antitumor efficacy. J. Immunol., *162:* 989–994, 1999.
28. Valmori, D., Dutoit, V., Schnuriger, V., Quiquerez, A. L., Pittet, M. J., Guillaume, P., Rubio-Godoy, V., Walker, P. R., Rimoldi, D., Lienard, D., Cerottini, J. C., Romero, P., and Dietrich, P. Y. Vaccination with a Melan-A peptide selects an oligoclonal T cell population with increased functional avidity and tumor reactivity. J. Immunol., *168:* 4231–4240, 2002.
29. Kurashige, T., Noguchi, Y., Saika, T., Ono, T., Nagata, Y., Jungbluth, A., Ritter, G., Chen, Y. T., Stockert, E., Tsushima, T., Kumon, H., Old, L. J., and Nakayama, E. Ny-ESO-1 expression and immunogenicity associated with transitional cell carcinoma: correlation with tumor grade. Cancer Res., *61:* 4671–4674, 2001.
30. Spiotto, M. T., Yu, P., Rowley, D. A., Nishimura, M. I., Meredith, S. C., Gajewski, T. F., Fu, Y. X., and Schreiber, H. Increasing tumor antigen expression overcomes "ignorance" to solid tumors via crosspresentation by bone marrow-derived stromal cells. Immunity, *17:* 737–747, 2002.
31. Lee, P. P., Yee, C., Savage, P. A., Fong, L., Brockstedt, D., Weber, J. S., Johnson, D., Swetter, S., Thompson, J., Greenberg, P. D., Roederer, M., and Davis, M. M. Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. Nat. Med., *5:* 677–685, 1999.
32. Pittet, M. J., Speiser, D. E., Lienard, D., Valmori, D., Guillaume, P., Dutoit, V., Rimoldi, D., Lejeune, F., Cerottini, J. C., and Romero, P. Expansion and functional maturation of human tumor antigen-specific CD8+ T cells after vaccination with antigenic peptide. Clin. Cancer Res., *7:* 796s–803s, 2001.
33. Valmori, D., Scheibenbogen, C., Dutoit, V., Nagorsen, D., Asemissen, A. M., Rubio-Godoy, V., Rimoldi, D., Guillaume, P., Romero, P., Schadendorf, D., Lipp, M., Dietrich, P. Y., Thiel, E., Cerottini, J. C., Lienard, D., and Keilholz, U. Circulating Tumor-reactive CD8(+) T cells in melanoma patients contain a CD45RA(+)CCR7(−) effector subset exerting ex vivo tumor-specific cytolytic activity. Cancer Res., *62:* 1743–1750, 2002.
34. Rosenberg, S. A. Progress in human tumour immunology and immunotherapy. Nature (Lond.), *411:* 380–384, 2001.

Tumor-reactive, SSX-2-specific CD8$^+$ T Cells Are Selectively Expanded during Immune Responses to Antigen-expressing Tumors in Melanoma Patients

Maha Ayyoub, Donata Rimoldi, Philippe Guillaume, et al.

Cancer Res 2003;63:5601-5606.