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

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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-2₁₁–₄⁹, and is recognized by specific CTL in an HLA-A2 restricted fashion. Here, we have used fluorescent HLA-A₂/SSX-₂₁₁–₄⁹ peptide multimeric complexes to analyze the response to SSX-₂₁₁–₄⁹ 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-₂₁₁–₄⁹ 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-₂₁₁–₄⁹ frequently occur in SSX-2-expressing melanomas and suggest that SSX-₂₁₁–₄⁹-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 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 non-tumor 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-₂₁₁–₄⁹. SSX-₂₁₁–₄⁹-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-₂₁₁–₄⁹, we analyzed the relative frequency at which SSX-₂₁₁–₄⁹-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-₂₁₁–₄⁹ multimeric complexes (multimers thereafter), we could clearly detect SSX-₂₁₁–₄⁹-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-₂₁₁–₄⁹ multimer⁺ T-cell populations displayed a wide range of functional avidity of antigen recognition and tumor reactivity. Interestingly, SSX-₂₁₁–₄⁹-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.
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 HMB-45 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-241–49-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 × 10^6/well) were stimulated with peptide (1 μM) in the presence of irradiated allogeneic 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 nonspecific 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-241–49 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 [35]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 μM) 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 × [(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-241–49. 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 Abs 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-241–49-specific CD8+ T-Cell Responses in HLA-A2 Melanoma Patients and Healthy Donors. To evaluate the frequency at which SSX-241–49-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-241–49-specific CD8+ T-cell response.

Fluorescent HLA-A2/SSX-241–49 multimers (19) were used to assess the presence of SSX-241–49-specific CD8+ T cells in TILs or TILN, cultured during 2 weeks in the presence of cytokines but without the addition of peptide SSX-241–49, 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 5 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-241–49 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-241–49. Results obtained for representative populations are shown in Fig. 2. The peptide concentration giving half-maximal lysis (EC50) for all populations tested is given in Table 2. The EC50 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-241–49 with very high functional avidity (EC50 < 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,

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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

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sample</th>
<th>RT-PCR</th>
<th>%SSX-2(_{41-49}) multimer (^+) CD8(^+) T cells</th>
<th>IVC</th>
<th>Ex vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAU 50</td>
<td>Sub. cut. 93</td>
<td>+/-</td>
<td>PBMC</td>
<td>0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LAU 156</td>
<td>Me 275*</td>
<td>+</td>
<td>TILN, 95</td>
<td>0.64</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LAU 233</td>
<td>LN, 96</td>
<td>-</td>
<td>PBMC</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LAU 321</td>
<td>Me 203*</td>
<td>+</td>
<td>PBMC</td>
<td>0.03</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LAU 343</td>
<td>LN, 99</td>
<td>+</td>
<td>PBMC</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LAU 567</td>
<td>LN, 01</td>
<td>+/-</td>
<td>PBMC</td>
<td>2.73</td>
<td>1.3</td>
</tr>
<tr>
<td>LAU 672</td>
<td>LN, 02</td>
<td>+</td>
<td>TILN, 01</td>
<td>0.6</td>
<td>0.56</td>
</tr>
<tr>
<td>LAU 4</td>
<td>Cut., 94</td>
<td>-</td>
<td>PBMC</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LAU 42</td>
<td>LN, 98</td>
<td>+</td>
<td>PBMC</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LAU 97</td>
<td>LN, 96</td>
<td>-</td>
<td>PBMC</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LAU 142</td>
<td>Primary, 95</td>
<td>-</td>
<td>PBMC</td>
<td>0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LAU 155</td>
<td>Liver met., 95</td>
<td>-</td>
<td>PBMC</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LAU 165</td>
<td>LN, 96</td>
<td>-</td>
<td>PBMC</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LAU 169</td>
<td>Me 280.M2*</td>
<td>-</td>
<td>PBMC</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LAU 270</td>
<td>Sub. cut., 95</td>
<td>-</td>
<td>PBMC</td>
<td>0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LAU 465</td>
<td>LN, 99</td>
<td>-</td>
<td>PBMC</td>
<td>0.54</td>
<td>&lt;0.01</td>
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<tr>
<td>HD 1</td>
<td>-</td>
<td>-</td>
<td>PBMC</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HD 2</td>
<td>-</td>
<td>-</td>
<td>PBMC</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
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<tr>
<td>HD 3</td>
<td>-</td>
<td>-</td>
<td>PBMC</td>
<td>&lt;0.01</td>
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<tr>
<td>HD 4</td>
<td>-</td>
<td>-</td>
<td>PBMC</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HD 5</td>
<td>-</td>
<td>-</td>
<td>PBMC</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HD 6</td>
<td>-</td>
<td>-</td>
<td>PBMC</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

\(^a\) 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
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 melanoma cell lines (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 \(\mu\)M). 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.

**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 after 567 and LAU 672, corresponding to the samples assessed previously and at the last 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-\(\gamma\) 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
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 an 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 ≤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.

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 (~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...
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