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# An immunodominant SSX-2–derived epitope recognized by CD4+ T cells in association with HLA-DR

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**Ectopic gene expression in tumors versus normal somatic tissues provides opportunities for the specific immunotargeting of cancer cells. *SSX* gene products are expressed in tumors of different histological types and can be recognized by tumor-reactive CTLs from cancer patients. Here, we report the identification of an SSX-2–derived immunodominant T cell epitope recognized by CD4+ T cells from melanoma patients in association with HLA-DR. The epitope maps to the 37–58 region of the protein, encompassing the sequence of the previously defined HLA-A2–restricted immunodominant epitope SSX-$2_{41–49}$. SSX-$2_{37–58}$–specific CD4+ T cells were detected among circulating lymphocytes from the majority of melanoma patients analyzed and among tumor-infiltrating lymphocytes, but not in healthy donors. Together, our data suggest a dominant role of the 37–58 sequence in the induction of cellular CD4+ T cell responses against SSX antigens and will be instrumental for both the onset and the monitoring of upcoming cancer-vaccine trials using SSX-derived immunogens.**

## Introduction

Antigens encoded by the *SSX* gene family members are emerging as targets of increasing interest for immunotherapy of cancer (1). Expression of the members of this multigene family in cancer cells can occur as the result of at least two distinct mechanisms. The first is through chromosomal translocations involving the X chromosome where the gene family is localized. Indeed, *SSX-1* and *SSX-2* genes were initially described as the fusion partners found in the common t(X;18)(p11.2;q11.2) chromosomal translocation in synovial sarcoma (2, 3). In addition, ectopic *SSX* gene expression can be detected in variable proportions of tumors of other histological types, similarly to expression of other members of the so-called cancer/testis antigen (CTA) group (4), to which the *SSX* gene family belongs. The expression of these genes is developmentally regulated, being mostly restricted to gametogenic cells but silent in adult normal tissues, and possibly occurs in cancer cells as the result of the aberrant activation of a gametogenic protein expression program (5). Similar to some other CTAs, SSX-2 is expressed in a wide variety of tumors (6–9).

The SSX-2 antigen is naturally immunogenic. It was identified as a tumor antigen by SEREX (serological identification of antigens by recombinant expression cloning) analysis of serum from a melanoma patient (10), and specific antibodies were detected in 10% of melanoma patients. Spontaneous immunoresponses to CTAs are highly specific, as they exclusively develop in patients bearing antigen-expressing tumors, but generally appear late in the disease evolution, are of limited magnitude, and often fail to control tumor progression. This supports the concept that the clinical course of the disease, in these patients, could be impacted by active immunotherapy using CTAs as immunogens. In preparation for such trials, we have recently undertaken the identification and characterization of SSX-2–specific T cell responses. We have previously identified the first SSX-2–derived epitope. It is located in the 41–49 region of the SSX-2 protein and is recognized by tumor-reactive CD8+ T lymphocytes in association with the frequently expressed MHC class I allele HLA-A2 (11, 12). More recently, we reported frequent, high-affinity CD8+ T cell responses to this epitope in HLA-A2+ melanoma patients (13). In this study, we have analyzed the CD4+ T cell response to SSX-2, using circulating lymphocytes from a melanoma patient bearing an antigen-expressing tumor, stimulated with antigen-loaded autologous DCs. This allowed the identification of a CD4+ T cell epitope mapping to the 37–58 region of the protein and recognized by specific T cells in association with HLA-DR. Together, the findings described indicate that the 37–58 sequence may play a dominant role in the induction of cellular CD4+ T cell responses to SSX antigens, and its identification might be key to the development of SSX-based vaccination trials.

**Nonstandard abbreviations used:** American Society for Histocompatibility and Immunogenetics (ASHI); cancer/testis antigen (CTA); National Marrow Donor Program (NMDP); recombinant human (rh); tumor-infiltrated lymph nodes (TILN).

**Conflict of interest:** The authors have declared that no conflict of interest exists.

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## Results

*Isolation of SSX-2–specific CD4+ T cells from the circulating lymphocytes of an antigen-expressing melanoma patient.* We have previously detected, in melanoma patient LAU 672 (bearing an SSX-2–expressing tumor), a spontaneous CD8+ T cell response to the HLA-A2–restricted epitope SSX-$2_{41–49}$ (13). With the aim of analyzing a potential CD4+ T cell response to SSX-2 in this patient, we isolated T cells from this patient's PBMCs by magnetic cell sorting and stimulated them in vitro with autologous monocyte-derived DCs loaded with SSX-2 recombinant protein. Two weeks later, cultures were tested

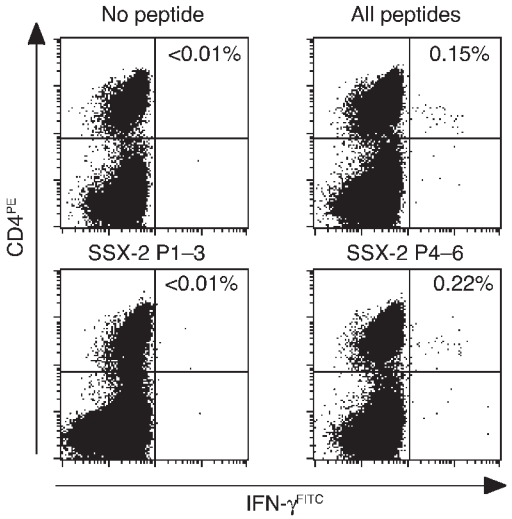



**Figure 1**

Detection of SSX-2–specific CD4+ T cells among circulating T lymphocytes from patient LAU 672 after stimulation with SSX-2–loaded autologous DCs. The presence of specific CD4+ T cells in the culture from patient LAU 672 was assessed by staining with phycoerythrin-labeled anti-CD4 mAB and intracellular staining with anti–IFN-γ mAb after incubation in the absence of added peptide or after stimulation with a pool containing all overlapping peptides spanning the SSX-2 protein, or subpools containing three peptides each, as indicated. Numbers in the upper right quadrants are the percentage of cytokine-producing cells among CD4+ T cells. Results for all subpools are summarized in Table 1. PE, phycoerythrin.

using a pool containing SSX-2 overlapping peptides spanning the SSX-2 protein sequence (11), or subpools containing three peptides each. The presence of specific CD4+ T cells was assessed by intracellular staining with IFN-γ and CD4-specific mAb's (Figure 1 and Table 1). One peptide pool (P4–6, containing peptides SSX-$2_{37–58}$, SSX-$2_{49–70}$, and SSX-$2_{61–82}$) stimulated a significant proportion of IFN-γ–secreting CD4+ T cells, as compared with samples containing either no peptide or other peptide mixtures (Figure 1 and Table 1). It is noteworthy that the proportion of IFN-γ–secreting CD4+ T cells in the P4–6–stimulated culture was equivalent to that obtained upon stimulation with the peptide mix containing all overlapping peptides (Figure 1). SSX-2–specific CD4+ T cells were isolated from the culture by magnetic cell sorting guided by cytokine secretion and were expanded as bulk populations or cloned under limiting-dilution conditions. The obtained CD4+ T cell populations were used to further define the epitope. Reactivity to single peptides in the subpool P4–6 was assessed using these populations. As shown in Figure 2A for one representative clone, this analysis revealed that SSX-$2_{37–58}$ was the active peptide, whereas no significant activity was detected in response to peptides SSX-$2_{49–70}$ and SSX-$2_{61–82}$.

*SSX-$2_{37–58}$ is recognized by specific CD4+ T cells from patient LAU 672 in the context of HLA-DR11.* To identify the MHC class II restricting element used by SSX-$2_{37–58}$–specific CD4+ T cells from patient LAU 672, peptide-presentation experiments were performed in the presence of antibodies that specifically block the recognition of antigens restricted by different MHC class II elements (HLA-DR, -DP, or -DQ). Anti–HLA-DR antibodies almost completely inhibited peptide recognition by T cells. In contrast, only partial inhibition was observed using anti–HLA-DP or anti–HLA-DQ antibodies (Figure 2B), which was considered nonspecific, as it was similarly observed when using an MHC class I–restricted CD8+ T cell clone (not shown). To establish the presenting allele(s), we first determined the HLA-DR alleles of the patient. Patient LAU 672 expressed *HLA-DRB1*0301* and *DRB1*1101*. We then assessed presentation by partially matched APCs from other melanoma patients (Table 2). In the case of two patients expressing *DRB1*1101* but not *HLA-DRB1*0301*, we obtained efficient presentation of the peptide to specific CD4+ T cells from patient LAU 672, whereas in the case of two patients expressing *HLA-DRB1*0301* but not *DRB1*1101*, no presentation was observed. This suggests that *DRB1*1101* is the presenting molecule in the case of patient LAU 672. However, we cannot exclude that other DRB gene products, in addition to DRB1, can also present the identified epitope to specific CD4+ T cells. We then tested the presenting capacity of PBMCs from a series of 20 healthy donors. We obtained presentation for eight of them. To gain insight into the nature of the restricting alleles, the presenting donors were molecularly typed. Five of the donors shared the *DRB1*1101* allele with patient LAU 672 (Table 2). The remaining three expressed the *DRB1*1104* allele, which differs from *DRB1*1101* at a single amino acid at position 86 of the molecule's β chain. To further assess antigen recognition in the context of *DRB1*1101* and *DRB1*1104* alleles, we used homozygous EBV-transformed cells pulsed with serial peptide dilutions as stimulators in IFN-γ secretion experiments. As illustrated in Figure 2C, both JBUSH (*DRB1*1101* homozygous) and JO528239 (*DRB1*1104* homozygous) were able to present peptide SSX-$2_{37–58}$ to specific CD4+ T cells. Peptide recognition, however, was slightly more efficient in association with the patient's autologous allele. In contrast, no recognition was detected with COX (*DRB1*0301* homozygous) used as an internal control.

*Identification of the optimal peptide sequence recognized by SSX-2–specific DRB1*1101-restricted CD4+ T cells and assessment of recognition of homologous regions in other SSX family members.* To more precisely define the SSX-2–derived peptide optimally recognized by DRB1*1101-restricted specific CD4+ T cells from patient LAU 672, we analyzed the capacity of SSX-$2_{37–58}$ relative to that of truncated peptide variants to stimulate IFN-γ secretion by specific T cells, in a peptide-titration assay. As illustrated in Figure 3, A and B, truncation of the first eight amino acids at the N-terminus did not significantly affect recognition, as peptide 45–58 was recognized similarly to 37–58. Further truncation of the peptide N-terminus resulted in decreased peptide recognition. Truncation of the first two C-terminal amino acids

**Table 1**

Assessment of SSX-2–specific CD4+ T cells among circulating lymphocytes from patient LAU 672

| Peptides | % CD4+ IFN-γ+ |
|---|---|
| None | <0.01 |
| All peptides | 0.15 |
| SSX-2 P1–3: 1–22, 13–34, 25–46 | <0.01 |
| SSX-2 P4–6: 37–58, 49–70, 61–82 | 0.22 |
| SSX-2 P7–9: 73–94, 87–105, 97–118 | <0.01 |
| SSX-2 P10–12: 109–130, 121–142, 133–154 | <0.01 |
| SSX-2 P13–15: 145–166, 157–178, 169–188 | <0.01 |

The presence of specific CD4+ T cells among circulating T lymphocytes from patient LAU 672 after stimulation with SSX-2–loaded autologous DCs was assessed as described in Figure 1. Significant values are underlined. Values were considered significant when the percentage of CD4+ IFN-γ–secreting cells in the peptide-stimulating culture was threefold higher than that in the unstimulated culture.

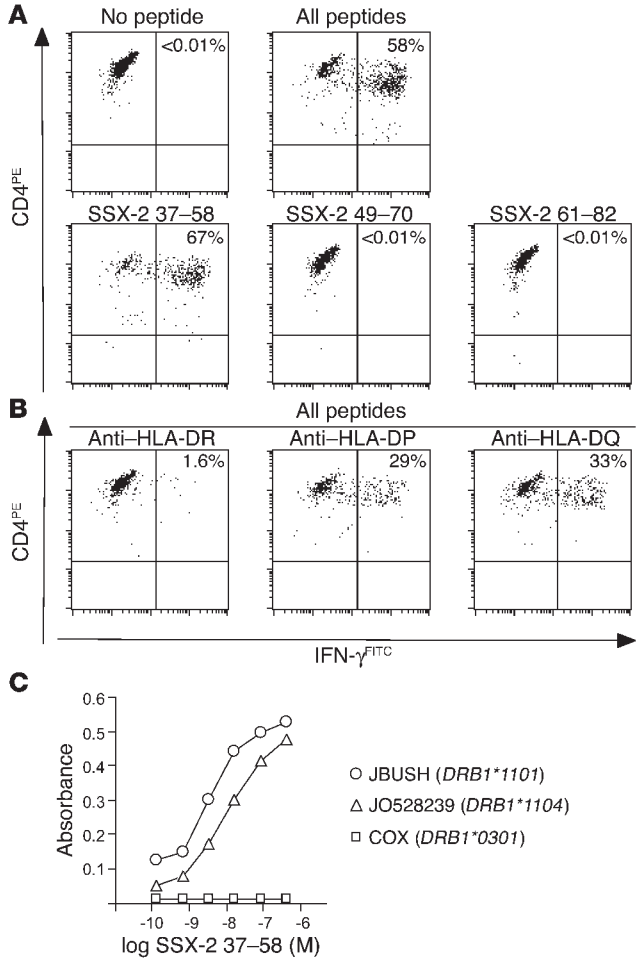


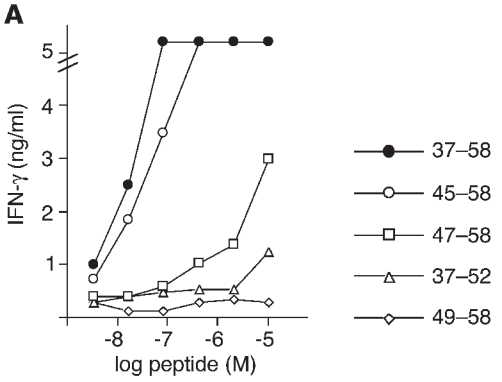

**Figure 2**
Identification of the active peptide and recognition in the context of HLA-DR. (**A**) Intracellular IFN-γ secretion by SSX-2–specific CD4+ T cells (clone 1B2) was assessed upon stimulation with a pool containing all overlapping peptides spanning the SSX-2 protein or single peptides in the active P4–6 subpool. (**B**) Peptide recognition was assessed either in the absence or in the presence of anti–HLA-DR, -DP, or -DQ antibodies. (**C**) The ability of homozygous EBV-transformed cell lines pulsed with serial dilutions of peptide SSX-$2_{37–58}$ to stimulate specific CD4+ T cells was assessed by ELISA measurement of IFN-γ secretion in the culture supernatant.

already resulted in a significant reduction of peptide activity, which was completely lost after removal of four additional amino acids. This analysis thus allowed the definition of the optimal epitope recognized by DRB1*1101-restricted CD4+ T cells. Peptide SSX-$2_{45–59}$ (which was recognized by specific CD4+ T cells similarly to SSX-$2_{45–58}$; Figure 3D) corresponds to the peptide sequence that, in this region of the protein, is predicted to optimally bind to DRB1*1101 using the prediction program developed by H.-G. Rammensee and colleagues (ref. 14; access via http://www.syfpeithi.de) (Figure 3C). Taking into account the overall SSX-2 sequence as per the presence of peptides with high DRB1*1101-binding potential, SSX-$2_{45–59}$ ranks second after SSX-$2_{22–36}$. As a high degree of homology exists between SSX-2 and other SSX family members, it is possible that some of the homologous peptides in the sequence of other SSX proteins also contain DRB1*1101-restricted epitopes. In support of this, SSX-$5_{45–59}$ ranks second among all SSX-5 15-mers for binding to DRB1*1101, and SSX-$1_{45–59}$ ranks third (Figure 3C). Also in line with this hypothesis, we obtained efficient cross-recognition of the SSX-5 homologous peptide using SSX-2–specific CD4+ T cells, whereas the SSX-1 homologous peptide was only marginally cross-recognized (Figure 3D). This implies that the 45–59 epitope could also be relevant to the analysis of SSX-5–induced responses.

*Assessment of SSX-$2_{37–58}$–specific CD4+ T cells in melanoma patients and healthy donors.* To get further insight into the spontaneous immunogenicity of the identified epitope, we assessed the responsiveness to SSX-$2_{37–58}$ using PBMCs from 18 additional melanoma patients as well as from the eight previously identified healthy donors expressing DR11 (Table 2). After two cycles of in vitro stimulation with peptide SSX-$2_{37–58}$, specific CD4+ T cells were detectable in 11 of 19 patients but in none of the healthy donors (Table 3 and Figure 4A). Responder patients included 9 of 11 patients expressing DR11 and, importantly, also two of eight DR11-negative patients. Both of these latter patients expressed HLA-DR3 and HLA-DR7, suggesting that SSX-$2_{37–58}$ could also contain an epitope recognized by specific CD4+ T cells from these patients in association with at least one of these frequently expressed alleles. In addition, in both of these patients, SSX-2 expression was detected in the autologous tumor sample and/or tumor cell line. Among the nine DR11-expressing patients with detectable specific responses to SSX-$2_{37–58}$, six had detectable SSX-2 expression in the autologous tumor sample, no tumor sample was available for two of the remaining patients, and for one patient (LAU 53), no SSX-2 expression was detected in the autologous tumor sample. In this sample, however, expression of SSX-5 was clearly detected (not shown). In the case of patient LAU 567 (DRB1*1101-positive and bearing an SSX-2–expressing tumor), who had, among all analyzed patients, the strongest response to SSX-$2_{37–58}$, a tumor-infiltrated lymph node sample was also available for analysis. A single-cell suspension from this lymph node was cultured for 2 weeks in the presence of recombinant human (rh) IL-2 and rhIL-7, but without the addition of SSX-2 antigen. Remarkably, CD4+ T cells specifically producing IFN-γ in response to stimulation with peptide SSX-$2_{37–58}$ were clearly detected in this

**Table 2**
The ability of molecularly typed APCs to present peptide SSX-$2_{37–58}$ to specific, HLA-DR–restricted, CD4+ T cells was assessed by intracellular IFN-γ staining

| APC | *HLA-DRB1* | SSX-$2_{37–58}$ presentation |
|---|---|---|
| LAU 672 | *0301; *1101 | + |
| LAU 567 | *1101; *1501 | + |
| LAU 14 | *1101; *X | + |
| LAU 203 | *0301; *1501 | – |
| LAU 465 | *0301; *0401 | – |
| HD 053 | *0401; *1101 | + |
| HD 1541 | *0901; *1101 | + |
| HD 503 | *1104; *1502 | + |
| HD 5646 | *0301; *1101 | + |
| HD 6504 | *1104; * 1401 | + |
| HD 303 | *1101; *1501 | + |
| HD 680 | *1104; *1505 | + |
| HD 830 | *0301; * 1101 | + |




B

| Truncation variant | | Relative activity |
|---|---|---|
| **WEKMKASEKIFYVYMKRKYEAM** | **37–58** | **1** |
| KMKASEKIFYVYMKRKYEAM | 39–58 | 0.4 |
| KASEKIFYVYMKRKYEAM | 41–58 | 2 |
| SEKIFYVYMKRKYEAM | 43–58 | 0.4 |
| KIFYVYMKRKYEAM | 45–58 | 0.7 |
| IFYVYMKRKYEAM | 46–58 | 0.009 |
| FYVYMKRKYEAM | 47–58 | 0.004 |
| VYMKRKYEAM | 49–58 | <0.001 |
| WEKMKASEKIFYVYMKRKYE | 37–56 | 0.007 |
| WEKMKASEKIFYVYMKRK | 37–54 | 0.007 |
| WEKMKASEKIFYVYMK | 37–52 | <0.001 |
| WEKMKASEKIFYVY | 37–50 | <0.001 |

C

Predicted binding to DRB1*1101

| Peptide | Sequence | Binding score | Ranking |
|---|---|---|---|
| SSX-$2_{45–59}$ | KIFYVYMKRKYEAMT | 24 | 2nd |
| SSX-$1_{45–59}$ | KI**S**YVYMKR**N**Y**K**AMT | 24 | 3rd |
| SSX-$4_{45–59}$ | KI**V**YVYMK**L**N**E**Y**E**MT | 16 | 13th |
| SSX-$5_{45–59}$ | KI**I**YVYMKRKYEAMT | 24 | 2nd |

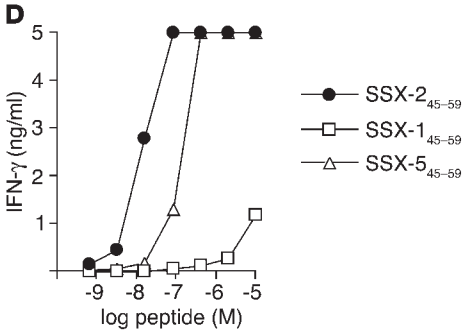



**Figure 3**
Determination of the minimal sequence optimally recognized by SSX-2–specific CD4+ T cells and assessment of cross-recognition of homologous peptides from other SSX antigens. (**A** and **B**) Synthetic peptides truncated at the N- or C- terminus of the SSX-$2_{37–58}$ sequence were used to determine the optimal length of the epitope recognized by SSX-2–specific CD4+ T cells. Peptide activity was calculated relative to that of SSX-$2_{37–58}$ in peptide-titration experiments. (**C**) Binding score and ranking of SSX-$2_{45–59}$–homologous peptides from other SSX antigens were calculated using the SYFPEITHI binding prediction program (http://www.syfpeithi.de). (**D**) Cross-recognition of 45–59 homologous peptides from SSX-1 and -5 by SSX-2–specific CD4+ T cells was assessed in peptide-titration experiments by ELISA measurement of IFN-γ secretion in the culture supernatant.

culture and could be isolated by cytokine-secretion sorting and expanded as polyclonal or monoclonal populations (Figure 4B).

*The natural T cell epitope recognized by SSX-$2_{37–58}$–reactive CD4+ T cells is not presented by antigen-expressing tumor cells but is efficiently processed and presented by professional APCs.* To assess whether the T cell epitope recognized by SSX-$2_{37–58}$–reactive CD4+ T cells was naturally presented on the surface of tumor cells, we selected a tumor cell line from patient LAU 567 (T567A) (13). T567A constitutively expressed detectable levels of HLA-DR molecules at the cell surface. In addition, HLA-DR expression was significantly enhanced after treatment with IFN-γ for 48 hours (Figure 5A). T567A cells, however, were not significantly recognized by SSX-$2_{37–58}$–specific CD4+ T cells from patient LAU 672, irrespectively of IFN-γ treatment, unless the peptide was added exogenously (Figure 5B). Similar results were obtained with autologous clones derived from TILN from patient LAU 567 (not shown). Recognition of the endogenous antigen by a previously derived SSX-$2_{41–49}$–specific HLA-A2–restricted CD8+ T cell clone was observed, as expected, and increased moderately after treatment with IFN-γ (Figure 5B). In contrast, no reactivity was detected using a control CD4+ T cell clone that failed to recognize SSX-$2_{37–58}$ (not shown). Tumor recognition by SSX-$2_{37–58}$–specific CD4+ T cells was further assessed using seven additional DR11-expressing melanoma cell lines, including three that express SSX-2 (Figure 5C). All cell lines were treated with IFN-γ for 48 hours and analyzed, after transfection with an SSX-2–encoding plasmid, or without transfection. As an internal control, recognition was assessed using SSX-$2_{41–49}$–specific CD8+ T cells. As illustrated in Figure 5C, the results of this experiment confirmed those obtained with T567A, namely, the lack of recognition of the endogenous antigen by SSX-2–specific CD4+ T cells. We then assessed the ability of professional APCs to process the SSX-2 antigen and present the epitope to SSX-$2_{37–58}$–reactive CD4+ T cells. As illustrated in Figure 6, autologous DCs from patient LAU 672 were able to present the recombinant SSX-2 protein to specific CD4+ T cells. The latter were also significantly stimulated by DCs loaded with a lysate of the SSX-2–expressing tumor cell line SK-MEL-37. It is noteworthy that SK-MEL-37 cells were not recognized by SSX-$2_{37–58}$–specific T cells. No presentation was obtained using the lysate from an SSX-2–negative tumor cell line (NA8-MEL).

## Discussion

Because tumor-specific CD8+ CTLs are able to recognize and kill tumor cells, resulting, in some instances, in the regression of large tumor masses in vivo, most efforts have, until recently, concentrated on the identification of tumor antigen–derived CD8+ T cell epitopes. Although tumor-reactive CD8+ T cells are, indeed, the main effector arm of the adaptive immune system in response to tumors, it has become increasingly clear that integrated CD8+ and CD4+ T cell responses are essential for efficient immune responses to tumors to occur in vivo (15). Tumor antigen–specific CD4+ T cells play multiple roles in mediating antitumor functions. They can provide help for priming and maintenance of tumor antigen–specific CD8+ T cells (16, 17), activate B cells for production of tumor antigen–specific antibodies that might contribute to anti-

**Table 3**
Assessment of SSX-$2_{37–58}$–specific $CD4^+$ T cells in melanoma patients and healthy donors

| Patient code | DR11 expression | SSX-2 expression in: | | SSX-$2_{37–58}$–specific cells in peptide-stimulated circulating $CD4^+$ T cells | | | |
|---|---|---|---|---|---|---|---|
| | | Tumor sample | Tumor line | IVS 1 | | IVS 2 | |
| | | | | –p | +p | –p | +p |
| LAU 672 | + | LN (+++)[A] | Not avail. | <0.01 | <0.01 | 0.03 | **0.12**[B] |
| LAU 567 | + | LN (+/–) | T567A (++) | 0.03 | **0.12** | 0.04 | **2.40** |
| LAU 321 | + | LN (+) | Not avail. | <0.01 | 0.01 | 0.02 | **0.07** |
| LAU 177 | + | Met (++) | Me 279.1 (+++) | <0.01 | <0.01 | 0.03 | **0.21** |
| LAU 431 | + | LN (+/–) | Not avail. | <0.01 | <0.01 | 0.01 | **0.15** |
| LAU 242 | + | LN (+/–) | Me 324 (–) | <0.01 | <0.01 | 0.01 | **0.27** |
| LAU 348 | + | Not avail. | Not avail. | <0.01 | <0.01 | 0.01 | **0.04** |
| LAU 390 | + | Not avail. | Not avail. | <0.01 | <0.01 | 0.02 | **0.19** |
| LAU 155 | + | Met (–) | Not avail. | <0.01 | <0.01 | 0.01 | 0.02 |
| LAU 165 | + | LN (–) | Me 280 (–) | 0.02 | 0.02 | 0.01 | 0.02 |
| LAU 53 | + | Met (–) | Me 312 (–) | <0.01 | <0.01 | <0.01 | **0.08** |
| LAU 50 | – | Met (+/–) | Me 275 (+++) | 0.01 | 0.01 | <0.01 | 0.01 |
| LAU 156 | – | Met (++) | Not avail. | 0.01 | <0.01 | 0.01 | 0.01 |
| LAU 149 | – | LN (+) | Me 260 (++) | <0.01 | <0.01 | 0.01 | 0.01 |
| LAU 343 | – | LN (++) | T343A (+++) | 0.01 | 0.01 | 0.02 | 0.01 |
| LAU 233 | – | LN (–) | Me 305 (+) | <0.01 | <0.01 | 0.01 | **0.12** |
| LAU 198 | – | LN (–) | T198A (+) | 0.01 | <0.01 | 0.01 | 0.02 |
| LAU 331 | – | LN (++) | T331A (+) | <0.01 | <0.01 | 0.01 | **0.10** |
| LAU 441 | – | Met (+++) | Not avail. | <0.01 | <0.01 | 0.01 | 0.01 |
| **Donor** | | | | **SSX-$2_{37–58}$–specific cells in peptide-stimulated circulating $CD4^+$ T cells** | | | |
| | | | | **IVS 1** | | **IVS 2** | |
| | | | | **–p** | **+p** | **–p** | **+p** |
| HD 053 | | | | <0.01 | <0.01 | <0.01 | <0.01 |
| HD 1541 | | | | <0.01 | <0.01 | <0.01 | <0.01 |
| HD 503 | | | | 0.02 | 0.02 | 0.01 | <0.01 |
| HD 5646 | | | | 0.01 | 0.01 | 0.01 | <0.01 |
| HD 6504 | | | | <0.01 | <0.01 | <0.01 | 0.02 |
| HD 303 | | | | 0.01 | <0.01 | 0.02 | <0.01 |
| HD 680 | | | | 0.01 | 0.01 | <0.01 | 0.01 |
| HD 830 | | | | <0.01 | <0.01 | <0.01 | <0.01 |

IVS, in vitro stimulation. Not avail., not available. LN, lymph node. Met, metastasis. [A]Semiquantitative assessment of the antigen-expression level was based on the intensity of the corresponding RT-PCR product compared with that obtained by amplification of mRNA serial dilutions of a reference tumor cell line (SK-MEL-37). Expression levels were scored as follows: –, not detectable; +/–, <1%; +, 1–10%; ++, 10–50%; +++, 50–200% of the level found in the reference cell line. [B]Significant values are shown in boldface. Values were considered significant when the percentage of $CD4^+$ IFN-γ–secreting cells in the peptide-stimulated culture (+p) was threefold higher than that in the corresponding unstimulated culture (–p).

tumor immunity (18, 19), or exert more direct effects in the effector phase of tumor rejection, either through indirect mechanisms (20–22) or, in some instances, through direct recognition of tumor cells (23, 24). Therefore, because of the importance of incorporating $CD4^+$ T cell epitopes into tumor vaccines, the identification of MHC class II–restricted, tumor antigen–derived sequences recognized by specific $CD4^+$ T cells in association with frequently expressed MHC class II alleles is presently of great interest.

The identification of MHC class II–restricted epitopes recognized by tumor antigen–specific $CD4^+$ T cells, however, has proven more difficult than that of $CD8^+$ T cell epitopes. These difficulties can be mostly attributed to the scarcity, until recently, of effective identification methods combined with low in vivo frequencies of $CD4^+$ T cells (25, 26). Great efforts have been made for the development of such approaches, including the use of MHC class II binding prediction programs (14), elution of MHC class II–bound peptides from tumor cells (27), peptide purification from tumor cell lysates (28), and, in particular, targeting of tumor antigens to the endogenous antigen-presentation pathway (29). As a consequence of these efforts, several tumor antigen–derived $CD4^+$ T cell epitopes have been identified (15).

The analysis of tumor antigen–specific T cell responses that spontaneously arise in cancer patients expressing a given tumor antigen in their lesions is of particular interest, as it allows the identification of T cell epitopes that are physiologically relevant and, additionally, provides the opportunity to analyze the molecular mechanisms leading to such responses in vivo. For this reason, we initially concentrated our analysis on patient LAU 672, who had a spontaneous response to the HLA-A2–restricted $CD8^+$ T cell epitope SSX-$2_{41–49}$ (13). After a single in vitro stimulation with autologous DCs loaded with SSX-2 recombinant protein, we assessed the presence of specific $CD4^+$ T cells among circulating lymphocytes from this patient using a set of 20- to 22-amino-acid-long peptides spanning the SSX-2 protein sequence and overlapping by

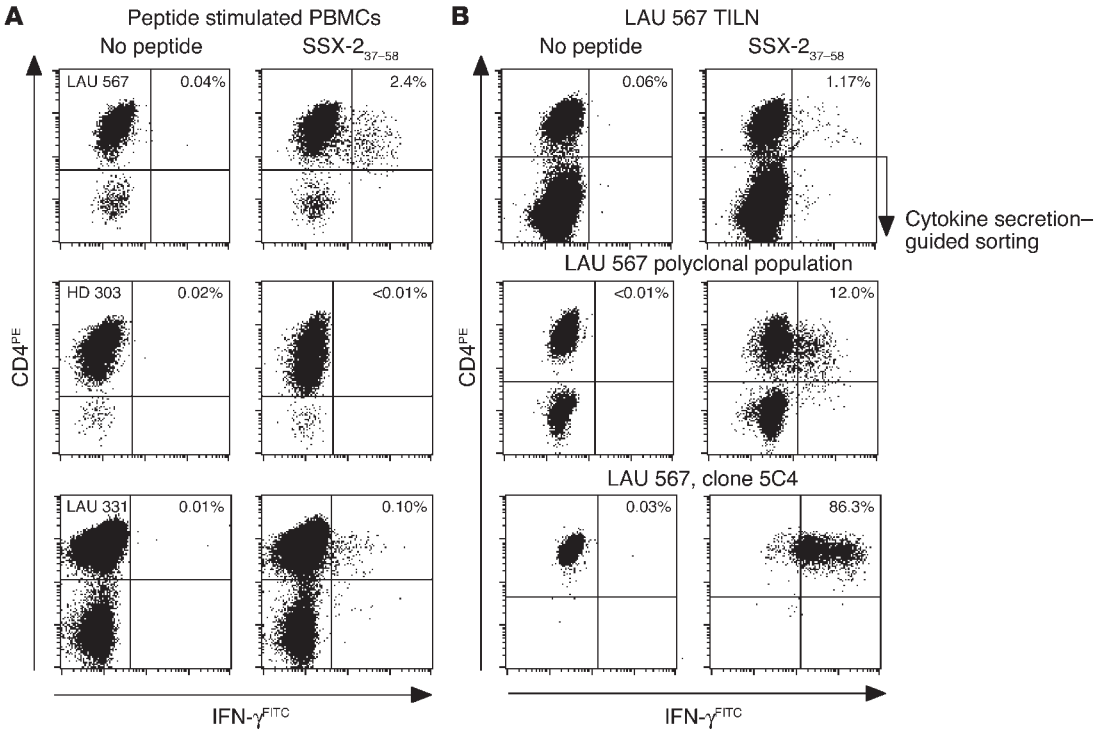



**Figure 4**

Assessment of SSX-$2_{37–58}$–specific CD4+ T cells among circulating lymphocytes and tumor-infiltrated lymph nodes (TILN). (**A**) The presence of specific CD4+ T cells among peptide-stimulated PBMCs was assessed by intracellular staining with anti–IFN-γ antibodies after incubation in the absence or the presence of peptide SSX-$2_{37–58}$. (**B**) SSX-$2_{37–58}$–specific CD4+ T cells were similarly assessed in TILN, enriched by cytokine secretion–guided cell sorting, and expanded in vitro as polyclonal or monoclonal populations. Numbers in the upper right quadrants are the percentage of cytokine-producing cells among CD4+ T cells.

ten amino acids. With this strategy, using synthetic peptides in the test phase instead of using the recombinant SSX-2 protein, we avoided the detection of activities directed against bacterial contaminants present in the recombinant protein preparation, a problem previously encountered by others (30). From the active culture, which specifically reacted to SSX-$2_{37–58}$, we isolated specific CD4+ T cells using a cytokine secretion–based cell sorting procedure (31) and used specific monoclonal populations to further characterize the identified epitope.

As revealed by the analysis of truncated peptides in the 37–58 region of SSX-2, the peptide corresponding to the optimal epitope recognized by the isolated SSX-2–specific CD4+ T cells is located between SSX-2 residues 45 and 58, a region that partially overlaps the previously defined HLA-A2–restricted immunodominant CD8+ T cell epitope (SSX-$2_{41–49}$; ref. 11). Remarkably, SSX-$2_{37–58}$ also appeared to be an immunodominant epitope for CD4+ T cells, as we could detect specific responses in 11 of 19 melanoma patients analyzed (58%). This situation is reminiscent of recent findings with another CTA, NY-ESO-1, in which immunodominant CD8+ and CD4+ T cell epitopes also overlap (32, 33). Recognition of the epitope located in the 37–58 region of SSX-2 by specific CD4+ T cells was HLA-DR restricted. Analysis of the molecular typing of both melanoma patients and healthy donors, in relation to their capacity to present the epitope to specific CD4+ T cells, initially brought us to the conclusion that recognition of the identified epitope was restricted by DR11. The prevalence of DR11 in different populations is in the 15–20% range (34). We observed efficient recognition of SSX-$2_{37–58}$ by DR11-restricted CD4+ T cells in association with *DRB1*1101* and *DRB1*1104*. These two alleles differ only at a single amino acid position (β86) located in a pocket of the molecule that is critical for peptide binding (35, 36). β86 is G in *DRB1*1101* and V in *DRB1*1104*. The V/G β86 dimorphism is highly conserved, occurs in most HLA-DR alleles, and influences both peptide binding and recognition. In the case of the *DRB1*1101* and *DRB1*1104* alleles, in particular, the impact of V/G β86 dimorphism on peptide binding appears to be limited, but the antigenic complex formed by a given peptide with each allele has been shown to be conformationally distinguishable by specific CD4+ T cells (37, 38).

We detected SSX-$2_{37–58}$–specific CD4+ T cells in two DR11-negative patients. Both patients expressed DR3 and DR7, indicating that the peptide could also be recognized by specific CD4+ T cells in association with one of these frequently expressed MHC class II molecules, further increasing the significance of this peptide. It is noteworthy that, using the SYFPEITHI prediction program (http://www.syfpeithi.de), we obtained relatively low binding scores for SSX-$2_{45–59}$ to HLA-DR alleles other than *DRB1*1101*, including *DRB1*0101*, *DRB1*0301*, *DRB1*0401*, *DRB1*0701*, and *DRB1*1501*, which made it unlikely that the minimal DR11-binding peptide would exhibit a "promiscuous" binding to HLA-DR. However, for some other partially overlapping peptides we obtained relatively high ranking and binding scores. For example, among all SSX-2–derived 15-mers, peptide SSX-$2_{37–51}$ ranks third and fourth for binding HLA-DRB1*0301 and HLA-DRB1*0701, respectively. Further analysis is warranted to characterize additional epitopes in this

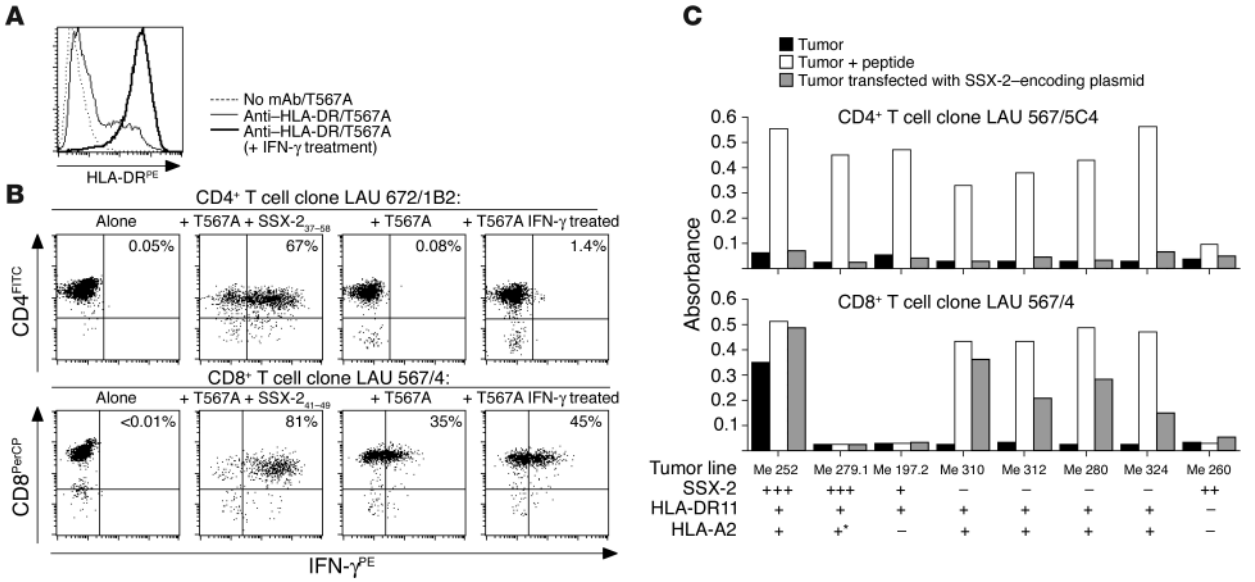



**Figure 5**

SSX-2–specific CD4+ T cells do not directly recognize SSX-2–expressing tumor cells. (**A**) Surface expression of HLA-DR on the melanoma cell line T567A was assessed using HLA-DR–specific mAb. (**B**) Recognition of T567A by SSX-2–specific CD4+ T cells was assessed by intracellular staining with anti–IFN-γ antibodies, in the absence or the presence of exogenously added peptide. Where indicated, cells were treated with IFN-γ (200 IU/ml) for 48 hours. Recognition was similarly assessed using a CD8+ T cell clone specific for the previously described HLA-A2–restricted CD8+ T cell epitope SSX-$2_{41–49}$. PerCP, peridinin chlorophyll protein. (**C**) Recognition of melanoma cell lines by SSX-2–specific CD4+ or CD8+ T cells, in the absence or the presence of exogenously added peptide, as indicated, was assessed by ELISA measurement of IFN-γ secretion in the culture supernatant. Prior to the test, tumor cells were treated with IFN-γ for 48 hours. Where indicated, tumor cells were transfected with a plasmid encoding full-length SSX-2. *The Me 279.1 tumor cell line had no surface expression of MHC class I molecules as assessed by staining with anti–HLA-ABC mAb.

region of the protein. Importantly, as a high degree of homology exists between SSX-2 and other SSX family members, it is possible that the homologous peptides in the sequence of other SSX proteins can also bind to and be recognized in association with one or more DRB1 alleles. In support of this hypothesis is our finding that SSX-2–specific DR11-restricted CD4+ T cells efficiently cross-recognize SSX-$5_{45–59}$. Thus, it is likely that both the presence of binding motifs specific for different DRB1 alleles and the cross-reactivity with other SSX family members contribute to the immunodominant character of this sequence, making it particularly attractive for the development of SSX-based cancer vaccines.

In support of the physiological relevance of the identified epitope, SSX-$2_{37–58}$–specific CD4+ T cells were isolated from TILN from patient LAU 567 that had been cultured in medium containing cytokines but in the absence of any added SSX-2 antigen. This demonstrates that the SSX-$2_{37–58}$–specific CD4+ T cells detected in this patient had spontaneously developed in response to the SSX-2 antigen present in the autologous tumor. It is noteworthy that SSX-$2_{37–58}$–reactive CD4+ T cells failed to recognize SSX-2–expressing tumor cells. In contrast, they efficiently recognized the native antigen, given in the form of recombinant protein or lysate from antigen-expressing tumor cells, upon processing and presentation by DCs. This indicates that processing and presentation of tumor-derived SSX-2 antigen by autologous professional APCs, through the exogenous pathway, was the mechanism through which spontaneous responses to SSX-$2_{37–58}$ occurred in vivo. CD4+ T cells that recognize other CTA-derived epitopes have been previously identified (32, 39). Interestingly, in the case of another CTA (MAGE-A3), both CD4+ T cell epitopes that are and that are not presented by antigen-expressing tumors have been described (30, 39, 40). As direct recognition of tumor cells is only one and, most likely, not the dominant mechanism through which tumor antigen–specific CD4+ T cells contribute to tumor rejection, the lack of recognition of endogenous SSX-2 antigen by SSX-$2_{37–58}$–specific CD4+ T cells does not speak against a major potential role of this epitope in the tumoricidal immune response to antigen-expressing cancer cells. On the other hand, the identification of an SSX-2–derived CD4+ T cell epitope that is not expressed by tumor cells does not necessarily imply that other epitopes from this antigen are not presented through the endogenous MHC class II presentation pathway.

In conclusion, we report the identification of an immunodominant CD4+ T cell epitope from the CTA SSX-2 that is presented to specific CD4+ T cells by frequently expressed HLA-DR alleles and is the target of frequent spontaneous CD4+ T cell responses in melanoma patients. Together, our data suggest a dominant role of the 37–58 region of SSX antigens in the induction of specific CD4+ T cell responses in cancer patients bearing antigen-expressing tumors. This finding is likely to contribute significantly to the development of SSX-based vaccine trials.

## Methods

*Cells and tissue culture*. Peripheral blood was obtained from healthy donors (New York City Blood Bank, New York, New York, USA) and melanoma patients (Lausanne University Hospital, Lausanne, Switzerland) upon informed consent. Homozygous EBV-transformed cell lines were obtained from the National Marrow Donor Program/American Society for Histocompatibility and Immunogenetics (NMDP/ASHI) Cell Repository (access via http://www.ashi-hla.org). The NMDP/ASHI Cell Repository is supported by funding from the Office of Naval Research and the ASHI. Tumor

and EBV-transformed cell lines were maintained in RPMI1640 and Iscove's Modified Dulbecco's Medium (IMDM) (GIBCO Invitrogen Corp., Rockville, Maryland, USA), respectively, supplemented with 10% heat-inactivated FCS. Culture medium for lymphocytes was IMDM supplemented with 8% heat-inactivated pooled human serum (CTL medium), rhIL-2 (GlaxoSmithKline, Geneva, Switzerland), and rhIL-7 (R&D Systems Inc., Minneapolis, Minnesota, USA).

*Analysis of SSX gene expression*. Tissue samples and tumor cell lines were processed using an FP120 FastPrep Cell Disrupter (Savant Instruments Inc., Holbrook, New York, USA). Total cellular RNA was prepared from frozen tissue specimens or tumor cell lines using NucleoSpin RNA II extraction kit (Macherey-Nagel GmbH, Düren, Germany). cDNA synthesis was performed using Promega Reverse Transcription System A3500 (Promega Corp., Madison, Wisconsin, USA). Integrity of cDNA was tested by amplification of β-actin in a 35-cycle PCR reaction. mRNA expression of SSX genes in tumor-tissue samples or tumor cell lines was assessed using previously described oligonucleotide primers and conditions (1).

*Generation of DCs, recombinant proteins, tumor lysates, and antigen loading*. Monocyte-derived DCs were prepared from $CD14^+$ monocytes isolated from PBMCs by magnetic cell sorting using MiniMACS (Miltenyi Biotec Inc., Sunnyvale, California, USA). Highly enriched $CD14^+$ cells (consisting of more than 95% $CD14^+$ cells) were cultured in CTL medium containing 1,000 U/ml of rhGM-CSF and 1,000 U/ml of rhIL-4 (R&D Systems Inc.) for 6 days. At the end of this period, the culture contained greater than 90% $HLA\text{-}DR^+$ $CD83^+$ cells. SSX-2 and NY-ESO-1 proteins were expressed in *Escherichia coli* as full-length with a six-histidine tag at the amino-terminus (41). The proteins were purified from solubilized inclusion bodies by nickel chelate affinity chromatography (Chelating Sepharose FF; Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) using a pH gradient and eluted in 8 M urea, 100 mM phosphate, and 10 mM Tris at pH 4.5. The purified proteins were reactive with anti–SSX-2 and anti–NY-ESO-1 mAb's by Western blot analysis; purity was greater than 80% by SDS-PAGE. Tumor cells ($2 \times 10^5$) were lysed in 200 μl of complete RPMI by ten cycles of rapid freezing and thawing. Where indicated, DCs were incubated with proteins or lysates for 12 hours and washed prior to their use in stimulation or antigen-recognition assay.

*Generation of SSX-2–specific $CD4^+$ T cells*. In vitro stimulation of SSX-2–specific T cells was carried out as described previously (33). Briefly, $1 \times 10^6$ to $2 \times 10^6$ $CD3^+$ T cells, highly enriched (>90%) from PBMCs by magnetic cell sorting using a MiniMACS device (Miltenyi Biotec Inc.), were stimulated with autologous DCs previously incubated with the SSX-2 protein (5 μg/ml) for 12 hours. Two weeks later the culture was tested using a set of partially overlapping peptides spanning the entire SSX-2 protein sequence (2 μM each) (11). For peptide-stimulation experiments, $CD4^+$ T cells were similarly enriched from PBMCs using MiniMACS and stimulated in vitro with peptide $SSX\text{-}2_{37\text{–}58}$ (10 μM) and irradiated cells (30 Gy) from the $CD4^-$ fraction as a source of autologous APCs. $CD4^+$ T cells secreting cytokines in response to peptide stimulation were isolated by cytokine-guided magnetic cell sorting using a cytokine-secretion detection kit (Miltenyi Biotec Inc.) and cloned by limiting-dilution culture in the presence of phytohemagglutinin (Sigma-Aldrich, St. Louis, Missouri, USA), allogeneic irradiated PBMCs, and rhIL-2 as described (33). Clones were subsequently expanded by periodic stimulation (every 3–4 weeks) under the same conditions.

*Antigen-recognition assays*. For detection of intracellular cytokine secretion, T cells were stimulated in the absence or the presence of peptides at the described dose for 4–6 hours. Where indicated, APCs preincubated or not preincubated with antigen were extensively washed and added at a 1:1 T cell/APC ratio. One hour after the beginning of the incubation, brefeldin A (20 μg/ml; Sigma-Aldrich, Steinheim, Germany) was added to inhibit cytokine secretion. At the end of the incubation period, cells were stained with anti-CD4 and/or anti-CD8 mAb (BD Biosciences Pharmingen, San Diego, California, USA) for 20 minutes at 4°C and fixed using formaldehyde. Cells were then permeabilized using saponin (Sigma-Aldrich, St. Louis, Missouri, USA; 0.1% in PBS containing 5% FCS), stained by incubation with mAb against IFN-γ (BD Pharmingen), and analyzed by flow cytometry. Data analysis was performed using CellQuest software (BD Biosciences, San Jose, California, USA). For detection of cytokine secretion in the culture

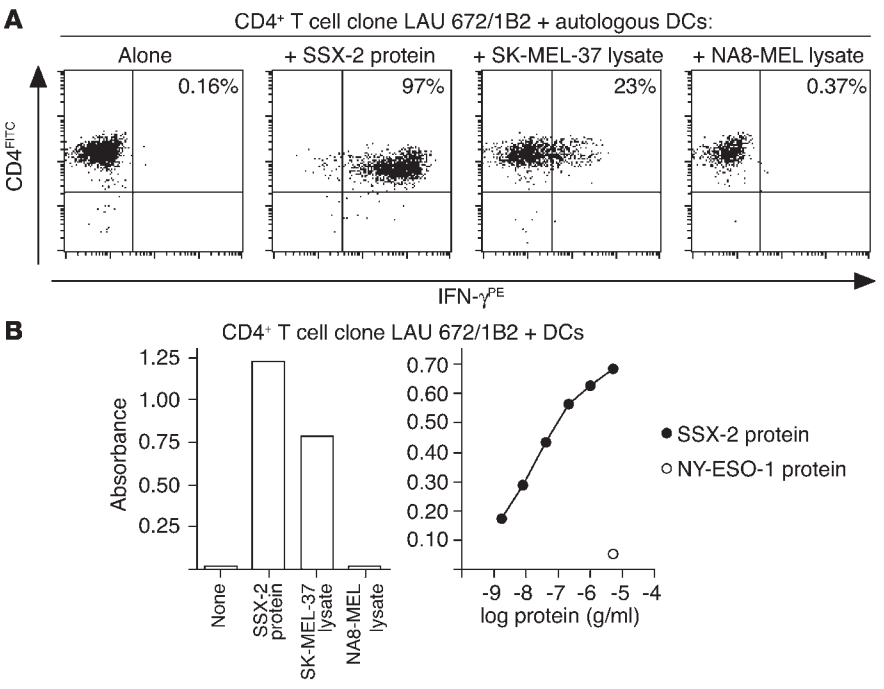



**Figure 6**

SSX-2–specific $CD4^+$ T cells efficiently recognize the SSX-2 native antigen upon processing and presentation by professional APCs. Processing and presentation of SSX-2 antigen by DCs after incubation with recombinant SSX-2 protein (or NY-ESO-1 protein as negative control), or with lysates from tumor cell lines SK-MEL-37 (SSX-2–expressing) or NA8-MEL (SSX-2–negative), was assessed by intracellular staining with anti–IFN-γ antibodies (**A**) or by ELISA measurement of IFN-γ secretion in the culture supernatant (**B**).

supernatant, 10,000 T cells were incubated in the absence or the presence of peptides at the described dose in 96-well round-bottom plates in 200 μl/well of CTL medium containing 20 U/ml rhIL-2. Where indicated, stimulating cells (10,000 per well), preincubated or not preincubated with antigen and extensively washed, were added. In some experiments, tumor cells were used as stimulators. Where indicated, tumor cells were transiently transfected with SSX-2–encoding recombinant pcDNA3.1 vector using FuGENE according to the manufacturer's instructions (Roche Diagnostics, Rotkreuz, Switzerland). After 24 hours of incubation at 37°C, culture supernatants were collected, and the content of IFN-γ was determined by ELISA (BioSource Europe SA, Fleurus, Belgium).

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We dedicate this work to our beloved colleague, Matthew Scanlan, who suddenly passed away shortly before publication of this research article, leaving all of us in the deepest sorrow.

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