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$CD4^+$ T Cell Responses to SSX-4 in Melanoma Patients[1]

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Genes of the synovial sarcoma X breakpoint (SSX) family are expressed in different human tumors, including melanomas, but not in adult somatic tissues. Because of their specific expression at the tumor site, SSX-encoded Ags are potential targets for anticancer immunotherapy. In this study, we have analyzed $CD4^+$ T cell responses directed against the Ag encoded by SSX-4. Upon in vitro stimulation of PBMC from four melanoma patients bearing Ag-expressing tumors with a pool of long peptides spanning the protein sequence, we detected and isolated SSX-4-specific $CD4^+$ T cells recognizing several distinct antigenic sequences, mostly restricted by frequently expressed HLA class II alleles. The majority of the identified sequences were located within the Krüppel-associated box domain in the N-terminal region of the protein, indicating a high potential immunogenicity of this region. Together our data document the existence of $CD4^+$ T cells specific for multiple SSX-4 derived sequences in circulating lymphocytes from melanoma patients and encourage further studies to assess the impact of SSX-4-specific T cell responses on disease evolution in cancer patients. *The Journal of Immunology,* **2005, 174: 5092–5099.**

The synovial sarcoma X breakpoint (*SSX*)[3] genes are located on the X chromosome and encode a family of highly homologous nuclear proteins. Two family members, *SSX-1* and *SSX-2* were initially identified as fusion partners of the *SYT* gene in t(X;18)-positive synovial sarcomas (1, 2). Later, serological analysis of tumor cDNA expression libraries (SEREX), revealed recognition of the *SSX-2* encoded Ag by Abs from cancer patients (3). Three additional homologous genes, *SSX-3*, *-4*, and *-5*, were identified (4, 5) either through screening of a testicular cDNA library with allogeneic patient serum (*SSX-3* (5)) or by analysis of homologous sequences amplified by SSX-specific primers (*SSX-3* (4), *SSX-4*, and *-5* (5)). Recently, *SSX* genes and pseudogenes have been further characterized, resulting in the definition of nine (*SSX-1* to *-9*) genes (6). Expression of the majority of the *SSX* genes, including *SSX-1* to *-5* and *SSX-7*, is restricted to gametogenic cells, not found in most adult normal tissues, but is detected in tumors of different histological types (7–9). Therefore, Ags of the SSX family are targets of interest for immunotherapy of cancer.

Among *SSX* genes, *SSX-1*, *-2*, *-4*, and *-5* are the most commonly expressed. Surveys of SSX gene expression in different human tumor types have shown expression of several family members in a significant proportion of tumors, although at variable levels depending on the particular histological type. In 325 specimens of human neoplasms from various histological origins, expression of SSX-4 was found in 15% of all tumors, including 10 of 48 (20.8%) malignant melanomas tested (7). More recently expression of SSX-4 has been reported by us (9) in 23% of sarcomas and by others in several different tumor types, such as 73% of hepatocellular carcinomas (8), 35% of primary lung cancers (10), 67% of neuroblastomas (11), and 20% of gastric carcinomas (12).

We have previously reported that one Ag of the family, SSX-2, is naturally immunogenic in cancer patients bearing Ag-expressing tumors, and identified both $CD8^+$ and $CD4^+$ SSX-2- derived T cell epitopes (13–15). However, no information was thus far available on T cell responses to other SSX Ags. Here, we report the analysis of $CD4^+$ T cell responses against SSX-4, in melanoma patients. Upon in vitro stimulation with a pool of long peptides spanning the protein sequence, we could detect and isolate SSX-4-specific $CD4^+$ T cells from four melanoma patients bearing Ag-expressing tumors. We isolated SSX-4-specific clonal $CD4^+$ T cell populations recognizing several distinct antigenic sequences restricted by different HLA class II alleles including some among the HLA-DR alleles more frequently expressed in several major ethnic groups. Interestingly, the majority of the identified sequences were located within the Krüppel-associated box (KRAB) repression domain in the N-terminal region of the protein.

Materials and Methods

Cells, tissue culture, and generation of SSX-4-specific $CD4^+$ T cells

Peripheral blood was obtained from healthy donors (New York City Blood Bank) and melanoma patients (Lausanne University Hospital, Switzerland) upon informed consent. Melanoma cell lines, anti-HLA-DR (D1.12), and -DP (B7.21.3) Abs were provided by Dr. D. Rimoldi (Ludwig Institute for Cancer Research, Lausanne, Switzerland). Cell lines were maintained in

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[3] Abbreviations used in this paper: SSX, synovial sarcoma X breakpoint; KRAB, Krüppel-associated box; DC, dendritic cell(s); rh, recombinant human.

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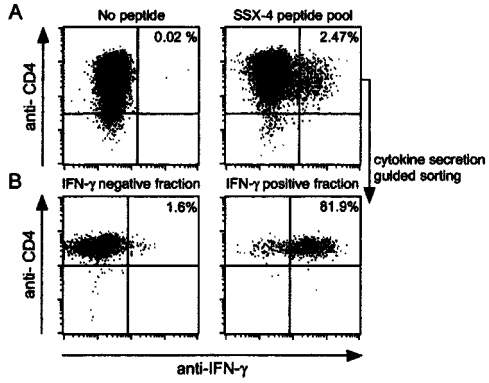

FIGURE 1. Assessment of SSX-4-specific $CD4^+$ T cell responses in circulating lymphocytes. *A*, The presence of specific $CD4^+$ T cells in the cultures from melanoma patients and healthy donors (shown for patient LAU 97) was assessed by intracellular staining with anti-IFN-γ-specific mAb after stimulation in the absence or presence of a pool containing overlapping peptides spanning the SSX-4 protein. Numbers in upper right quadrants are percentage of cytokine producing cells among $CD4^+$ T cells. *B*, IFN-γ-secreting cells were isolated from the culture stimulated with the SSX-4 peptide pool by cytokine secretion guided magnetic sorting.

RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS. Culture medium for lymphocytes was IMDM (Invitrogen Life Technologies) supplemented with 8% heat-inactivated pooled human serum (CTL medium), recombinant human (rh) IL-2 (Glaxo) and rhIL-7 (R&D Systems). Homozygous EBV-transformed B cell lines (EBV-B) were obtained from the National Marrow Donor Program/American Society for Histocompatibility and Immunogenetics (NMDP/ASHI) Cell Repository. In vitro stimulation of SSX-4-specific T cells was conducted as described previously (15). $CD4^+$ T cells were stimulated with autologous APCs in the presence of a pool of partially overlapping peptides spanning the entire SSX-4 protein sequence (2 μM each). The culture was tested 2 wk later using the same peptides, as indicated. $CD4^+$ T cells secreting IFN-γ in response to peptide stimulation were isolated using the cytokine secretion detection kit (Miltenyi Biotec) and cloned by limiting dilution culture as described (15). Clones were subsequently expanded by periodic (3–4 wk) stimulation under the same conditions.

Ag recognition assays

For intracellular cytokine secretion detection, T cells were stimulated in the absence or presence of peptides at the indicated dose during 4 h as described previously (15). Brefeldin A (10 μg/ml; Sigma-Aldrich) was added 1 h after the beginning of the incubation. After incubation, cells were stained with anti-CD4 mAb (BD Biosciences) for 20 min at 4°C and fixed using formaldehyde, permeabilized with saponin (0.1% in PBS and 5% FCS; Sigma-Aldrich), stained with anti-IFN-γ mAb (BD Pharmingen), and analyzed by flow cytometry. Data analysis was performed using Cell Quest software. IFN-γ secretion was assessed as described (15). T cells (10,000) were incubated in the absence or presence of peptides. Where indicated, APC (EBV-B cells, 10,000/well) preincubated or not with Ag and extensively washed, were added. In some experiments, tumor cells were used as APC. Where indicated, tumor cells were transiently transfected with SSX-4 cDNA, cloned into pcDNA3.1 vector, using FuGENE according to the manufacturer's instructions (Roche Diagnostics). After 24 h of incubation at 37°C, culture supernants were collected and the content of IFN-γ was determined by ELISA (BioSource International).

Generation of dendritic cells (DC) and recombinant proteins

Monocyte-derived DC were prepared from $CD14^+$ monocytes isolated from PBMC by magnetic cell sorting using miniMACS (Miltenyi Biotec) and cultured in CTL medium containing 1000 U/ml of rhGM-CSF and 1000 U/ml of rhIL-4 (R&D Systems) during 6 days. SSX-4 protein was expressed by cloning full-length cDNA into pQE30 (Qiagen) and synthesized in *Escherichia coli* with a six-histidine tag at the amino terminus. The protein was purified by nickel chelate affinity chromatography (His-Bind Resin; Novagen) by using a pH gradient and eluting in 8 M urea, 100 mM phosphate, and 10 mM Tris at pH 4.5, followed by step gradient dialysis to 2 mM urea in PBS. Where indicated, DC were incubated with proteins for 12 h and washed before their use in Ag recognition assay.

Results

Assessment of SSX-4-specific $CD4^+$ T cell responses in circulating lymphocytes from melanoma patients

To confirm expression of SSX-4 in melanoma, we assessed tumors from 61 melanoma patients. Tumor samples from 26 (42%) patients analyzed showed detectable expression of SSX. Of those, 16 (61%) (that is, 25% of the total number analyzed) were $SSX\text{-}4^+$. We analyzed $CD4^+$ T cell responses to SSX-4 in circulating lymphocytes from 4 of these patients. $CD4^+$ T cells were isolated from PBMC by magnetic cell sorting and stimulated in vitro with a pool containing 20 amino acid long peptides overlapping by 10 amino acids and spanning the SSX-4 protein sequence, in the presence of autologous APC. Cultures were tested using the SSX-4 peptide pool for the presence of specific, IFN-γ-secreting $CD4^+$ T cells, by staining with IFN-γ and CD4-specific mAbs (Fig. 1*A*). After the first cycle of in vitro stimulation (Table I), no clear responses were detectable, although the proportion of IFN-γ-secreting $CD4^+$ T cells was 2- to 3-fold higher in the presence than in the absence of the peptide pool in the case of three out of four patients. After the second cycle of in vitro stimulation, specific responses to the SSX-4 peptide pool were clearly detected for three out of four patients. Namely, for LAU 14, LAU 331, and LAU 97, a 20-, 7-, and 120-fold increased frequency of IFN-γ-secreting $CD4^+$ T cells was detected in the presence than in the absence of SSX-4 peptide pool, respectively. However, in the case of the fourth patient (LAU 672) the frequency of IFN-γ-secreting $CD4^+$ T cells obtained in the presence of the SSX-4 peptide pool was only 3-fold higher compared with background values. $CD4^+$ T cells specifically secreting IFN-γ in response to stimulation with SSX-4 peptides were isolated from the cultures using a cytokine secretion detection kit. In all cases, this resulted in the isolation of highly enriched populations of $CD4^+$ IFN-γ-secreting T cells (Fig. 1*B*).

Table I. *Assessment of SSX-4-specific $CD4^+$ T cell responses in circulating lymphocytes from melanoma patients and healthy donors*[a]

	IVS-1		IVS-2		$CD4^+$ IFN-γ^+ after Isolation	
Code	−P	+P	−P	+P	Negative fraction	Positive fraction
LAU 14	0.06[b]	0.06	0.02	0.40	5.8	64.1
LAU 672	0.11	0.27	0.01	0.03	0.5	75.9
LAU 331	0.02	0.06	0.07	0.54	2.1	87.9
LAU 97	0.02	0.05	0.02	2.47	1.6	81.9

[a] $CD4^+$ T cells from patients (LAU) were stimulated once (IVS-1), or twice (IVS-2), with a pool of 20 amino acid long and partially overlapping peptides encompassing the complete sequence of the SSX-4 Ag. Cultures were then assessed functionally in the absence (−P) or presence (+P) of the same peptide pool. IVS, in vitro stimulation; P, peptide pool.

[b] Percentage of $CD4^+$ IFN-γ-secreting cells.

Table II. *Assessment of active peptides in the SSX-4 peptide pool*[a]

SSX-4	LAU 97	LAU 331	LAU 14
No peptide	0.02[b]	0.03	0.03
All peptides	3.55	0.18	0.20
1–20	<0.01	<0.01	0.07
11–30	<0.01	0.02	<0.01
21–40	<0.01	0.02	0.04
31–50	<0.01	0.34	0.01
41–60	0.20	0.02	<0.01
51–70	<0.01	0.65	0.06
61–80	0.03	0.16	0.12
71–90	<0.01	0.05	0.03
81–100	0.02	0.02	<0.01
91–110	<0.01	0.02	0.03
101–120	<0.01	0.02	0.91
111–130	<0.01	0.01	0.01
121–140	<0.01	0.01	<0.01
131–150	<0.01	0.02	0.01
141–160	<0.01	0.01	0.03
151–170	9.43	0.01	0.01
161–180	2.25	<0.01	<0.01
171–188	0.01	0.02	<0.01

[a] The activity of individual SSX-4 peptides was assessed after the second cycle of in vitro stimulation with the SSX-4 peptide pool. Values at least 3-fold higher than baseline (no peptide) were considered significant and are underlined.

[b] Numbers are percentage of CD4$^+$ IFN-γ-secreting cells.

Assessment of active peptides in the SSX-4 peptide pool

For three of the four patients, enough cells were available from the cultures that had undergone two cycles of in vitro stimulation to assess reactivity to individual SSX-4 peptides. The results of this analysis are shown in Table II. For each patient, reactivity was detected toward more than one peptide, indicating recognition of multiple sequences. However, the active peptides were mostly different for each patient. For patient LAU 14, we identified two active peptides (61–80 and 101–120) at distinct locations of the protein sequence. For patient LAU 331, two active overlapping peptides (51–70 and 61–80) were identified. In addition, reactivity was also detected toward a third peptide at a different location (31–50). We obtained similar results in the case of patient LAU 97. Namely, the main activity was detected for peptide 151–170 with a lower proportion of IFN-γ-secreting CD4$^+$ T cells detected upon stimulation with the partially overlapping peptide 161–180. In addition, a third peptide at a distinct location, 41–60, stimulated IFN-γ production by a lower proportion of CD4$^+$ T cells. It is noteworthy that, in all cases, the proportion of IFN-γ-secreting CD4$^+$ T cells detected in the presence of the peptide mix containing all overlapping peptides was much lower than that of both the sum of individual activities and the most active peptide in the mixture, when tested individually. For example 9.43% of the cells in the culture from patient LAU 97 produced IFN-γ upon stimulation with peptide SSX-4 151–170, whereas the proportion of IFN-γ-secreting cells detected in the presence of the SSX-4 peptide pool was ~3 times lower (3.55%).

Isolation of clonal SSX-4-specific CD4$^+$ T cells and assessment of MHC class II restriction

The enriched populations of CD4$^+$ T cells secreting IFN-γ in response to stimulation with the SSX-4 peptide pool were cloned under limiting dilution conditions, and the obtained clonal CD4$^+$ T cell populations were used to further define and characterize the corresponding antigenic sequences. Peptide titration curves for representative clones specific for each of the identified sequences are shown in Fig. 2. In the majority of the cases, the CD4$^+$ T cell clones recognized the corresponding active peptide but not the neighboring overlapping peptides. However, in two cases, and in agreement with the findings reported in Table II, recognition of one of the overlapping peptides was observed. This was the case for SSX-4 51–70-specific clones from LAU 331, which recognized peptide SSX-4 61–80, and for SSX-4 151–170-specific clones from LAU 97, which recognized peptide SSX-4 161–180.

To identify the MHC class II restricting element used by SSX-4-specific CD4$^+$ T cell clones, we initially performed peptide presentation experiments in the presence of Abs known to specifically

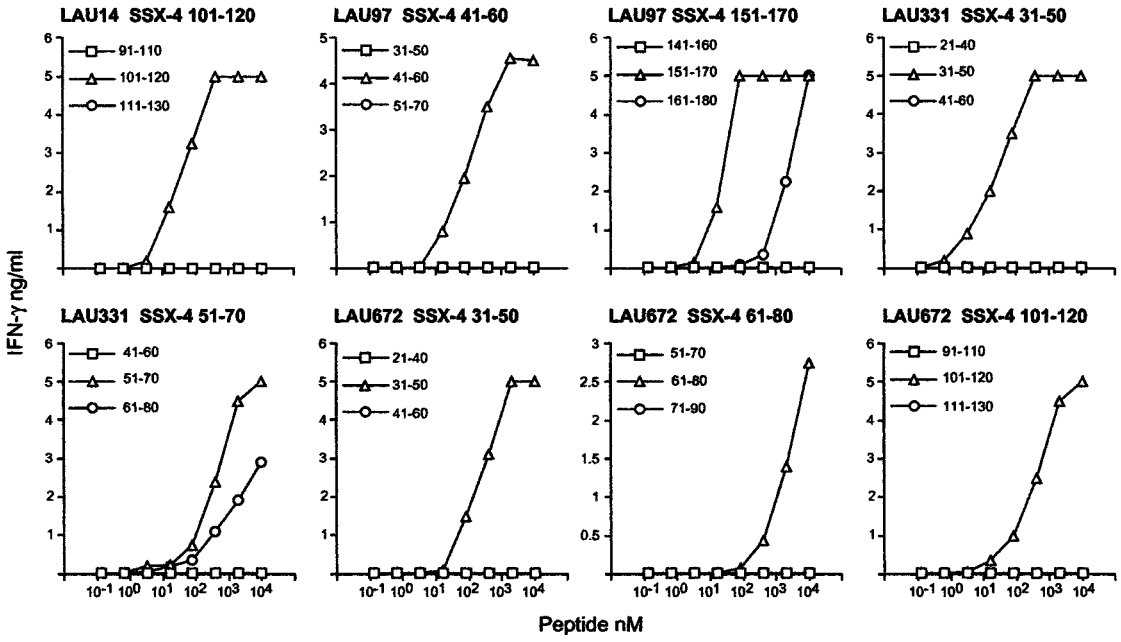

FIGURE 2. Recognition of SSX-4 peptides by specific CD4$^+$ T cell clones from melanoma patients. Ag recognition by specific CD4$^+$ T cell clones was assessed in the presence of graded peptide dilutions. For clones specific for each of the identified sequences, Ag recognition was assessed for the corresponding active peptide as well as for neighboring overlapping peptides. The concentration of IFN-γ in the culture supernatant was assessed by ELISA after 24 h of culture.

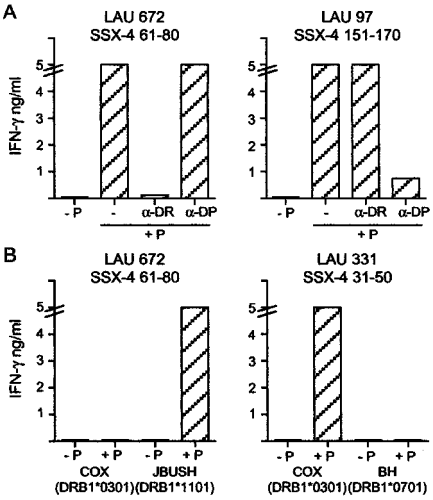

FIGURE 3. Determination of the MHC class II restricting elements and alleles. *A*, To determine the MHC class II restricting element, peptide recognition was assessed either in the absence or in the presence of anti-HLA-DR or -DP antibodies. *B*, The MHC class II restricting allele was determined by assessing the ability of molecularly typed EBV-B cells to present SSX-4 peptides to the corresponding $CD4^+$ T cell clones.

block Ag recognition restricted by different MHC class II molecules. An example of the data obtained with this analysis is shown in Fig. 3*A*, and results obtained for all clones are summarized in Table III. To establish the presenting allele(s), we first assessed by molecular typing the HLA-DR or HLA-DP alleles of the patients, depending on the restriction of the corresponding clones (Table III). We then assessed presentation by homozygous EBV-B cells expressing each of the patient's alleles. An example of these experiments is shown in Fig. 3*B*, and the results obtained for all clones are summarized in Tables III and IV.

The results obtained following this approach indicated the presence of two different antigenic sequences corresponding to the activity of peptide SSX-4 31–50. In the case of 31–50-specific clones from patient LAU 331 the restricting DRB1* allele was DRB1*0301. It is noteworthy that we have recently identified the homologous SSX-2 peptide (SSX-2 37–51) as a DRB1*0301 restricted epitope (16). When assessed in a peptide titration assay, peptide SSX-4 37–51 was well recognized by SSX-4 31–50-reactive $CD4^+$ T cells from LAU 331, although slightly less efficiently compared with peptide SSX-4 31–50 (3-fold). However, peptide SSX-2 37–51 was not recognized by SSX-4-specific $CD4^+$ T cells (not shown). Interestingly, in the case of 31–50-specific clones from patient LAU 672 and despite the fact that this patient also expressed DRB1*0301, DRB1*0301 homozygous EBV-B cells pulsed with SSX-4 31–50 were unable to present the peptide to specific clones. In contrast, efficient presentation was obtained using DRB1*1101 homozygous EBV-B cell lines. Presentation by the DRB3*0202 allele was excluded, as no presentation was obtained using 31227ABO (DRB1*1401, DRB3*0202 homozygous). From patient LAU 672, we obtained SSX-4-specific $CD4^+$ T cell clones recognizing two additional peptides 61–80 and 101–120, in association with HLA-DR. For both, based on results obtained with HLA-typed APC, DRB1*1101 was the restricting DR allele. In the case of SSX-4 101–120-specific $CD4^+$ T cells from LAU 14, DRB1*1101 was also the restricting DR allele. Peptide SSX-4 41–60 was recognized by specific clones from patient LAU 97 in association with HLA-DR. Based on the results obtained with HLA-typed APC, the candidate-restricting allele was DRB1*1501 (Table III). However, presentation through DRB5*0101 could not be formally excluded because of the lack of appropriate APC. In the case of peptide SSX-4 51–70, recognized by specific clones from patient LAU 331 in association with HLA-DR, the restricting DR allele was DRB1*0701 (Table III). Finally, peptide SSX-4 151–170 was recognized by $CD4^+$ T cells from LAU 97 in association with HLA-DP. The restricting DP allele was DPB1*1001.

Table III. *Determination of the HLA class II presenting allele using molecularly typed APC*

LAU 331 31–50[a]	COX (+)[b]	BH (−)	0MW (−)		
DRB1*0301–0701	DRB1*0301	DRB1*0701	DRB1*1301		
DRB3*0101	DRB3*0101		DRB3*0101		
DRB4*0101		DRB4*0101			
LAU 672 31–50	COX (−)	JBUSH (+)	LAU 14 (+)	0MW (−)	31227ABO (−)
DRB1*0301–1101	DRB1*0301	DRB1*1101	DRB1*1101	DRB1*1301	DRB1*1401
DRB3*0101–0202	DRB3*0101	DRB3*0202	DRB3*0202	DRB3*0101	DRB3*0202
LAU 97 41–60	0MW (−)	SCHU (+)	COX (−)		
DRB1*1301–1501	DRB1*1301	DRB1*1501	DRB1*0301		
DRB3*0101	DRB3*0101		DRB3*0101		
DRB5*0101		DRB5*0101			
LAU 331 51–70	COX (−)	BH (+)	0MW (−)	T7526 (−)	
DRB1*0301–0701	DRB1*0301	DRB1*0701	DRB1*1301	DRB1*0901	
DRB3*0101	DRB3*0101		DRB3*0101		
DRB4*0101		DRB4*0101		DRB4*0101	
LAU 672 61–80	COX (−)	JBUSH (+)	31227ABO (−)		
DRB1*0301–1101	DRB1*0301	DRB1*1101	DRB1*1401		
DRB3*0101–0202	DRB3*0101	DRB3*0202	DRB3*0202		
LAU 672 101–120	COX (−)	JBUSH (+)	31227ABO (−)		
DRB1*0301–1101	DRB1*0301	DRB1*1101	DRB1*1401		
DRB3*0101–0202	DRB3*0101	DRB3*0202	DRB3*0202		
LAU 14 101–120	JBUSH (+)	31227ABO (−)			
DRB1*1101	DRB1*1101	DRB1*1401			
DRB3*0202	DRB3*0202	DRB3*0202			
LAU 97 151–170	SCHU (−)	BM21 (+)			
DPB1*0402/1001	DPB1*0402	DPB1*1001			

[a] For each $CD4^+$ T cell specificity tested the code of the corresponding patient and the reactive SSX-4 peptide are shown.
[b] The ability of peptide-pulsed APC to present or not the appropriate peptide to the corresponding $CD4^+$ T cell clones is indicated as (+) or (−).

Table IV. *Identification of the restricting HLA class II allele(s) for SSX-4 specific CD4$^+$ T cell clones*[a]

Patient Code	HLA Class II Molecular Typing	Active Peptide	Restricting MHC Class II Element	Restricting MHC Class II Allele
LAU 14	DRB1*1101; DRB3*0202	101–120	DR	DRB1*1101
LAU 97	DRB1*1301/1501; DRB3*0101, DRB5*0101	41–60	DR	DRB1*1501 or DRB5*0101
	DPB1*0402/1001	151–170	DP	DPB1*1001
LAU 331	DRB1*0301/0701, DRB3*0101, DRB4*0101	31–50	DR	DRB1*0301
		51–70	DR	DRB1*0701
LAU 672	DRB1*0301/1101, DRB3*0101–0202	31–50	DR	DRB1*1101
		61–80	DR	DRB1*1101
		101–120	DR	DRB1*1101

[a] The restricting MHC class II element was assessed in peptide presentation experiments in the presence of Abs known to specifically block Ag recognition restricted by different MHC class II element. The restricting allele was determined in peptide presentation experiments using homozygous EVB-B expressing each of the patient's alleles. Examples of these experiments are shown in Fig. 3. The results obtained for all clones are summarized above and in Table III.

Recognition of naturally processed SSX-4 Ag by specific CD4$^+$ T cell clones

To assess the recognition of endogenously expressed SSX-4 Ag by specific CD4$^+$ T cells, we selected a panel of melanoma cell lines characterized in terms of expression of SSX-4, relevant MHC class II molecules, and HLA-DR expression levels (Fig. 4*A*). All tumor cell lines, with the exception of Me280, expressed detectable levels of HLA-DR molecules at the cell surface. In addition, for all cell lines with the exception of Me279, HLA-DR expression was significantly enhanced after treatment with IFN-γ. Recognition of endogenous SSX-4 Ag by specific CD4$^+$ T cells was assessed using the tumor cell lines as such or after treatment with IFN-γ and/or transfection with an SSX-4-encoding plasmid. Recognition of endogenous SSX-4 Ag by HLA-DP10-restricted CD4$^+$ T cells could not be assessed because of unavailability of appropriate tumor cell lines. However, in all the other cases, tumor cells were not significantly recognized by SSX-4-specific CD4$^+$ T cells, irrespective of IFN-γ treatment, and transfection with SSX-4-encoding plasmid, unless the peptide was added exogenously (Fig. 4*B*). We then assessed the ability of professional APC to process the SSX-4

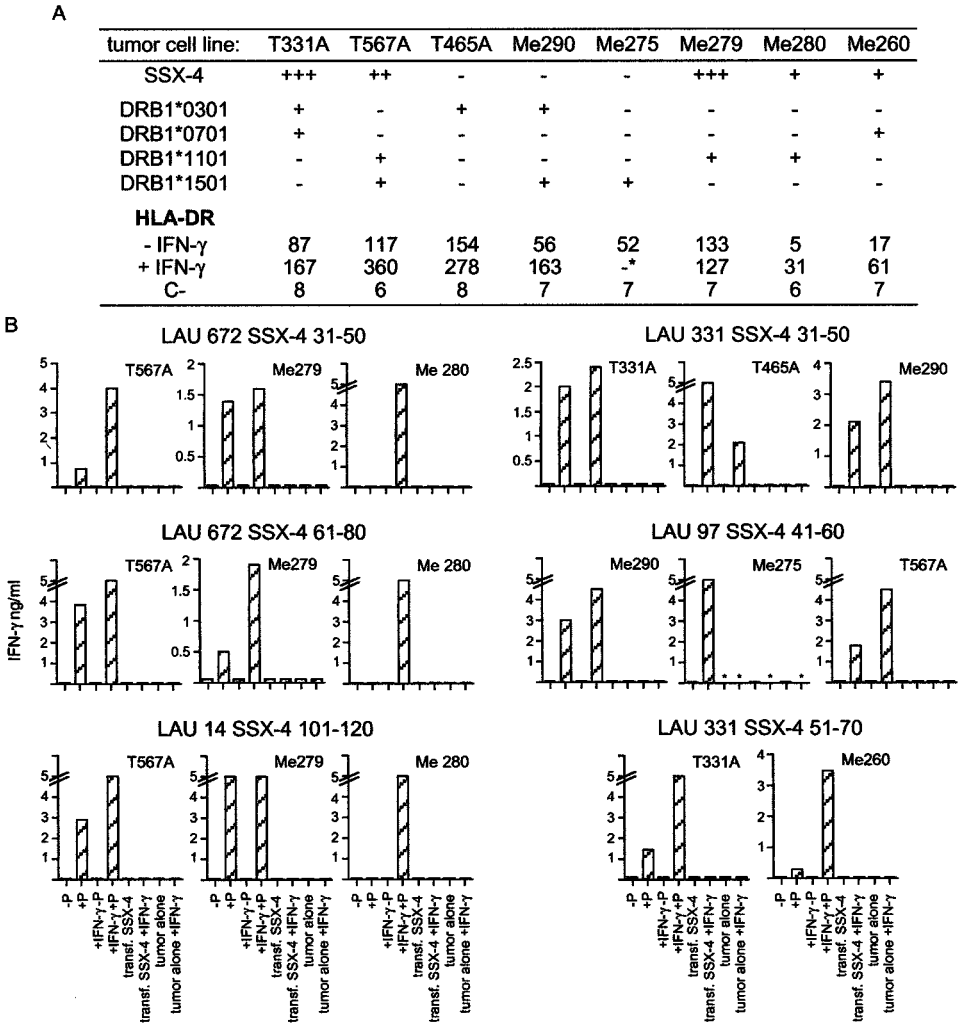

FIGURE 4. Lack of recognition of endogenous SSX-4 Ag by specific CD4$^+$ T cells. *A*, Expression of SSX-4 in tumor lines was assessed by PCR as described previously (9). HLA-DR alleles were determined by molecular typing. Surface expression of HLA-DR was assessed using specific mAb. Where indicated, cells were treated with IFN-γ (200 IU/ml) during 24 h. *B*, Recognition of melanoma cell lines, transfected or not with an SSX-4 encoding plasmid, after 24 h of incubation with SSX-4-specific CD4$^+$ T cells, was assessed by ELISA measurement of IFN-γ secretion in the culture supernatant, in the absence or presence of peptide. Where indicated, cells were treated with IFN-γ during 24 h and extensively washed before test. ∗, Me275 cells died upon treatment with IFN-γ.

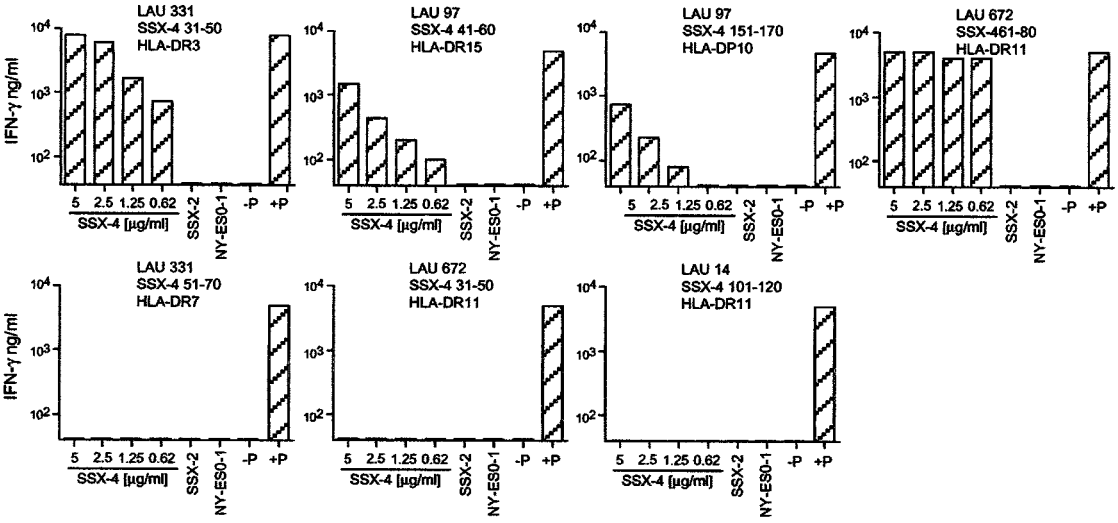

FIGURE 5. Processing and presentation of recombinant SSX-4 protein to specific $CD4^+$ T cells by professional APC. The ability of monocyte derived DC to process the SSX-4 protein and present the relevant antigenic sequences to specific $CD4^+$ T cells was assessed after 12 h of incubation of DC with soluble recombinant SSX-4 protein, at the indicated dose. Recombinant SSX-2 and NY-ESO-1 proteins (5 μg/ml) as well as the corresponding active peptide (2 μM) were used as internal controls.

Ag and present the different antigenic sequences to the corresponding $CD4^+$ T cells. As illustrated in Fig. 5, monocyte-derived DC were able to present the recombinant SSX-4 protein to specific $CD4^+$ T cells in the case of the following sequences: the DR3-restricted 31–50, the DR15-restricted 41–60 (or DRB5*0101), the DR11-restricted 61–80, and the DP10-restricted 151–170. In contrast, no significant recognition of the DR11-restricted 31–50 sequence, the DR7-restricted 51–70 sequence nor the DR11-restricted 101–120 sequence was obtained under these test conditions.

Discussion

Stimulation of both $CD4^+$ and $CD8^+$ tumor Ag-specific T cells is critical for generating potent antitumor immune responses in vivo. Tumor Ag-specific $CD4^+$ T cells have been shown to mediate antitumor immune responses through a variety of different mechanisms. They participate in the effector phase of tumor rejection both indirectly via macrophage/eosinophil activation and by producing inflammatory cytokines, such as IFN-γ, and sometimes also through direct killing of tumor cells (17, 18). In addition and importantly they exert helper functions for priming and maintenance of tumor Ag-specific $CD8^+$ T cells (17, 18) and for production of tumor Ag-specific Abs (19). Assessment of $CD4^+$ T cell responses to tumor Ags and identification of tumor Ag-derived sequences recognized by $CD4^+$ T cells in association with frequently expressed MHC class II alleles are, therefore, important elements for the implementation of cancer immunotherapy.

We analyzed SSX-4-specific $CD4^+$ T cell responses in patients bearing Ag-expressing tumors, using a panel of 20 amino acid long peptides spanning the entire SSX-4 sequence and overlapping by 10 amino acids. This approach offers several advantages, such as the following: it is not technically cumbersome, it allows the identification of $CD4^+$ T cell epitopes irrespective of their physiologic processing pathways, and it leads to a comprehensive assessment of the immunogenicity of the overall Ag. The peptides were used as a pool for in vitro stimulation of purified circulating $CD4^+$ T cells using cells from the $CD4^-$ fraction as APC and for a first screening of the elicited responses. After two cycles of in vitro stimulation, responses to the peptide pool were detected in four out of four patients. Single peptides in the pool were then used to define individual activities. This analysis revealed a potential limitation of the method, due to peptide competition for binding to the MHC molecule, that is likely to occur also in the in vitro stimulation phase but could be overcome by stimulating T cells with a mixture of APC incubated with single peptides from the pool.

For each of the detected specificities, $CD4^+$ T cells were isolated and cloned. We identified seven distinct antigenic sequences, six of which were restricted by HLA-DR. HLA-DR molecules account for >90% of the HLA class II isotypes expressed on APC, the HLA-DRB1 locus being highly polymorphic. However, the majority of the identified sequences were restricted by HLA-DR alleles frequently expressed in major ethnic groups. Three were restricted by DR11 (17% Caucasians, 18.1% Blacks, 4.9% Japanese, 19.4% Chinese, 18.1% Hispanics) one by DRB3 (17.7% Caucasians, 19.5% Blacks, 0.4% Japanese, 7.3% Chinese, 14.4% Hispanics) one by DR7 (26.2% Caucasians, 11.1% Blacks, 1% Japanese, 15% Chinese, 16.6% Hispanics) and one by DRB15 (19.9% Caucasians, 14.8% Blacks, 30.9% Japanese, 22% Chinese, 15.0% Hispanic) (20). We also identified one antigenic sequence restricted by HLA-DP10, which is less frequently expressed (2.2% French, 1.6% Canadians) (20). HLA-DP molecules have been generally scarcely studied. However, recently HLA-DP tumor Ag-derived T cell epitopes have been described (21, 22). HLA-DP molecules have been previously shown to present peptides derived from infectious agents and alloantigens (23, 24). It has also been suggested that some HLA-DP alleles may play a role in autoimmune diseases such as juvenile chronic arthritis (25). However, the functional role of HLA-DP (and HLA-DQ) molecules in the immune response in relation to that of HLA-DR is still unclear and subject to investigation.

Five of the seven antigenic sequences identified in this study are localized in the KRAB domain of SSX-4 (Fig. 6). Interestingly, the four previously defined SSX-2 T cell epitopes (13–16) are also located in the KRAB domain. Previous studies have indicated that the immunogenicity of antigenic sequences can be influenced by their location within defined protein stretches, most likely because of their accessibility to fragmentation by proteases in the Ag-processing pathway (26). Therefore, although structural data on SSX proteins are not yet available, our findings suggest that the KRAB

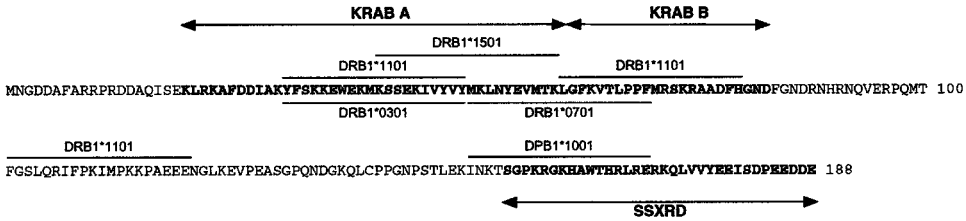

FIGURE 6. Distribution of antigenic sequences in SSX-4. Location of the previously described KRAB domain (divided in the A and B subdomains) and the SSXRD domain are indicated. For each antigenic sequence, both the location of the SSX-4 peptide used for its identification as well as the corresponding restricting allele are indicated.

domain could be particularly exposed to degradation by proteases resulting in a "hot spot" region for T cell recognition.

CD4$^+$ T cells specific for six of the seven identified sequences (the DP10-restricted epitope could not be evaluated because of lack of DP10-expressing targets) failed to recognize endogenously expressed SSX-4 Ag. Similar results were previously obtained for SSX-2-reactive CD4$^+$ T cells specific for three distinct epitopes (14–16). One possible explanation of these findings it that the nuclear localization of SSX proteins during most of the cell cycle (27, 28) could hamper their accessibility to the MHC class II endogenous processing pathway. However, since direct recognition of tumor cells is probably not the dominant mechanism through which tumor Ag-specific CD4$^+$ T cells contribute to tumor rejection in vivo (17, 18), lack of recognition of endogenous SSX Ags by specific CD4$^+$ T cells does not imply a lesser role of these cells in the immune response to SSX Ag-expressing tumors.

These results also indicate that in vivo processing and presentation of endogenous SSX Ags mainly occur through the exogenous pathway. In support of this, four of the seven identified SSX-4 antigenic sequences were efficiently processed and presented to specific CD4$^+$ T cells upon incubation of monocyte-derived DC with SSX-4 recombinant protein. In contrast, the three remaining sequences were not significantly presented under identical test conditions and could correspond to "cryptic" epitopes generated in vivo, possibly under inflammatory conditions and/or by APC distinct from those used in our test conditions (29–32). However, the interest of these sequences for vaccination remains to be established.

In conclusion, in this study, we have identified multiple SSX-4-derived antigenic sequences presented to CD4$^+$ T cells in association with frequently expressed alleles that collectively cover 35–80% of individuals from several major ethnic groups. The majority of these sequences are located in the KRAB domain suggesting a dominant role of this region of the protein in the induction of SSX-specific immune responses. The identification of these sequences will be highly instrumental for future studies addressing the relationship between SSX-4-specific T cell responses and disease evolution in cancer patients.

Disclosures

The authors have no financial conflict of interest.

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