

LATEST PAPERS

SEARCH for PAPERS

Printer-friendly PDF

Comment(s)

[>Abstract](#) [>Introduction](#) [>Results](#) [>Discussion](#) [>References](#) [>Materials & methods](#) [>Contact authors](#)

Cancer Immunity, Vol. 3, p. 18 (17 December 2003) Submitted: 12 November 2003. Accepted: 12 November 2003.
Contributed by: M Pfreundschuh

Identification of an HLA-A*02 restricted immunogenic peptide derived from the cancer testis antigen HOM-MEL-40/SSX2

Claudia Wagner¹, Frank Neumann¹, Boris Kubuschok¹, Evi Regitz¹, Axel Mischo¹, Stefan Stevanovic², Michael Friedrich³, Werner Schmidt³, Hans-Georg Rammensee², and Michael Pfreundschuh¹✉

¹Med. Klinik I, Saarland University Medical School, D-66424 Homburg, Germany

²Institute for Cell Biology, Department of Immunology, Eberhard-Karls-Universität, D-72074 Tübingen, Germany

³Universitäts-Frauenklinik, Saarland University Medical School, D-66424 Homburg, Germany

Keywords: human, breast cancer, T lymphocyte epitopes, SSX2, HLA-A2

Abstract

HOM-MEL-40/SSX2 is a SEREX-defined cancer testis antigen with frequent expression in various human neoplasms. To search for HLA-A*0201 restricted peptides that induce HOM-MEL-40/SSX2-specific CD8+ responses in breast cancer patients, we used the SYFPEITHI algorithm to identify three HOM-MEL-40/SSX2-derived nonamers with high binding affinity for HLA-A*0201, which has a prevalence of 40% in the Caucasian population. Of the three peptides, p41-49 and p103-111 but not p167-175 had been shown to be processed by the proteasome. Only stimulation with p103-111 induced HOM-MEL-40-specific CTLs in 5/7 patients with HOM-MEL-40/SSX2 positive breast cancers and in 6/11 healthy controls. HLA-A*0201 restriction of p103-111 was demonstrated by blocking with specific antibodies. The natural processing and presentation of p103-111 was demonstrated by the recognition of the HOM-MEL-40/SSX2 positive cell line SK-MEL-37 and of COS7/A2 cells transfected with HOM-MEL-40/SSX2 by p103-111 specific CD8+ cells. No correlation was found between CD8+ T-cell responses against p103-111 and anti-HOM-MEL-40/SSX2 antibody titers in the serum of patients, suggesting that CD8+ and B-cell responses against HOM-MEL-40/SSX2 are regulated independently. p103-111 holds promise as a broadly applicable peptide vaccine for patients with HOM-MEL-40/SSX2 positive neoplasms.

Introduction

According to their expression pattern and the specificity of the immune responses they evoke, antigens expressed by human neoplasms can be classified into different groups. These include the so-called "shared tumor antigens", the differentiation antigens (including the idiotypes of B-cell lymphomas), the products of viral, mutated, differentially spliced, over-expressed and amplified genes, as well as common autoantigens expressed by the malignant cells of a tumor (1). Most attractive candidates for vaccine development are the so-called

"shared tumor-specific antigens" (2) because they are expressed in a broad spectrum of different human neoplasms. "Shared tumor-specific antigens" include the CTL-reactive MAGE (3), BAGE (4) and GAGE (5) families, as well as the HOM-MEL-40/SSX2, the other SSX family members (6), NY-ESO-1 (2), HOM-TES-14/SCP-1 (7), CT-7 (8) and HOM-TES-85 (9), all of which have been defined using SEREX, the serological identification of antigens by recombinant expression cloning (10). It is enigmatic that the expression of all of the so-called "shared tumor antigens" in humans that have been molecularly defined to date by cellular (11) and serological techniques (12) is restricted to different types of cancers and normal testis. Therefore the term "cancer testis antigens" (CTAs) (2) or "cancer germline antigens" (13) has been coined for them and the term "cancer testis genes" for the genes encoding them.

HOM-MEL-40 was cloned by SEREX from a melanoma-derived cDNA library using autologous serum (10). The sequence of the *HOM-MEL-40* gene was identical with that of *SSX2*, which had been identified as one of the two genes involved in the t(X;18)(p11.2;11.2) translocation found in 70% of human synovial sarcomas (14). Expression analysis using Northern blot and RT-PCR demonstrated that *SSX2* is expressed in a significant proportion of human cancers such as melanomas (35%), head and neck cancers (35%), and colorectal carcinomas (12%) (15). Antibodies against *SSX2* were found in 10% of patients with melanoma (6). Thus, *SSX2* appears to be a promising candidate for vaccine development in a broad range of human cancers. With respect to the development of T-cell based vaccines, however, it is crucial to demonstrate that SEREX-defined antigens do not only elicit B-cell, but also T-cell responses in patients suffering from tumors expressing the antigen. Pursuing different strategies of what has been termed "reverse T-cell immunology", several MHC-I restricted epitopes derived from SEREX-defined antigens have been identified, most of them for the HLA-A*02 haplotype which is shared by 40% of the Caucasian population. "Reverse T-cell immunology" has in particular proved to be successful for the characterization of antigenic T-cell epitopes derived from NY-ESO-1, for which both MHC-I restricted (16) and MHC-II restricted T-cell epitopes have been defined (17, 18, 19, 20, 21, 22). However, since NY-ESO-1 is not at all expressed in certain types of human neoplasms, such as lymphomas (23), and only rarely in others, such as hepatocellular carcinomas (24), the demonstration of T-cell immunity against other SEREX-defined CTAs is warranted. Of the latter, *SSX2* appears to be a promising candidate. However, to date only one *SSX2*-derived peptide (*SSX2* p41-49) has been described (25). The definition of additional T-cell stimulating antigens is a prerequisite if the development of a polyvalent *SSX2* vaccine is the goal. We describe an HLA-A*02 restricted *HOM-MEL-40/SSX2*-derived peptide that elicits T-cell responses in the majority of HLA-A*0201 positive patients with *HOM-MEL-40/SSX2* positive tumors and might therefore be valuable alone or as part of a polyvalent *SSX2* peptide vaccine.

Results

Expression of *HOM-MEL-40/SSX2* in primary breast cancer

Fresh biopsy specimens of 65 consecutive HLA-A*0201 positive patients with primary breast cancer were tested for *HOM-MEL-40/SSX2* expression by RT-PCR. Of these, 7 (11%) expressed the *HOM-MEL-40/SSX2* antigen. No correlation was found between *HOM-MEL-40/SSX2* expression and the age of the patients or the stage of the tumor (Table 1).

Table 1. CD8+ T-cell response of patients with HOM-MEL-40/SSX2 positive breast cancers to HOM-MEL-40/SSX2-derived p103-111.

Patient	Age	Stage	Therapy at Time of Blood Sample	Anti-SSX2 Ab Reactivity	p103-111 Reactivity
G218	65	T1c, N1b3, M0, GIII	surgery	-	+
G221	61	T2, N1b4, Mx, GIII	surgery	-	+
G231	52	T1c, N0, M0, GIII	surgery	-	-
G237	69	T2, N1b3, M0, GIII	surgery	-	-
G244	59	T2, N1b1, M0, GII	surgery	-	+
G312	50	T3, N1b2, M1, GIII	chemotherapy	-	+
G313	73	T2, N0, M0, GII	surgery ^a	-	+

^aSurgery of second primary; previous therapy (8 years prior to second primary): segment resection of primary tumor followed by adjuvant chemotherapy plus radiotherapy followed by tamoxifen maintenance therapy.

Prediction of HOM-MEL-40/SSX2-derived epitopes reactive with T-cells from HLA-A*0201 positive patients

We used the SYFPEITHI algorithm to identify HOM-MEL-40/SSX2 peptides with a high binding affinity score for HLA-A*0201 molecules using the SYFPEITHI algorithm. SYFPEITHI predicted the following 3 peptides derived from HOM-MEL-40/SSX2 with a high binding probability: p41-49, p103-111 and p167-175. These peptides (Table 2) were synthesized and used for the stimulation experiments. To test whether the three peptides with the highest predicted binding affinity to HLA-A*0201 are appropriately processed by the proteasome, a set of 14 overlapping 22 amino acid-long peptides covering the 188 amino acids of the SSX2 protein were synthesized and incubated with standard proteasome purified from human erythrocytes, and the digested products were

analyzed by mass spectrometry (25). As reported previously (25), the results indicate that both p41-49 and p103-111, but not p165-173, are likely to be generated intracellularly by proteasomal degradation.

Table 2. HLA-A*0201 binding scores for the peptides used in the tests.

Antigen	Peptide	Amino Acid Sequence	Binding Score	Proteasomal Cleavage
HOM-MEL-40/SSX2	p41-49	KASEKIFYV	22	+
HOM-MEL-40/SSX2	p103-111	RLQGISPKI	23	+
HOM-MEL-40/SSX2	p167-175	RLRERKQL	22	-
SSX4	p103-111	SLQRIFPKI	23	n.d.
Human helicase	p148-156	YLLPAIVHI	30	n.d.
IMP	p58-66	GILGFVFTL	30	n.d.
CMV pp65	p495-503	NLVPMVATV	30	n.d.

Reactivity of HOM-MEL-40/SSX2-derived peptides with T-cells from patients with HOM-MEL-40/SSX2 expressing tumors

PBMCs from consecutive HLA-A*0201 positive breast cancer patients with a HOM-MEL-40/SSX2 expressing tumor were tested for reactivity with the HOM-MEL-40/SSX2-derived peptides p41-49, p103-111 and p167-175. Because none of the first three patients showed reproducible T-cell responses after stimulation with the HOM-MEL-40/SSX2 peptides p41-49 and p167-175, further investigations focused on p103-111. Furthermore, none of the 5 healthy donors tested showed T-cell reactions against these two peptides. In contrast, peptide p103-111 proved to be strongly immunogenic. The T-cells from 5/7 (71%) HLA-A*0201 positive patients tested showed a response upon stimulation with p103-111, as demonstrated by IFN-gamma release in an ELISPOT assay. As shown in Figure 1A, two patients (G218 and G221) showed a very strong reactivity, while the response of patients G244, G312, and G313 was less pronounced, yet clearly significant. In addition to HLA-A*0201 positive patients with HOM-MEL-40/SSX2-expressing tumors, eleven healthy HLA-A*0201 positive donors were tested for T-cell reactivity to p103-111. After stimulation with p103-111, six (GS13, GS15, GS17, GS27, GS28 and GS40) of the eleven donors (55%) showed a CD8+ T-cell reactivity as determined by IFN-gamma release in an ELISPOT assay (Figure 1B). The number of spots generated by the T-cells of healthy controls was in the range of what was observed for the patients responding the most, except for the two patients (G218 and G221) with the exceptionally high numbers of spots.

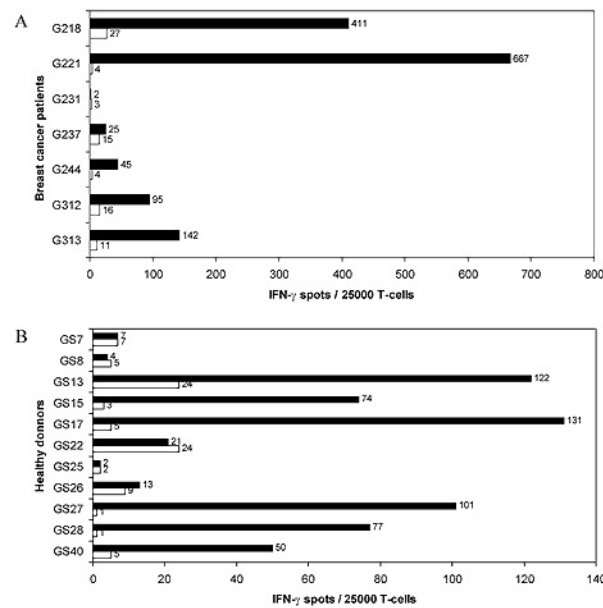


Figure 1. Specific recognition of HOM-MEL-40/SSX2-derived p103-111 pulsed targets by p103-111 stimulated T-cells from patients with HOM-MEL-40/SSX2 positive breast cancers (A) and HLA-A*0201 positive healthy controls (B). IFN-gamma production was measured by ELISPOT assay, as determined after three weeks of stimulation (i.e. after sensitization with peptide on days 1, 7 and 14). An HLA-A*02 binding, but non-immunogenic peptide from human helicase (p148-156) served as a negative control. The numbers of IFN-gamma spots per 25,000 T-cells arising from a reaction against target cells and SSX2 p103-111 (black), as well as against target cells and control peptide (white), are shown for each donor.

Cytotoxicity of p103-111 primed T-cells

To test the cytotoxic activity of p103-111-specific CD8+ T-cells, p103-111 prestimulated cells were used as effector cells in a europium release assay. As can be seen from Figure 2, p103-111-specific T-cells had a moderate cytotoxic activity against T2 cells loaded with p103-111 at E:T ratios ranging from 90:1 down to 20:1.

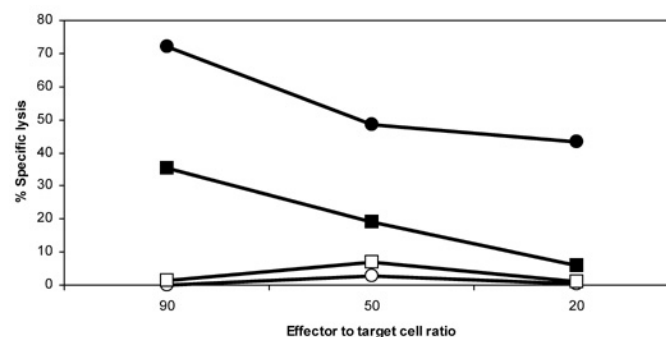


Figure 2. Cytotoxic activity of CD8+ T-cells stimulated with SSX2-derived p103-111. The lytic activity of T-cells was tested in a europium release assay. Specific lysis is shown at the indicated effector to target cell ratios, using 1000 labeled T2 cells as targets. Cells were pulsed with SSX2 p103-111 (filled boxes) or a control peptide (empty boxes). T-cells specific for the influenza matrix protein (IMP) derived peptide p58-66 were used as positive controls. Shown is the lysis of T2 cells loaded with IMP p58-66 (filled circles) or a control peptide (empty circles).

Specificity of the T-cell response to p103-111

To further delineate the specificity of the response against p103-111 by T-cells that had been sensitized with this peptide, p103-111 prestimulated T-cells were tested against T2 cells loaded with the HOM-MEL-40/SSX2-derived p103-111, SSX4-derived p103-111, CMV-derived p495-503 peptide, as well as a control peptide (p148-156 derived from human helicase). As shown in Figure 3, there was no cross reactivity between the SSX2- and SSX4-derived p103-111 peptides. Similarly, T-cells sensitized with the viral peptide reacted specifically with T2 cells loaded with CMV p495-503, but not with those loaded with the SSX-derived peptides. T-cells were derived from a patient with a tumor expressing HOM-MEL-40/SSX2 and SSX4. While T-cells specific for the SSX2 p103-111 peptide could be generated, the generation of T-cells specific for the SSX4 p103-111 peptide failed.

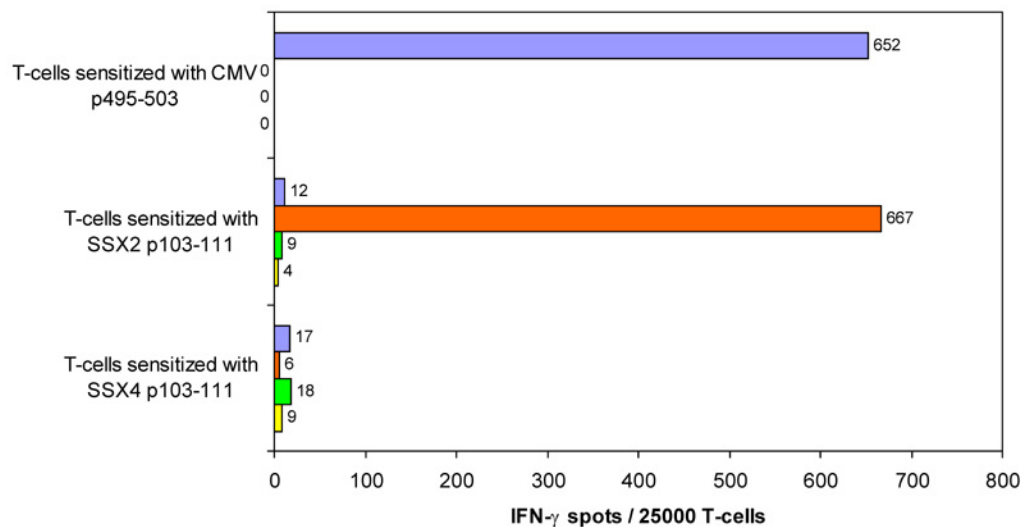


Figure 3. Specificity of HOM-MEL-40/SSX2 p103-111 stimulated CD8+ T-cells. The reactivity of T-cells prestimulated with SSX2-derived p103-111 was tested against peptides SSX2 p103-111 (red) and SSX4 p103-111 (green) by ELISPOT. T-cells prestimulated with SSX4 derived p103-111 were tested the same way to exclude any cross-reactivity. The peptides are located at the corresponding positions of the two antigens, which belong to the same cancer testis antigen family. T-cells were derived from a breast cancer patient with an SSX2 and SSX4 positive tumor. Peptide p148-156 derived from human helicase served as a negative control (yellow). T-cells specific for CMV p495-503 were used as a positive control (blue).

Demonstration of HLA-A*02 restriction of p103-111-specific T-cells

In addition to the fact that all patients responding to p103-111 stimulation had been preselected for HLA-A*0201 expression, definitive evidence for the HLA-A*0201 restriction of the T-cell response was obtained by blocking the T-cell response to p103-111 with the anti-human pan-HLA class I antibody W6/32 and the anti-HLA-A*02 antibody BB7.2. The specific response of T-cells to COS7/A2 cells loaded with HOM-MEL-40/SSX2-derived peptide was blocked by both the anti-pan-HLA class I antibody and the anti-HLA-A*02 antibody (Figure 4A). Similar results were observed when COS7/A2 cells transfected with SSX2 were used as targets (Figure 5A). Furthermore, CMV p495-503 was able to displace the SSX2 p103-111 peptide, as demonstrated by the fact that increasing concentrations of the viral peptide led to a decrease in specific reactions measured by ELISPOT assay (Figure 4B).

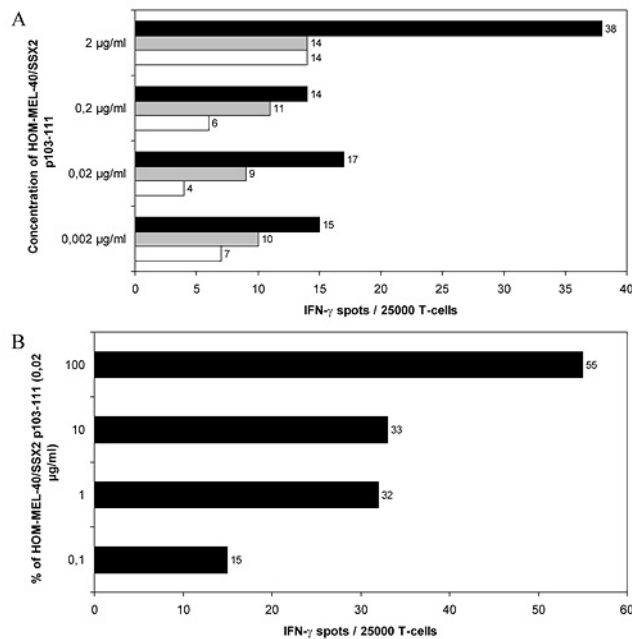


Figure 4. HLA-A*0201 restriction of SSX2-derived p103-111 stimulated CD8+ T-cells. (A) COS7/A2 cells were loaded with decreasing concentrations of SSX2 p103-111 peptide (black) ranging from 2 to 0.002 $\mu\text{g/ml}$. For blocking, the anti-pan human MHC-I antibody (clone W6/32) (grey) or the anti-HLA-A*02 antibody (clone BB7.2) (white) was added. (B) To displace SSX2 p103-111, T2 cells were loaded with a mix of SSX2 p103-111 peptide (0.02 $\mu\text{g/ml}$) and increasing concentrations of the HLA-A*0201 binding, CMV-derived p495-503 peptide. Reactions were measured by IFN-gamma ELISPOT assay.

Natural processing and presentation of the HOM-MEL-40/SSX2-derived p103-111 peptide

To demonstrate the natural processing and presentation of the HOM-MEL-40/SSX2-derived p103-111 peptide, T-cells that had been prestimulated with the peptide were challenged with COS7/A2 cells transfected with SSX2, as well as with SSX2 positive and negative cell lines. As can be seen from Figure 5 (panels A and B), p103-111 prestimulated T-cells reacted with SSX2-transfected COS7/A2 cells, but not with EGFP-transfected COS7/A2 cells. Similarly, p103-111 prestimulated T-cells reacted with the HLA-A*0201 positive cell line SK-MEL-37 that expresses HOM-MEL-40/SSX2. These reactions could be blocked by the anti-pan HLA class I antibody W6/32. In contrast, p103-111 prestimulated T-cells did not react with the SSX2 positive, HLA-A*02 negative cell line T47D, nor with the SSX2 negative cell lines BT 549 (HLA-A*02 positive) or ZR7530 (HLA-A*02 negative).

Correlation of MHC-I restricted T-cell and humoral anti-HOM-MEL-40/SSX2 responses

None of the seven patients tested for a T-cell response against the HOM-MEL-40/SSX2 p103-111 epitope had anti-HOM-MEL-40/SSX2 antibodies in their serum as determined by phage and recombinant antigen expression on yeast surface (RAYS) assays and none of the eleven healthy controls was HOM-MEL-40/SSX2 seropositive. Thus, no association between "T-cell responders" against p103-111 and "antibody responders" could be established.

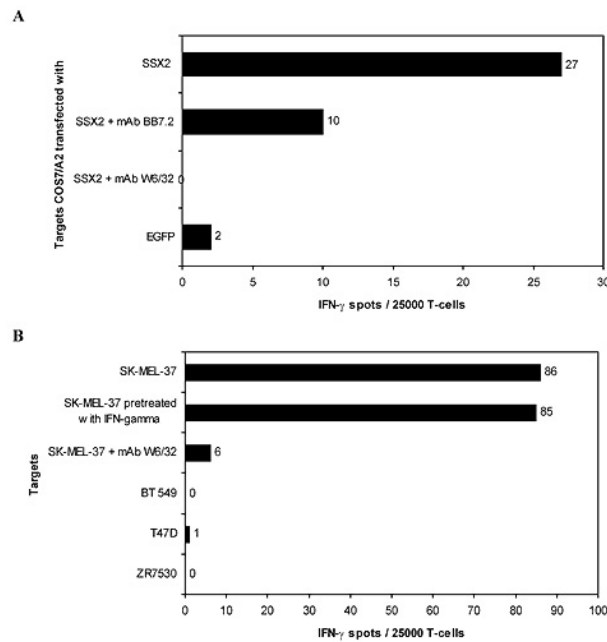


Figure 5. Natural processing and presentation of the HOM-MEL-40/SSX2-derived p103-111 peptide. (A) HLA-A*0201 restriction of SSX2-derived p103-111 stimulated CD8+ T-cells.

COS7/A2 cells transfected with HOM-MEL-40/SSX2 were used as targets and EGFP-transfected cells served as a negative control in the ELISPOT assay. The reaction was blocked by adding the anti-pan human MHC-I antibody (clone W6/32) or the anti-HLA-A*02 antibody (clone BB7.2). (B) Recognition of the HLA-A*02 positive, SSX2 positive SK-MEL-37 cell line. The recognition of the cell line by SSX2-derived p103-111 stimulated T-cells was demonstrated by ELISPOT assay. The effect of a pretreatment of the SK-MEL-37 cells with IFN-gamma was tested. In order to block the reaction against SK-MEL-37, the anti-pan human MHC-I antibody (clone W6/32) was added. The tumor cell lines BT 549 (HLA-A*02+/SSX2-), T47D (HLA-A*02-/SSX2+) and ZR7530 (HLA-A*02-/SSX2-) were used as negative controls.

Discussion

The reported frequent expression of HOM-MEL-40/SSX2 in a broad range of tumors highlighted this cancer-testis antigen as a potentially interesting target for immunotherapy of a broad spectrum of malignancies. The analysis of HOM-MEL-40/SSX2 expression in this large series of 65 consecutive newly diagnosed HLA-A*0201 positive breast cancer patients indicates that HOM-MEL-40/SSX2 is less frequently expressed than reported in previous studies. In contrast to the current study, which was restricted to a series of 65 newly diagnosed patients with breast cancer, patients with advanced metastatic and relapsed disease were included and even represented the majority of the patients (15). Therefore the lower incidence of HOM-MEL-40/SSX2 expression in this study might be explained by the previously reported observation that CTAs are often expressed in metastases of primary tumors that had been negative for the antigen.

The *HOM-MEL-40/SSX2* gene was cloned from a melanoma-derived cDNA library expressed in *E. coli* that was shown to code for an antigen detectable by high-titered IgG antibodies in the autologous serum from the melanoma patient. High-titered IgG antibodies imply cognate T-cell help; therefore the approach of reverse T-cell immunology should be most promising in patients with high-titered IgG serum antibodies. In a previous series of

16 melanoma patients (26), anti-SSX2 antibodies were found in 3 of 8 patients (38%) whose melanoma expressed HOM-MEL-40/SSX2, but not in healthy controls. In the current study, of the 7 patients with HOM-MEL-40/SSX2 positive breast cancers, anti-HOM-MEL-40/SSX2 antibodies were detected in none, suggesting that the frequency at which HOM-MEL-40/SSX2 positive breast carcinomas elicit antibody responses in the autologous host are lower than in melanomas. Due to the relative paucity of HOM-MEL-40/SSX2 expression in this series of 65 newly diagnosed breast cancer patients and the lack of HOM-MEL-40/SSX2 antibody positive patients, we did not restrict our analysis of CD8+ responses to antibody positive patients but included all patients with a HOM-MEL-40/SSX2 antigen positive tumor. Of the three HOM-MEL-40/SSX2-derived peptides with a high predicted binding affinity to HLA-A*0201, CD8+ T-cell responses against p103-111 could be detected in 5/7 patients, suggesting that this peptide is immunogenic in the majority of HLA-A*0201 positive individuals and patients with HOM-MEL-40/SSX2 positive tumors. When comparing antibody and CD8+ T-cell responses in these patients, no apparent correlations were detected. This is in line with observations with respect to cellular and humoral NY-ESO-1 responses (27), but in contrast to others (16, 28) suggesting a correlation between the demonstration of CD8+ responses and seropositivity; however, since the analysis of T-cell responses in these studies was restricted to a few seropositive patients, the claim of a correlation between CD8+ und antibody responses against CTAs does not withstand scrutiny (19). The lack of correlation between seropositivity and the demonstration of CD8+ T-cell responses is further supported by the observation in our study that CD8+ responses against HOM-MEL40/SSX2 were also detectable in 6/11 apparently healthy controls, all of which were antibody negative.

SSX2 is a member of the SSX multigene family which is composed of 9 genes, the products of which share 77-91% sequence homology (6, 29). All SSX genes are expressed in testis, but not in other normal tissues, except for SSX6, 8, and 9 which were not detected in any normal tissue (29) and all, with the exception of SSX3, 8, and 9 were found to be expressed in tumors or tumor cell lines (29). Based on their high degree of homology, it would be of interest to study the immunogenicity of the other members of the SSX family that are expressed in tumors. However, to date no antibody or cellular responses have been reported for members of the SSX gene family other than SSX1, 2, and 4. We also did not observe cross-reactions of T-cells specific for HOM-MEL-40/SSX2-derived p103-111 with the p103-111 homologue derived from SSX4, which differs from the former in 3 amino acids (Table 2).

The strategy of reverse immunology pursued in this study enables the prediction of potential HLA-class I ligands from any protein sequence *in silico*, based on the identification of appropriately positioned anchor residues specific for a given HLA class I allele (30, 31). The peptides that are predicted to bind to a given HLA class I molecule with the highest affinity are selected and their immunogenicity is then tested *in vitro*. Although this method allows a number of promising peptides to be identified, several of the latter were not presented by tumors as demonstrated by the poor recognition of cells expressing the corresponding protein of interest endogenously (32, 33, 34). Hence a crucial step for the application of reverse T-cell immunology is the demonstration that the CTLs generated can recognize target cells expressing the antigenic protein. The natural processing and presentation of p103-111 was proved by demonstrating that stimulated CTLs recognized both COS7/A2 cells transfected with HOM-MEL-40/SSX2, as well as SK-MEL-37, a cell line that endogenously expresses HOM-MEL-40/SSX2 and presents it in the context of HLA-A*0201, as shown by the specific blocking of the response by anti-pan-HLA class I antibody.

We were not able to generate CD8+ T-cell responses against all three HOM-MEL-40/SSX2 peptides predicted to bind with high affinity to HLA-A*0201 by the SYPEITHI algorithm. The failure to detect T-cells responding to p167-175 might be explained by the fact that this epitope is not processed by the proteasome and that the human T-cell repertoire might be devoid of T-cells with this specificity. In contrast, our failure to detect T-cell reactivity in patients with SSX2 positive breast cancers against p41-49 is more enigmatic. That p41-49 reactivity is induced *in vivo* is shown by recent findings that p41-49-specific CD8+ T-cell were readily detectable in a tumor-infiltrated lymph node population by multimer staining (25). Moreover, CTL lines isolated from this lymph node by multimer-guided cell sorting were able to lyse HLA-A*0201 positive tumor cells expressing SSX2. However, to date p41-49

reactive CD8+ T-cells have not been detected in additional patients. It remains to be determined whether only a minority of HLA-A*0201 patients can respond to p41-49 or whether specific conditions that are operative *in vivo* in a lymph node infiltrated by HOM-MEL-40/SSX2 tumor (or more specifically melanoma) cells, but that can not be simulated *in vitro*, are necessary to stimulate T-cells with specificity for p41-49. Similarly, the possibility that stimulation with peptide-loaded autologous DCs, instead of PBMCs, might induce p41-49-specific CD8+ T-cells with an affinity that is strong enough to recognize HOM-MEL-40/SSX2 positive targets can not be excluded.

Not all individuals tested in this study and expressing the HLA-A*0201 subtype showed a T-cell response to the p103-111 epitope *in vitro*. Individual differences in the T-cell reactions of patients against epitopes from tumor antigens are well-known: a possible reason for this might be the low number of T-cells used for the stimulations, so that no T-cells were generated that recognize the relevant epitope. Furthermore, PBMCs were used as antigen presenting cells. Therefore, it might well be that stimulations with peptide-loaded DCs instead of PBMCs might increase the percentage of p103-111 responders, further supporting the potential value of p103-111 as a promising target for HOM-MEL-40/SSX2 vaccine development.

Acknowledgements

We thank Claudia Schormann and Evi Regitz for excellent technical assistance, the operation theatre team at the Universitäts-Frauenklinik (Homburg) and the blood bank team at the Universitätsklinik Homburg for excellent co-operation, as well as Dr. Elke Jäger (Krankenhaus Nordwest, Frankfurt/Main, Germany). This study was supported by BIOMED II (CT BMH4-C98-3589) of the European Commission, Pf-135/7-1 and by Kompetenznetz Maligne Lymphome (TP 11) of the BMBF.

References

1. Preuss KD, Zwick C, Bormann C, Neumann F, Pfreundschuh M. Analysis of the B-cell repertoire against antigens expressed by human neoplasms. *Immunol Rev* 2002; **188**: 43-50. (PMID: 12445280)
2. Chen YT, Scanlan MJ, Sahin U, Tureci O, Gure AO, Tsang S, Williamson B, Stockert E, Pfreundschuh M, Old LJ. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc Natl Acad Sci USA* 1997; **94**: 1914-8. (PMID: 9050879)
3. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, van den Eynde B, Knuth A, Boon T. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 1991; **254**: 1643-7. (PMID: 1840703)
4. Boel P, Wildmann C, Sensi ML, Brasseur R, Renauld JC, Coulie P, Boon T, van der Bruggen P. BAGE: a new gene encoding an antigen recognized on human melanomas by cytolytic T lymphocytes. *Immunity* 1995; **2**: 167-75. (PMID: 7895173)
5. van den Eynde B, Peeters O, De Backer O, Gaugler B, Lucas S, Boon T. A new family of genes coding for an antigen recognized by autologous cytolytic T lymphocytes on a human melanoma. *J Exp Med* 1995; **182**: 689-98. (PMID: 7544395)
6. Gure AO, Tureci O, Sahin U, Tsang S, Scanlan MJ, Jager E, Knuth A, Pfreundschuh M, Old LJ, Chen YT. SSX: a multigene family with several members transcribed in normal testis and human cancer. *Int J Cancer* 1997; **72**: 965-71. (PMID: 9378559)
7. Tureci O, Sahin U, Zwick C, Koslowski M, Seitz G, Pfreundschuh M. Identification of a meiosis-specific protein as a member of the class of cancer/testis antigens. *Proc Natl Acad Sci USA* 1998; **95**: 5211-6. (PMID: 9560255)
8. Chen YT, Gure AO, Tsang S, Stockert E, Jager E, Knuth A, Old LJ. Identification of multiple cancer/testis antigens by allogeneic

antibody screening of a melanoma cell line library. *Proc Natl Acad Sci USA* 1998; **95**: 6919-23. (PMID: 9618514)

9. Tureci O, Sahin U, Koslowski M, Buss B, Bell C, Ballweber P, Zwick C, Eberle T, Zuber M, Villena-Heinsen C, Seitz G, Pfreundschuh M. A novel tumour associated leucine zipper protein targeting to sites of gene transcription and splicing. *Oncogene* 2002; **21**: 3879-88. (PMID: 12032826)

10. Sahin U, Tureci O, Schmitt H, Cochlovius B, Johannes T, Schmits R, Stenner F, Luo G, Schobert I, Pfreundschuh M. Human neoplasms elicit multiple specific immune responses in the autologous host. *Proc Natl Acad Sci USA* 1995; **92**: 11810-3. (PMID: 8524854)

11. Van den Eynde BJ, van der Bruggen P. T cell defined tumor antigens. *Curr Opin Immunol* 1997; **9**: 684-93. (PMID: 9368778)

12. Tureci O, Sahin U, Pfreundschuh M. Serological analysis of human tumor antigens: molecular definition and implications. *Mol Med Today* 1997; **3**: 342-9. (PMID: 9269687)

13. Schultz ES, Lethe B, Cambiaso CL, Van Snick J, Chaux P, Corthals J, Heirman C, Thielemans K, Boon T, van der BP. A MAGE-A3 peptide presented by HLA-DP4 is recognized on tumor cells by CD4+ cytolytic T lymphocytes. *Cancer Res* 2000; **60**: 6272-5. (PMID: 11103782)

14. Clark J, Rocques PJ, Crew AJ, Gill S, Shipley J, Chan AM, Gusterson BA, Cooper CS. Identification of novel genes, SYT and SSX, involved in the t(X;18)(p11.2;q11.2) translocation found in human synovial sarcoma. *Nat Genet* 1994; **7**: 502-8. (PMID: 7951320)

15. Tureci O, Chen YT, Sahin U, Gure AO, Zwick C, Villena C, Tsang S, Seitz G, Old LJ, Pfreundschuh M. Expression of SSX genes in human tumors. *Int J Cancer* 1998; **77**: 19-23. (PMID: 9639388)

16. Jager E, Chen YT, Drijfhout JW, Karbach J, Ringhoffer M, Jager D, Arand M, Wada H, Noguchi Y, Stockert E, Old LJ, Knuth A. Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. *J Exp Med* 1998; **187**: 265-70. (PMID: 9432985)

17. Jager E, Gnjatich S, Nagata Y, Stockert E, Jager D, Karbach J, Neumann A, Rieckenberg J, Chen YT, Ritter G, Hoffman E, Arand M, Old LJ, Knuth A. Induction of primary NY-ESO-1 immunity: CD8+ T lymphocyte and antibody responses in peptide-vaccinated patients with NY-ESO-1+ cancers. *Proc Natl Acad Sci USA* 2000; **97**: 12198-203. (PMID: 11027314)

18. Jager E, Jager D, Karbach J, Chen YT, Ritter G, Nagata Y, Gnjatich S, Stockert E, Arand M, Old LJ, Knuth A. Identification of NY-ESO-1 epitopes presented by human histocompatibility antigen (HLA)-DRB4*0101-0103 and recognized by CD4(+) T lymphocytes of patients with NY-ESO-1-expressing melanoma. *J Exp Med* 2000; **191**: 625-30. (PMID: 10684854)

19. Zarour HM, Storkus WJ, Brusic V, Williams E, Kirkwood JM. NY-ESO-1 encodes DRB1*0401-restricted epitopes recognized by melanoma- reactive CD4+ T cells. *Cancer Res* 2000; **60**: 4946-52. (PMID: 10987311)

20. Zarour HM, Maillere B, Brusic V, Coval K, Williams E, Pouvelle-Moratille S, Castelli F, Land S, Bennouna J, Logan T, Kirkwood JM. NY-ESO-1 119-143 is a promiscuous major histocompatibility complex class II T-helper epitope recognized by Th1- and Th2-type tumor-reactive CD4+ T cells. *Cancer Res* 2002; **62**: 213-8. (PMID: 11782380)

21. Zeng G, Touloukian CE, Wang X, Restifo NP, Rosenberg SA, Wang RF. Identification of CD4+ T cell epitopes from NY-ESO-1 presented by HLA- DR molecules. *J Immunol* 2000; **165**: 1153-9. (PMID: 10878395)

22. Zeng G, Wang X, Robbins PF, Rosenberg SA, Wang RF. CD4(+) T cell recognition of MHC class II-restricted epitopes from NY-ESO-1 presented by a prevalent HLA DP4 allele: association with NY-ESO- 1 antibody production. *Proc Natl Acad Sci USA* 2001; **98**: 3964-9. (PMID: 11259659)

23. Xie X, Wacker HH, Huang S, Regitz E, Preuss KD, Romeike B, Parwaresch R, Tiemann M, Pfreundschuh M. Differential expression of cancer testis genes in histological subtypes of non-Hodgkin's lymphomas. *Clin Cancer Res* 2003; **9**: 167-73. (PMID: 12538465)

24. Luo G, Huang S, Xie X, Stockert E, Chen YT, Kubuschok B, Pfreundschuh M. Expression of cancer-testis genes in human hepatocellular carcinomas. *Cancer Immunol* 2002; **2**: 11. (PMID: 12747756)

25. Ayyoub M, Stevanovic S, Sahin U, Guillaume P, Servis C, Rimoldi D, Valmori D, Romero P, Cerottini JC, Rammensee HG, Pfreundschuh M, Speiser D, Levy F. Proteasome-assisted identification of a SSX-2-derived epitope recognized by tumor-reactive CTL infiltrating metastatic melanoma. *J Immunol* 2002; **168**: 1717-22. (PMID: 11823502)

26. Tureci O, Sahin U, Schobert I, Koslowski M, Schmitt H, Schild HJ, Stenner F, Seitz G, Rammensee HG, Pfreundschuh M. The SSX-2 gene, which is involved in the t(X;18) translocation of synovial sarcomas, codes for the human tumor antigen HOM-MEL-40. *Cancer Res* 1996; **56**: 4766-72. (PMID: 8840996)

27. Valmori D, Dutoit V, Lienard D, Rimoldi D, Pittet MJ, Champagne P, Ellefsen K, Sahin U, Speiser D, Lejeune F, Cerottini JC, Romero P. Naturally occurring human lymphocyte antigen-A2 restricted CD8+ T-cell response to the cancer testis antigen NY-ESO-1 in melanoma patients. *Cancer Res* 2000; **60**: 4499-506. (PMID: 10969798)

28. Dutoit V, Taub RN, Papadopoulos KP, Talbot S, Keohan ML, Brehm M, Gnjjatic S, Harris PE, Bisikirska B, Guillaume P, Cerottini JC, Hesdorffer CS, Old LJ, Valmori D. Multiepitope CD8(+) T cell response to a NY-ESO-1 peptide vaccine results in imprecise tumor targeting. *J Clin Invest* 2002; **110**: 1813-22. (PMID: 12488431)
29. Gure AO, Wei IJ, Old LJ, Chen YT. The SSX gene family: characterization of 9 complete genes. *Int J Cancer* 2002; **101**: 448-53. (PMID: 12216073)
30. Boon T, van der Bruggen P. Human tumor antigens recognized by T lymphocytes. *J Exp Med* 1996; **183**: 725-9. (PMID: 8642276)
31. Rammensee H, Bachmann J, Emmerich NP, Bacher OA, Stevanovic S. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics* 1999; **50**: 213-9. (PMID: 10602881)
32. Valmori D, Gileadi U, Servis C, Dunbar PR, Cerottini JC, Romero P, Cerundolo V, Levy F. Modulation of proteasomal activity required for the generation of a cytotoxic T lymphocyte-defined peptide derived from the tumor antigen MAGE-3. *J Exp Med* 1999; **189**: 895-906. (PMID: 10075973)
33. Zaks TZ, Rosenberg SA. Immunization with a peptide epitope (p369-377) from HER-2/neu leads to peptide-specific cytotoxic T lymphocytes that fail to recognize HER-2/neu+ tumors. *Cancer Res* 1998; **58**: 4902-8. (PMID: 9809997)
34. Ayyoub M, Migliaccio M, Guillaume P, Lienard D, Cerottini JC, Romero P, Levy F, Speiser DE, Valmori D. Lack of tumor recognition by hTERT peptide 540-548-specific CD8(+) T cells from melanoma patients reveals inefficient antigen processing. *Eur J Immunol* 2001; **31**: 2642-51. (PMID: 11536162)
35. SYFPEITHI algorithm. URL: <http://www.syfpeithi.de/>
36. Lang KS, Moris A, Gouttefangeas C, Walter S, Teichgraber V, Miller M, Wernet D, Hamprecht K, Rammensee HG, Stevanovic S. High frequency of human cytomegalovirus (HCMV)-specific CD8+ T cells detected in a healthy CMV-seropositive donor. *Cell Mol Life Sci* 2002; **59**: 1076-80. (PMID: 12169019)
37. Mischo A, Wadle A, Watzig K, Jager D, Stockert E, Santiago D, Ritter G, Regitz E, Jager E, Knuth A, Old L, Pfreundschuh M, Renner C. Recombinant antigen expression on yeast surface (RAYS) for the detection of serological immune responses in cancer patients. *Cancer Immun* 2003; **3**: 5. (PMID: 12828452)
38. Blomberg K, Granberg C, Hemmila I, Lovgren T. Europium-labelled target cells in an assay of natural killer cell activity. I. A novel non-radioactive method based on time-resolved fluorescence. *J Immunol Methods* 1986; **86**: 225-9. (PMID: 3456003)
39. Blomberg K, Granberg C, Hemmila I, Lovgren T. Europium-labelled target cells in an assay of natural killer cell activity. II. A novel non-radioactive method based on time-resolved fluorescence. significance and specificity of the method. *J Immunol Methods* 1986; **92**: 117-23. (PMID: 3745921)
40. Patel AK, Boyd PN. An improved assay for antibody dependent cellular cytotoxicity based on time resolved fluorometry. *J Immunol Methods* 1995; **184**: 29-38. (PMID: 7622867)

Materials and methods

Ethical considerations

The study was approved by the local ethics review committee (Ethikkommission der Ärztekammer des Saarlandes) and was performed in accordance with the declaration of Helsinki. Recombinant DNA work was carried out with the permission and according to the regulations of the local authorities (Regierung des Saarlandes).

Patients

A total of 11 HLA-A*0201 positive healthy donors and 65 breast cancer patients were included in this study, and informed written consent was obtained from all. Blood samples from patients were obtained perioperatively in most of the patients before the initiation of any chemotherapy, except for patient G312 from whom blood was obtained after the first cycle of chemotherapy (4x EC, 3x CMF).

Cell lines

The SSX2-expressing SK-MEL-37 melanoma cell line was kindly provided by Dr. Elisabeth Stockert (LICR, New York). SK-MEL-37 is positive for SSX2 both at the mRNA and at the protein level as demonstrated by immunocytology (unpublished results). The BT 549 (HLA-A2 positive, SSX2 negative), T47D (HLA-A2 negative, SSX2 positive) and ZR7549 (HLA-A2 negative, SSX2 negative) breast cancer cell lines were kindly provided by Dr. Brigitte Gückel (Dept. Gynecology, University of Tübingen, Germany); the latter two served as controls. The HLA-A*02 positive and TAP-deficient cell line T2 was used in the tests, as well as COS7/A2, a cell line stably transfected with the human HLA-A*0201 molecule (kindly provided by Prof. Dr. Dolores Schendel, IMI, GSF Forschungszentrum für Umwelt und Gesundheit, München, Germany). Cell lines were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine and 1% penicillin/streptomycin (GIBCO, Invitrogen, Karlsruhe, Germany). In the case of SK-MEL-37 cells pretreated with IFN-gamma, the medium containing hrIFN-gamma (PeproTech, Rocky Hill, New York) at a concentration of 100 U/ml was changed every two days. Cells were used in the tests after 7 days.

Analysis of SSX2 mRNA expression by tumors

Fresh tumor biopsies were frozen within 15 min after surgical excision. SSX2 mRNA was analyzed by RT-PCR using the conditions described previously ([26](#)).

Prediction of HLA-binding peptides by the SYFPEITHI algorithm

Peptides were derived on the basis of the previously published SSX2 sequence ([26](#)). Potential HLA-A*0201 ligands were selected using a matrix pattern suitable for the calculation of nonamer or decamer peptides matching the HLA-A*0201 motif ([35](#)).

Synthetic peptides

Peptides predicted to bind with high affinity to HLA-A*0201 (Table 2) were synthesized following the Fmoc/tBu strategy ([36](#)). Purity was >90% as assessed by HPLC and mass spectrometry. All peptides were dissolved completely in a mixture of water and DMSO. The concentration for each peptide during pulsing was 2 µg/ml with the DMSO concentration during APC pulsing remaining <1% (v/v).

In vitro stimulation of T-cells with peptides

PBMCs from HLA-A*0201 positive patients and healthy donors were isolated by Ficoll-Paque™ PLUS separation (Amersham Pharmacia Biotech, Uppsala, Sweden), followed by enrichment of CD8+ cells by MACS (Miltenyi Biotech, Bergisch-Gladbach, Germany). The CD8 negative fraction was used as antigen presenting cells (APCs). CD8 negative cells were washed and resuspended in serum-free X-VIVO 15 medium (Biowhittaker Europe, Verviers, Belgium) and were pulsed with peptide at a concentration of 2 µg/ml for 1 h and irradiated with 30 Gy. To remove excess peptide, cells were washed and resuspended in X-VIVO 15 medium. 5x10⁵ peptide-loaded APCs were mixed with 5x10⁵ CD8+ cells per well in a 96-well flat-bottom plate (Nunc, Wiesbaden, Germany) and incubated at 37°C to generate specific T-cells. Cells were grown in 200 µl X-VIVO 15 supplemented with 10% human AB serum (Biowhittaker), 2 mM L-glutamine (Gibco, Invitrogen, Karlsruhe, Germany) and 10 ng/ml hrIL-7 (R&D Systems, Wiesbaden-Nordenstadt, Germany). The next day hrIL-2 (R&D Systems) was added for a final concentration of 20 U/ml. T-cells were restimulated every 7 days with autologous peptide pulsed PBMCs following the same procedure. The specificity of the T-cells was tested by IFN-gamma ELISPOT assay on day 20. Lytic activity was determined by europium release assay on day 5 after the third restimulation.

IFN-gamma ELISPOT assay

IFN-gamma ELISPOT assays were performed in nitrocellulose-lined 96-well microplates (MultiScreen-HA MAHAN 45, Millipore, Bedford, MA) that had been coated overnight at 4°C with anti-human IFN-gamma capture antibody (Mabtech, Nacka, Sweden) and washed six times. The assay procedure followed the manufacturer's instructions with an additional blocking step with 10% (v/v) heat-inactivated human AB serum for 1 h at 37°C. After washing the plates once, 2.5×10^4 effector T-cells and 5×10^4 target cells per well were incubated for 14-18 h at 37°C. Cells were removed and plates were washed six times. Reactions were developed with a second (biotinylated) anti-human IFN-gamma antibody (Mabtech). After another washing step the plates were incubated for 1 h with alkaline phosphatase conjugated streptavidin (Roche Diagnostics, Mannheim, Germany) diluted 1:2000 in PBS. IFN-gamma spots were visualized using the AP Conjugate Substrate Kit (BIO-RAD Laboratories, Hercules, CA, U.S.A.) following the manufacturer's instructions. Spots were counted using a BIOREADER 3000 Pro (BIOSYS, Karben, Germany).

Peptide-pulsed T2 (TAP-deficient) or COS7/A2 cells served as targets. To this end, T2 cells were loaded with peptide at a concentration of 2 µg/ml in serum-free X-VIVO 15 medium for 1 h at 37°C and irradiated with 120 Gy. Unbound peptide was removed by washing. An HLA-A*02 restricted high-affinity, but non-immunogenic peptide derived from human helicase (p148-156) served as a negative control. T-cells specific for the IMP-derived p58-66 or CMV-derived p495-503 viral peptides were used as positive controls. COS7/A2 cells were pulsed with peptide at concentrations of 2, 0.2, 0.02 and 0.002 µg/ml. Blocking with an anti-pan human MHC-I antibody (clone W6/32, DAKO, Glostrup, Denmark) and an anti-HLA-A*02 antibody (tissue culture supernatant of the clone BB7.2) was used as a control for the HLA-A*02 restriction of T-cell responses. To this end, COS7/A2 cells were incubated with antibody diluted 1:10 (the BB7.2 supernatant was diluted 1:4) in serum-free X-VIVO 15 medium for 30 min at 4°C. Peptide was added at the concentrations indicated above for 1 h at 37°C. Unbound peptide was washed and cells were resuspended in medium containing the antibody specified. To displace the SSX2 p103-111 peptide (0.02 µg/ml), CMV p495-503 peptide was added at the indicated concentrations, resulting in the SSX2 p103-111 peptide making up 100%, 10%, 1% and 0.1% of the total peptide concentration. For ELISPOT assays with cell lines, the SK-MEL-37 melanoma cell line, as well as the breast cancer cell lines BT 549, T 47D and ZR 7530 (untreated or pretreated with 100 U/ml IFN-gamma) were used as stimulators at a concentration of 5×10^4 cells per well after irradiation with 120 Gy. Furthermore, COS7/A2 cells transiently transfected with SSX2 or EGFP were used as targets. All tests were run in duplicate.

Detection of serum antibodies against HOM-MEL-40/SSX2

Serum antibodies against HOM-MEL-40/SSX2 were assessed by phage assay ([26](#)) or by recombinant antigen expression on yeast surface (RAYS) ([37](#)) at a serum dilution of 1:100.

Cytotoxicity assay

Cytotoxicity was assessed using T2 cells labeled with europium (2 mM) by electroporation prior to pulsing with peptides. Europium release assays were performed as described elsewhere ([38](#), [39](#), [40](#)) with moderate modifications. Briefly, effector T-cells were incubated with 1000 labeled target cells in 150 µl serum-free X-VIVO 15 medium for 2 h at 37°C with effector to target ratios ranging from 1:90 to 1:20. Europium release in the supernatant was measured. 10 µl of supernatant was added to 100 µl of Enhancement Solution (Wallac/Perkin Elmer, Freiburg, Germany) in a 96-well fluoronunc plate (Nunc, Wiesbaden, Germany) and incubated 10 min while shaking. Fluorescence was measured using the fluorescence counter Wallac VICTOR2 (1420 Multichannel Counter, Wallac / Perkin Elmer, Freiburg, Germany). All tests were run in duplicate. The percent specific lysis was calculated as: $100 \times [(\text{experimental} - \text{spontaneous release}) / (\text{total} - \text{spontaneous release})]$.

Transient transfection and IFN-gamma release assay

SSX2- and *EGFP*-encoding cDNA was cloned in pcDNA3.1 plasmid (Invitrogen Life Technologies, Basel, Switzerland). COS-7/A2 cells were transiently transfected with SSX2- or *EGFP*-encoding plasmids using Polyfect Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Transfected cells were tested for their ability to stimulate the release of IFN-gamma by peptide specific CTLs.

Contact

Address correspondence to:

Michael Pfreundschuh
Med. Klinik I
Saarland University Medical School
D-66421 Homburg
Germany
Tel.: + 49 6841 16 23002
Fax: + 49 6841 16 23101
E-mail: michael.pfreundschuh@uniklinik-saarland.de

Copyright © 2003 by Michael Pfreundschuh