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# Recombinant antigen expression on yeast surface (RAYS) for the detection of serological immune responses in cancer patients

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## Abstract

The serological analysis of antigens by recombinant expression cloning (SEREX) has identified a multitude of new tumor antigens in many different tumor entities. These antigens can be grouped into different classes according to their specificities, with cancer/testis antigens appearing to be the most attractive candidates for vaccine development. The observation that CD8 and CD4 T-cell responses against cancer/testis antigens such as NY-ESO-1 correlate with the presence of specific antibodies demonstrates the importance of serological monitoring patients participating in vaccine trials. However, all serological assays available (Western blot, phage display and ELISA) are hampered by the fact that the protein cannot be analyzed in its natural conformation. We have thus developed a yeast display system where the antigen is expressed on the yeast surface (RAYS), allowing for a more natural folding of the protein. To validate this approach we displayed the A33 colorectal cancer antigen on the yeast cell surface and demonstrated specific binding by an A33 monoclonal antibody recognizing a conformation-dependent epitope on the A33 antigen. We then compared RAYS with the more commonly used ELISA and Western blot serological monitoring methods by analyzing 50 sera from cancer patients with known NY-ESO-1 antibody status and 10 sera from patients with unknown SSX2 antibody status in a blind fashion. RAYS appears at least equivalent to both ELISA and Western blotting for the monitoring of antibodies against NY-ESO-1 as regards specificity and sensitivity, while antibodies against SSX2 were detected more frequently by RAYS than by ELISA or phage display.

## Introduction

The screening of tumor-derived gene expression libraries with high-titered IgG antibodies obtained from the sera of cancer patients represents a hallmark in modern tumor serology as it enables the straightforward identification of potential tumor-related antigens. This method, known as the "serological identification of antigens by

recombinant expression cloning" (SEREX), is now widely being used by tumor immunologists (1, 2). So far, SEREX has led to the identification of a multitude of new cancer antigens in many different tumor entities and the international Cancer Immunome database (3) holds more than 1,200 different gene entries of potentially cancer-related antigens identified in this manner. The importance of this technology goes beyond the serological identification of potential tumor antigens in allowing to create a link between T- and B-cell immunology, as strong antibody responses are considered to rely on the presence of T-cell help (4, 5). Thus, the analysis of the B-cell repertoire helps to identify antigens that might become potential targets for immunotherapy with cancer vaccines aiming to induce cytotoxic T-cell responses. The NY-ESO-1 antigen is an example of this concept, as it was initially identified by SEREX and was then detected by T-cell epitope cloning (6, 7). NY-ESO-1 has become one of the most actively investigated cancer antigens in early clinical trials. Studies have been initiated to learn how to optimally immunize and induce cellular immune responses against NY-ESO-1 by using different vaccination approaches, including vaccination with peptides, protein, DNA, bacterial and viral vectors.

However, evidence accumulated over recent years suggests that the antigen repertoire detectable by the conventional SEREX approach is limited (8). This might in part be attributed to the fact that potential antigens that are subject to posttranslational modifications remain undetected in a bacterial expression system which is generally not capable of modifying recombinantly expressed proteins post-translationally. Posttranslational modifications play an important role in the proper function of many proteins and also effect their immunogenicity. Hence, the characterization of such modifications is also very important for the development of recombinant protein-based vaccines (9). The spectrum of protein posttranslational modifications ranges from glycosylation, lipidation, phosphorylation, methylation, acylation, citrullination, deimination, to posttranslational truncation (10, 11). The modification of peptides during natural processing has also been described (12, 13).

To overcome some of the inherent problems with the conventional SEREX system, we attempted to establish a eukaryotic expression system in yeast for SEREX, designated as "recombinant antigen expression on yeast surface" (RAYS). Yeast has the advantage that recombinant proteins can be expressed on the cell surface in a more naturally folded, partially glycosylated (14, 15) manner. Therefore, antibody responses directed primarily against conformational epitopes that would not be detected by the conventional SEREX method may be more readily detected by RAYS, due to the improved accessibility of the epitopes when the antigen is displayed on the yeast surface.

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# Results

### Expression and detection of the A33 antigen on yeast cells

To develop the system and demonstrate the capacity of yeast to present tumor-associated antigens in their naturally folded state, we decided to express the colorectal cancer antigen A33 on the surface of yeast (Figure 1). Only yeast transformed with cDNA coding for the extracellular domain of the A33 antigen and induced for protein expression were stained with a monoclonal anti-A33 antibody. The staining was specific as antibody binding to non-induced yeast or yeast expressing unrelated antigens could not be detected.

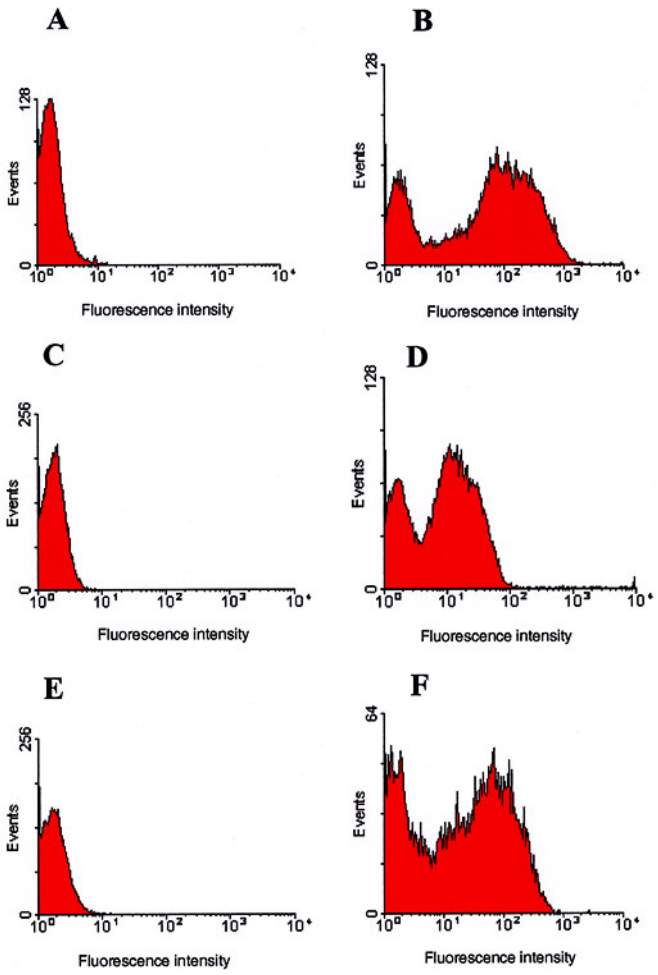


**Figure 1. Recombinant antigen expression on yeast surface.** The extracellular domain of A33 (A, B) or full length NY-ESO-1 (C-F) was cloned into the pYD1 vector and protein expression induced for at least 24 h (B, D, F). Yeast induced to express an irrelevant antigen served as negative controls (A, C, E). Samples were either stained with a monoclonal anti-A33 antibody (A, B), a monoclonal anti-NY-ESO-1 antibody (C, D) or serum from patients known to react with NY-ESO-1 protein at a dilution of 1:100 (E, F). All assays were carried out at least in triplicate. Isotype-matched control antibodies did not show any specific binding (data not shown).

### Expression of NY-ESO-1 protein on yeast cells

To test the broader applicability of the yeast display system for the detection of specific antibody responses to predefined targets, full length NY-ESO-1 protein was expressed on the yeast surface. Correct antigen expression was confirmed using a murine monoclonal antibody against NY-ESO-1 and sera from patients known to have specific antibody responses against the target antigen (Figure 1). Titration of the sera typically reveals three levels of binding, strong (filled diamonds), medium (filled triangles) and low (filled octagons, filled squares) (Figure 2). For some of the patient sera, a positive signal could be detected with serum dilutions of up to 1:10,000. Again, no binding was detected to non-induced yeast or yeast expressing an unrelated antigen.

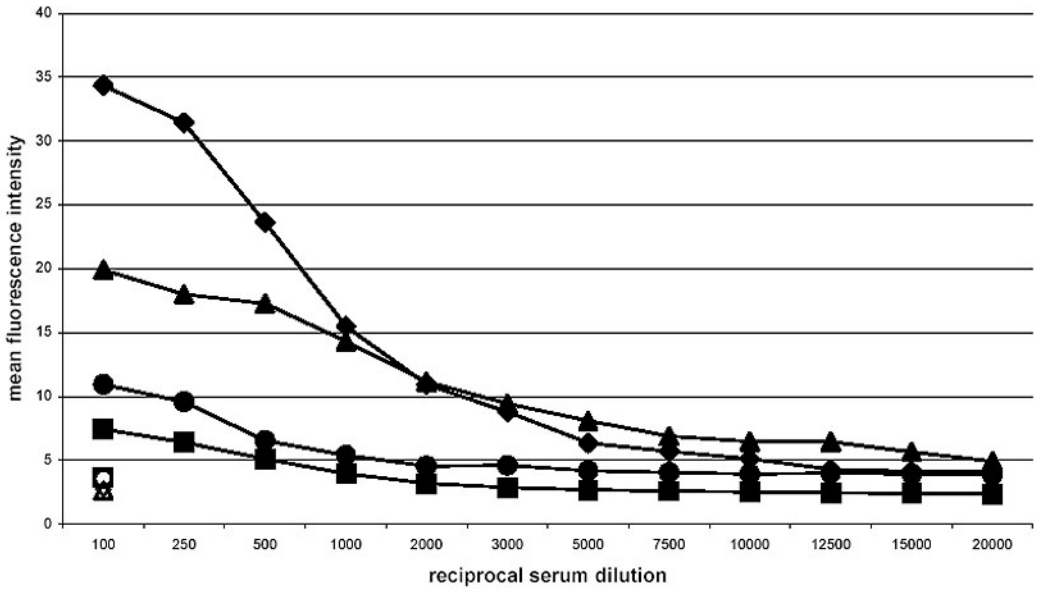


**Figure 2. Pattern of serological immune responses.** Sera from four patients known to have anti-NY-ESO-1 antibody responses were either analyzed on yeast induced to express NY-ESO-1 protein (filled symbols) or an irrelevant antigen as negative controls (open symbols). The reciprocal dilution factor is shown on the x-axis. According to prior experiments (data not shown), a sample at a particular dilution was determined to be positive if the ratio between the fluorescence intensity on NY-ESO-1 protein expressing yeast and that on non-induced yeast exceeded two. All assays were repeated three times. Sera from patients without NY-ESO-1 antibody responses were used as controls (data not shown).

### Comparative analysis of different expression systems for the detection of anti-NY-ESO-1 humoral responses in blood samples from cancer patients

Western blot assays and ELISAs have previously been standardized to detect NY-ESO-1 serum antibody. Both methods yield consistent results for large series of serum samples (16, 17). In this study, we set out to compare these assays against the yeast display system for the routine detection of anti-NY-ESO-1 antibody responses in the sera from cancer patients. In addition, the immuno-phage plaque assay forming part of the old SEREX method was run in parallel (Table 1). Of the 50 serum samples analyzed, 25 were determined to be positive by RAYS analysis, with one sample (F42) revealing a high unspecific background. The same result was obtained by Western blotting, with a good correlation between the intensity of the signals obtained in the two assays. ELISA failed to detect three positive samples. Of these, two (samples F22 and F30) were weak according to RAYS while the other (sample F3) gave a signal of intermediate intensity by Western blotting. Phage analysis was the least sensitive assay, identifying a total of 20 positive samples, but failing to detect six samples (F3, F14, F15, F28, F29, F30) that were positive according to RAYS/Western blotting. One sample (F4) was determined to be positive only by phage display.

**Table 1. Comparison of different serological technologies for the detection of anti-NY-ESO-1 immune responses[a].**

| Sample No. | RAYS[b] | | ELISA[c] | Western Blot[d] | Phage Display[d] |
|---|---|---|---|---|---|
| | FACS | Ratio | | | |
| F 1 | **49.68** | **21.6** | **1284** | **+++** | **+** |
| F 2 | 3.12 | 1.28 | 421 | - | - |
| F 3 | **11.63** | **4.02** | 410 | **++** | - |
| F 4 | 3.2 | 1.47 | 589 | - | **+** |
| F 5 | **12.88** | **5.37** | **1064** | **++** | **+** |
| F 6 | **57.11** | **16.22** | **1008** | **+++** | **+** |
| F 7 | 2.78 | 1.22 | 201 | - | - |
| F 8 | 2.97 | 1.28 | 260 | - | - |
| F 9 | 2.89 | 1.23 | 316 | - | - |
| F 10 | **9.49** | **4.12** | **884** | **+** | **+** |
| F 11 | **6.12** | **2.18** | **742** | **+** | **+** |
| F 12 | **11.45** | **3.78** | **1003** | **++** | **+** |
| F 13 | **35.38** | **15.72** | **1537** | **++** | **+** |
| F 14 | **3.3** | **2.64** | **658** | **+** | - |
| F 15 | **5.57** | **2.71** | **808** | **+** | - |
| F 16 | **5.13** | **2.26** | **534** | **+** | **+** |
| F 17 | **28.84** | **13.54** | **1389** | **+++** | **+** |
| F 18 | **8.03** | **2.84** | **812** | **++** | **+** |
| F 19 | **6.65** | **3.69** | **1161** | **+** | **+** |
| F 20 | **62.04** | **27.45** | **1037** | **+++** | **+** |
| F 21 | **63.75** | **27.48** | **1014** | **+++** | **+** |
| F 22 | **4.22** | **2.11** | 449 | **+** | **+** |
| F 23 | **18.95** | **7.52** | **1122** | **+++** | **+** |
| F 24 | **47.55** | **19.02** | **997** | **+++** | **+** |
| F 25 | **13.67** | **5.69** | **1595** | **+++** | **+** |
| F 26 | **31.23** | **13.17** | **1299** | **++** | **+** |
| F 27 | **59.12** | **26.6** | **1340** | **+++** | **+** |
| F 28 | **2.72** | **2.16** | **772** | **+** | - |
| F 29 | **9.1** | **3.8** | **874** | **+** | - |
| F 30 | **6.49** | **2.58** | 274 | **(+)** | - |
| F 31 | 3 | 1.15 | 246 | - | - |
| F 32 | 2.85 | 1.84 | 302 | - | - |
| F 33 | 3 | 1.26 | 215 | - | - |
| F 34 | 3 | 1.16 | 137 | - | - |
| F 35 | 3.99 | 1.54 | 280 | - | - |
| F 36 | 4.5 | 1.48 | 429 | - | - |
| F 37 | 3.2 | 1.22 | 593 | - | - |
| F 38 | 3.4 | 1.27 | 271 | - | - |
| F 39 | 3.3 | 1.34 | 274 | - | - |
| F 40 | 3.7 | 1.35 | 225 | - | - |
| F 41 | 3 | 1.18 | 133 | - | - |
| F 42 | <0 | 1.52 | 312 | - | - |
| F 43 | 4.1 | 1.45 | 194 | - | - |
| F 44 | 3.1 | 0.8 | 453 | - | - |
| F 45 | 3.4 | 1.31 | 224 | - | - |
| F 46 | 3.4 | 1.29 | 346 | - | - |
| F 47 | 3.4 | 1.27 | 352 | - | - |
| F 48 | 2.2 | 1.6 | 360 | - | - |
| F 49 | 3.1 | 1.18 | 287 | - | - |
| F 50 | 3.5 | 0.34 | 199 | - | - |

[a]Fifty serum samples from patients with different malignancies were analyzed in a blind fashion. Serum samples were diluted 1:100 for RAYS and phage display, 1:400 for ELISA and 1:250 for Western blot analysis. Individual values highlighted in bold are considered positive.
[b]Antibody binding to yeast was determined by FACS and the mean fluorescence intensity values are given. The RAYS ratio was determined by dividing the fluorescence intensity obtained by the individual serum sample on NY-ESO-1 protein positive yeast by the fluorescence intensity that was obtained on yeast expressing an irrelevant antigen. Samples with a ratio of two or more were considered positive.
[c]The mean ELISA values (OD at 405 nm) of triplicate analyses are given.
[d]The intensity of the signal detected by Western blotting or phage analysis was determined visually.

### Detection of anti-SSX-2 antibody responses

To extend the analysis beyond NY-ESO-1, we decided to study the immune responses against another member of the cancer/testis antigen family, the SSX-2 antigen (Table 2). Detection of anti-SSX-2 immune responses by ELISA and phage analysis turned out to be difficult as results varied considerably. Ten serum samples from cancer patients were analyzed by ELISA and RAYS. Only 1 of the 10 sera was reactive by ELISA (sample # 1). In contrast, 4 of the 10 sera (samples #1, 5, 6, 10) gave signals significantly exceeding background staining activity in RAYS. Specific immune responses to SSX-2 in SSX-2 positive patients were detected with serum dilutions of up to 1:10,000 (data not shown).

**Table 2. Comparison of different serological technologies for the detection of anti-SSX-2 immune responses[a].**

| Sample No. | RAYS | | ELISA |
|---|---|---|---|
| | FACS | Ratio | |
| 1 | **46.9** | **20.9** | **486** |
| 2 | 2 | 0.75 | 191 |
| 3 | 3 | 0.72 | 118 |
| 4 | 2 | 0.82 | 239 |
| 5 | **15.1** | **6.5** | 187 |
| 6 | **80.1** | **30.23** | 230 |
| 7 | 3 | 0.87 | 154 |
| 8 | 2 | 0.83 | 230 |
| 9 | 4 | 0.74 | 134 |
| 10 | **29.5** | **10.5** | 189 |

[a]Serum samples from 10 patients with solid cancers were analyzed for the presence of anti-SSX-2 antibodies by ELISA (1:400 dilution) and RAYS (1:100 dilution) as described. Positive samples are highlighted in bold. The intensity of the signal obtained by the individual assay and the yeast ratio were determined as described for Table 1. The ELISA data shown are mean values of triplicate analyses.

## Discussion

Transferring the SEREX technology into a eukaryotic expression system can potentially open a new area for the identification of tumor antigens. The A33 antigen used in this paper is an example where the monoclonal antibody generated by mouse hybridoma technology only recognizes the antigen in a properly folded state, either on the cell surface or under non-reducing conditions (18, 19). The antibody does not bind to the reduced or linear A33 antigen. The fact that the antibody binds to the A33 antigen when expressed on the yeast surface is a clear indication that the antigen is properly folded under the conditions studied. This establishes the yeast system as an important tool to study antibody-antigen interactions, especially if correct folding or cell surface presentation of the antigen is required. The A33 antigen is not the only tumor antigen for which an antibody directed against a conformational epitope exists (20). The observation that antibodies recognize a significant proportion of B-cell epitopes in a conformational-dependent manner is not new (21). There is good evidence in the literature that a protective polyclonal immunity in patients harboring either infections or malignant diseases is directed either at the tertiary folded antigen structure or even at protein modifications caused by glycosylation (22, 23).

Having demonstrated the capability of a eukaryotic expression system to display the colon cancer associated A33 antigen in a properly folded state, we set out next to analyze the immune response in cancer patients, aiming to overcome the current limitations of bacterial- and phage-based systems. RAYS was shown to be a usefulness system to analyze immune responses against the well-characterised cancer/testis (CT) antigen NY-ESO-1 (6), as it yielded highly reproducible results and low inter-test variation (data not shown). Concerning the specificity of the system, we compared RAYS against well-established prokaryotic systems such as ELISA, Western blotting and phage display (1, 5, 16). The results obtained by RAYS and Western blotting were not only identical, but showed a correlation between the intensity of the signals obtained in the two assays. Moreover, RAYS was superior to ELISA or phage display assays as regards the identification of positive immune responses. The sensitivity of the yeast and the established bacterial expression systems differed. For the bacterial system, positive signals can be detected with serum dilutions of up to 1:100,000 using either Western blotting or ELISA (16). In our yeast system the detection limit is around 1:5,000 to 1:10,000 for NY-ESO-1. However, the yeast system was more sensitive for the detection of immune responses against other tumor antigens, such as SSX-2, than the ELISA. Therefore, the difference in detection levels cannot be explained solely by a limitation of the individual system, but may depend mainly on the tumor antigen chosen for analysis. RAYS is currently being extended for the analysis of immune responses against other CT antigens for which the detection of positive signals is difficult to obtain in a reliable fashion, such as other SSX family members (24, 25), and other antigens for which immune responses are extremely rare, such as against the MAGE family members (16, 26). Interestingly, we found 4 out of 10 positive immune responses against SSX-2 by RAYS compared to only one positive response detected by ELISA. The SSX-2 gene was initially found to be involved in the t(X;18) translocation of synovial sarcomas, but it can be found by RT-PCR in approx. 20% of all cancers with the highest expression levels (72%) in neuroblastoma (27). However, serological immune responses which are much more rare (approx. 10%) are detected with some ambiguity as regards the consistency of the results (28). Serum samples used for the initial identification of SSX-2 in the mid-90s turned out to be negative over the years when measured using recombinant protein by ELISA (data not shown). These samples were still highly positive when analyzed by RAYS; this indicates that the currently available recombinant bacterial protein does not display the entire spectrum of epitopes recognized by anti-SSX-2 immune responses. Using a eukaryotic expression system might reveal if immune responses are really rare or whether currently used assays are inadequate to reveal the whole picture.

The RAYS technology can be extended to the screening of cDNA libraries derived from all types of tissue. Recently, the group from Wittrup demonstrated the feasibility of large-scale antibody display on the surface of yeast and proved the versatility of the yeast display system (29). This opens the perspective that RAYS will enable the identification of a new flavor of tumor antigens that have escaped detection to date because they elicit immune responses against post-translational modified or conformational epitopes that are not accessible to the conventional SEREX approach.

## Abbreviations

RAYS, recombinant antigen expression on yeast surface; SEREX, serological analysis of antigens by recombinant expression cloning

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# Materials and methods

### Patients

All patients in this study were treated in the outpatient departments of the II. Medizinische Klinik at Krankenhaus Nordwest in Frankfurt or of the I. Medizinische Klinik in Homburg, Saarland Medical School.

### Strains and media

*Escherichia coli* DH5alpha was used as a host for recombinant DNA manipulation and was grown in LB medium [1% (w/v tryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) sodium chloride] containing 50 µg/ml ampicillin. The *Saccharomyces cerevisiae* EBY100 strain (13) was used as host for yeast surface display. Yeast were grown either in YPD medium (1% yeast extract, 2% polypeptone, 2% dextrose) or in YNB/CAA-Glu [2% dextrose, 0.67% yeast nitrogen base, 0.5% Casamino acids (Difco)] at 30°C with constant agitation to an approx. OD at 600 nm of 4. To induce surface protein expression, yeast were pelleted by centrifugation, resuspended to an OD at 600 nm of 0.6 in YNB/CAA-Gal and grown with agitation at 20°C for 48 h.

### Construction of plasmids and yeast transformation

The pYD1 plasmid was part of the pYD1 yeast display vector kit from Invitrogen (Leiden, The Netherlands). cDNA coding for NY-ESO-1 (primers 5'-GGG **GAA TTC** CAG GCC GAA GGC CGG GGGC AC-3' and 5'-GGG **CTC GAG** TTA GCG CCT CTG CCC TGA GGG-3') and SSX-2 (primers 5'-GGG GGG **GAA TTC** AAC GGA GAC GAC GCC TTT G-3' and 5'-CTA **CTC GAG** TTA CTC GTC ATC TTC CTC AGG GTC-3') were amplified by PCR from phage supernatant. The extracellular domain of the A33 glycoprotein (primers 5'-GGG GGG **GAA TTC** ATC TCT GTG GAA ACT CCG CAG-3' and 5'-GGG GGG **CTC GAG** GTT CAT GGA GGG AGA TCT GAC-3') was amplified from a peripheral blood library. All inserts were cloned into the *Eco*RI/*Xho*I sites of pYD1.

The plasmids pYD1, pYD1-NY-ESO-1, pYD1-SSX2 and pYD1-A33 were introduced into *S. cerevisiae* EBY100 using a commercially available transformation kit (EasyComp, Invitrogen, The Netherlands) according to the manufacturer's recommendations. Thereafter, yeast were spread on MD-Leu plates (0.67% yeast nitrogen base, 2% dextrose, 0.01% leucine, 1.5% agar) for the selection of transformed clones.

### Pre-absorption of human sera

Induced yeast containing the empty pYD1 vector were pelleted and washed twice with PBS. Patients' sera were diluted 1:50 in PBS and mixed with approx. 1/10 volume of pelleted yeast. This preabsorption mix was agitated overnight at 4°C to eliminate unspecific binding to yeast surface molecules. After pelleting, the supernatant was recovered and diluted with PBS to a final serum dilution of 1:100. Sodium azide [0.05% (w/v)] was added for preservation.

### Immunofluorescence staining

Yeast cells were collected by centrifugation at 2000 x *g* for 5 min and washed with PBS. Yeast containing pYD1 as a control and yeast containing either pYD1-NY-ESO-1, pYD1-A33 or pYD1-SSX-2 were incubated at room temperature with 100 µl of pre-absorbed serum (1:100 dilution) for 30 min with occasional agitation. After washing, secondary biotinylated anti-human-IgG Fcgamma specific serum (Dianova, Hamburg, Germany) diluted 1:200 was added and incubated for 30 min at room temperature with occasional agitation. The A33 antigen was stained using a humanized anti-A33-antibody (18) diluted 1:300 and the same secondary reagents. A murine monoclonal anti-NY-ESO-1 antibody was used to demonstrate NY-ESO-1 expression on the yeast surface. Antibody binding was detected using a biotinylated anti-murine IgG Fcgamma specific serum (Dianova, Hamburg, Germany) in the same manner as described above.

### Flow cytometry

Labeled yeast cell suspensions were analyzed by flow cytometry (FACScan, Becton Dickinson, Heidelberg, Germany) and the data analyzed using Cell Quest software according to the manufacturer's guidelines. For each sample, 30,000 cells were collected. The ratio between the intensity of the signal measured on antigen expressing (induced) and non-induced (pYD1) yeast was calculated for each individual serum sample. A sample was considered to be positive if this ratio was greater than or equal to 2.

### Western blot analysis

Serum antibody responses against recombinant NY-ESO-1 protein were tested by standard Western blot analysis (30). Briefly, 1 µg of purified recombinant NY-ESO-1 protein was diluted in SDS and electrophoresed on a 15% SDS polyacrylamide gel. In all assays, the recombinant NY-ESO-1 ‘short’ protein (amino acids 10-121; molecular weight 14 kDa) was used, as it was previously shown that the full length and recombinant NY-ESO-1 ‘short’ proteins are recognized equally well by serum IgG antibodies (15). After blotting overnight on a

nitrocellulose filter (0.45 µm, Sartorius, Göttingen, Germany) and blocking with 3% BSA in TBS, blots were incubated with either patients' sera at 1:250, 1:1,000, and 1:10,000 dilutions or the E978 mouse monoclonal antibody, diluted 1:250, against NY-ESO-1 as a positive control (15). To show that recognition of the recombinant NY-ESO-1 'short' protein (14 kDa) by serum IgG antibodies corresponds to recognition of the naturally expressed NY-ESO-1 protein (22 kDa), 5 µl of lysate of the NY-ESO-1-expressing melanoma cell line NW-MEL-38 was used. Serum antibodies binding to NY-ESO-1 were detected by incubation with goat anti-human IgG (Fc-spec.; Sigma, Munich, Germany) diluted 1:10,000, or in the case of E978 with goat anti-mouse IgG (Bio-Rad, Munich, Germany) diluted 1:3,000, and visualized with NBT/X-phosphate (Sigma, Munich, Germany). Serum samples were considered positive for NY-ESO-1 antibody when reactivity with the 14 kDa recombinant NY-ESO-1 'short' protein and/or the naturally expressed 22 kDa NY-ESO-1 protein was detectable.

**Enzyme-linked immunosorbent assays (ELISAs)**

10 µl/well of 1 µg/ml recombinant protein in coating buffer (15 mM $Na_2CO3$, 30 mM $NaHCO_3$, pH 9.6, with 0.02% $NaN_3$) was adsorbed overnight at 4°C to TC microwell plates 60 × 10 (Nunc, Roskilde, Denmark). The plates were then washed with PBS and blocked overnight at 4°C with 10 µl/well of 2% BSA in PBS. After washing, 10 µl/well of serum dilutions in 2% BSA were added and the plates incubated for 2 h at room temperature. The plates were then washed and 10 µl/well diluted secondary antibody (goat anti-human IgG-AP; Southern Biotechnology, Birmingham, AL, USA) in 2% BSA was added. Following incubation for 1 h at room temperature, the plates were washed, incubated with 10 µl/well of substrate solution (Attophose substrate; JBL Scientific, San Louis Obispo, CA, USA) for 25 min at room temperature, and immediately read using a CytoFluor 2350 (Millipore, Bedford, MA, USA). For the serological survey of human sera, sera were tested over a range of serial 4-fold dilutions from 1:100 to 1:100,000. A positive reaction was defined as an OD value for a serum diluted 1:400 that exceeds the mean OD value of sera from normal donors by three standard deviations.

**Immunoscreening**

A phage assay described by us in detail previously (1) was used to detect IgG reactivity against *E. coli* transfectants. *XLI MRF* bacteria transfected with recombinant ZAP Express phages were plated onto Luria-Bertani agar plates. Expression of recombinant proteins in lytic phage plaques on the bacterial lawn was induced with IPTG (Roche, Mannheim, Germany). Plates were incubated at 37°C until plaques were visible and then blotted onto nitrocellulose membranes. The membranes were blocked with 5% low-fat milk in Tris-buffered saline and incubated with a 1:250 dilution of the patient's serum that had been preabsorbed with transfected *E. coli* lysates. Serum antibodies binding to recombinant proteins expressed in lytic plaques were detected by incubation with alkaline phosphatase-conjugated goat anti-human IgG and visualized by staining with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Sigma, Munich, Germany).

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