Abstract. Background/Aim: To evaluate cancer/testis (CT) antigens as targets for immunotherapy or vaccine approaches in prostate cancer. Patients and Methods: We investigated the antibody response in 181 patients with prostate cancer, 83 benign prostate hyperplasia (BPH) patients, and 39 healthy donors against 13 different CT antigens recombinantly expressed on yeast surface (RAYS) and compared the results to antigen expression in tumor tissue. We then used the yeast clone expressing the most promising antigen directly as a vaccine to elicit potent cellular immunity. Results: The antibody response to NY-ESO-1 was more frequent (20%) and strong compared to other investigated antigens, and was associated with progressive disease. Interestingly, it was also detected in several BPH patients (9%). Feeding dendritic cells with NY-ESO-1-expressing yeast cells resulted in efficient HLA presentation and activation of specific CD3+ T-cells. Conclusion: The RAYS approach offers a fast means of analyzing serological autoreactivity in cancer patients and serves as an effective anticancer vaccine platform.

Prostate cancer (PCA) is the most common cancer affecting men in the Western world. Whereas for organ-confined PCA, healing can be achieved in a high percentage by radical surgery or radiation, there is currently no known cure for metastatic prostate cancer. Although growth of PCA cells is initially dependent on androgens, tumor cells become androgen insensitive over time resulting in further tumor progression of so-called hormone-refractory prostate cancer. For this reason, androgen deprivation is the first choice of treatment, followed by palliative docetaxel-based chemotherapy as second line. Especially for metastatic and hormone-refractory PCA, new therapeutic approaches are desperately needed. A promising goal is the identification of tumor antigens as target structures for antibody and vaccine approaches (1).

The concept of immunising cancer patients against specific tumor-associated antigens (TAA) has thrilled immunologists for the last two decades and remains very attractive although the definite proof of its efficacy is still missing (2). A prerequisite for the development of tumor-specific immunotherapeutic strategies is the existence of tumor antigens, i.e., genes that are either exclusively or preferentially expressed by malignant compared to normal tissues. Cancer/testis (CT) antigens represent a promising class of tumor antigens because of their restricted expression pattern (3). Furthermore, it has been demonstrated for some of these antigens that they not only elicit a humoral B-cell response but also a cellular CD8+/CD4+ T-cell response (4-6). Prime candidates for developing vaccine trials among these CT antigens are synovial sarcoma, X breakpoint 2 (SSX-2), cancer/testis antigen 1B (NY-ESO-1), and melanoma antigen family A, 3 (MAGE-3).

In this study, we used recombinant antigen expression on yeast surface (RAYS) as a versatile tool for both the detection of humoral response to CT antigens in PCA patients and the rapid production of an anticancer vaccine. Firstly, we investigated the prevalence of antibody responses to known CT antigens in PCA and benign prostate hyperplasia (BPH) patients and analyzed the antigen expression by immunohistochemistry. Based on our results, we directly used the CT antigen-expressing yeast as a vaccine with the potential to induce HLA-dependent peptide presentation on the surface of antigen-presenting cells (APCs), inducing potent cytotoxic T-cell activation. This crucial and early step is necessary in the process of achieving effective therapeutic anticancer vaccines.
Patients and Methods

Patient samples and study group population. This study was approved by the local Ethical Review Board and performed according to the Declaration of Helsinki. After informed consent, serum samples were collected from 181 patients suffering from pathologically confirmed PCA, 83 age-matched patients with BPH and from 39 male healthy donors. Gleason score, pathological stage, histological diagnosis and TNM classification were performed according to the guidelines of the Union International contre Cancer 2002 (7).

Expression of CT antigens on yeast surface (RAYS). The amplified coding regions of the CT antigens GAGE-1 to -3, NY-ESO-1, SSX-1, SSX-2, SSX-3, SSX-4, SSX-5, SCP-1, LAGE-1a, LAGE-1b, and CT-7 were cloned into the multiple cloning site of the pYD1 plasmid and antigens expressed on yeast surface as described elsewhere (8). To determine the best surface expression of the CT antigens used, we cultured the different yeast strains in 11 shaking flasks for a time period of 120 h, frequently measuring the NY-ESO-1 expression. Protein production in batch culture was compared by fed batch cultivation replacing 80% of the used medium every day.

Serological analysis by flow cytometry. Detection of immunological response at single sera level was performed using 30,000 yeast cells displaying the various CT antigens on their cell surface. All preabsorbed sera were diluted 1:100 and the staining procedure was performed as described elsewhere (8-10). All antigens carried a His-tag which was detected using the specific biotinylated anti-penta-His antibody (1:200, room temperature (RT), 15 min, Qiagen, Hilden, Hamburg, Germany). FACS analysis was carried out using WinMDI 2.8 software (The Scripps Research Institute, San Diego, USA). Ratios of the mean fluorescence of antigen-expressing yeast and mock-transformed yeast were calculated for each serum. A serum was considered responsive when this ratio exceeded the mean value of the healthy control group’s ratio by more than 3 times the standard deviation (11). To analyze phagocytosis, yeast cells were labelled with the visible fluorescent dye Vybrant CFDA SE (Invitrogen, Leiden, the Netherlands) following the manufacturer’s guide. After staining, arrays and tissue sections were analyzed using a Nikon Eclipse TE2000-U light microscope.

Expression of CT antigens on yeast surface (RAYS). The amplified coding regions of the CT antigens GAGE-1 to -3, NY-ESO-1, SSX-1, SSX-2, SSX-3, SSX-4, SSX-5, SCP-1, LAGE-1a, LAGE-1b, and CT-7 were cloned into the multiple cloning site of the pYD1 plasmid and antigens expressed on yeast surface as described elsewhere (8). To determine the best surface expression of the CT antigens used, we cultured the different yeast strains in 11 shaking flasks for a time period of 120 h, frequently measuring the NY-ESO-1 expression. Protein production in batch culture was compared by fed batch cultivation replacing 80% of the used medium every day.

Serological analysis by Western blot analysis. Serum antibody responses against NY ESO 1 protein were tested by standard Western blot analysis (8). Briefly, 500 ng of bacterially expressed NY-ESO-1 protein were loaded per lane, separated by SDS-polyacrylamid-gелеlectrophoresis and blotted on a Nylon membrane (PVDF) (Bio-Rad, Munich, Germany). The strength of the NY-ESO-1-specific sera response was measured by a dilution series of previously determined positive and negative sera (1:200/500/1,000/ 5,000/20,000). Mouse monoclonal anti-NY-ESO-1 antibody (1:5,000, Upstate, Charlottsville, USA) and an irrelevant rabbit anti-SSX-2 antibody (1:1,000 Aviva Systems Biology, San Diego, CA) served as controls. NY-ESO-1-specific antibodies, whether from human or from mouse, were detected by specific F(ab’2)-conjugated peroxidase antibodies (1:20,000, Dianova, Hamburg, Germany). Enhanced chemoluminescence kit (GE Healthcare, Germany) was used for detection.

Immunohistochemistry and microscopy. Immunohistochemical staining of NY-ESO-1 protein was carried out on paraffin-embedded sections with mouse monoclonal anti-NY-ESO-1 antibody (clone E978) as described elsewhere (11). Tissue arrays (CC19-01-005, CC19-01-003) were obtained from Cybrdi (Frederick, USA). They contained 39 different pathologically confirmed PCA patient tissue samples arranged either in duplicates or triplicates. Additionally, paraffin-embedded tissue sections obtained from 14 seropositive PCA patients and 7 seropositive BPH patients were stained following the same procedure. After staining, arrays and tissue sections were analyzed using a Nikon Eclipse TE2000-U light microscope.

NY-ESO-1 processing and presentation after yeast uptake. Monocyte-derived macrophages were generated according to protocols previously reported (12). After feeding with NY-ESO-1 protein expressing yeasts, HLA-A0201*/NY-ESO-1157-165 complexes were detected by biotinylated Fab-antibody 3MA4E5 (13) (45 min, 4˚C, 10 μg/ml) and phycoerythrin-conjugated steptavidin (30 min, 4˚C, 1:200) (Invitrogen;) using a FACSFlow cytometer (BD Clontech, Heidelberg, Germany) or Nikon Eclipse microscope.

T-Cell activation after yeast uptake by APCs was recorded by ELISPOT assays (14). NY-ESO-1 peptide-presenting dendritic cells (10⁴ cells per well) were detected by a CD8+ HLA-A2/NY-ESO-1157-165-specific T-cell clone (15). DCs and T-cells were co-cultured at a target:effector cell ratio of 1:1. DCs were co-incubated with either protein (10 μg bacterial expressed NY-ESO-1 combined with CpG (OD2006; Invitrogen)) or yeast for 24 h at 37˚C. For the visualization of T-cell activation, supernatants were removed and interferon-γ (IFN-γ) spots were stained using IFN-γ detection antibody (1:1,000, 2 h, 37˚C) (Dako, Copenhagen, Denmark) or Fluorescein isothiocyanate-conjugated anti-human IFN-γ (1:500, 2 h, 37˚C) (BD Biosciences, Heidelberg, Germany) and demonstrated by flow cytometry (Figure 1A). Next, we analyzed a series of 1:100 diluted sera from 70 PCA patients, 14 BPH patients, and 39 healthy donors for the presence of CT antigen-specific antibodies. The strongest and most frequent antibody reactions were observed against the NY-ESO-1 (20%), SCP-1 (8.5%), LAGE-1a (4.3%) and LAGE-1b (5.7%) antigens (Figure 1B, Table I). A few sera also contained IgGs specific for CT-7 (3%), GAGE-2 and SSX-1 (1.4%). In contrast, no antibody responses could be detected in the sera from the control groups, with the sole exception of that from two BPH patients, which responded to NY-ESO-1.

Expanded analysis of NY-ESO-1 specific serum response in patients. The outstanding sera reactivity against NY-ESO-1 antigen prompted us to analyze the sera response in more detail. For this purpose, we increased the number of PCA (n=181) and BPH sera (n=83). We further divided the PCA patients into groups of organ-confined disease (n=150) and
Figure 1. A: Serological immune response against CT antigens in prostate carcinoma CT antigen expression on yeast cell was ensured by flow cytometry (C-terminal penta His-tag). Vital yeast cell populations expressing the different CT antigens (grey solid curve) or the corresponding not induced cells (black open curve) are displayed: A: CT-7, B: GAGE-1, C: GAGE-2, D: GAGE-3, E: NY-ESO-1, F: LAGE-1a, G: LAGE-1b, H: SCP-1, I: SSX-1, J: SSX-2, K: SSX-3, L: SSX-4, M: SSX-5. B: Dots in the diagram stand for specific IgG response of 70 PCA, 14 BPH and 39 healthy donor (N) sera against the 13 used CT antigens. The horizontal bars indicate the range for positive sera activity specific for each different antigen (3× standard deviation plus mean value of control sera). A: CT-7, B: GAGE-1, C: GAGE-2, D: GAGE-3, E: NY-ESO-1, F: LAGE-1a, G: LAGE-1b, H: SCP-1, I: SSX-1, J: SSX-2, K: SSX-3, L: SSX-4, M: SSX-5. C: Detailed analysis of antibody response against CT antigen NY-ESO-1 with 264 prostatic disease sera (BPH n=83, PCA n=150, metPCA: advanced prostate carcinoma adv. n=31, healthy control donors n=39). Dots reflect NY-ESO-1-specific IgG responses. The horizontal bar indicates cut-off for positive sera activity range. D: Strength of NY-ESO-1 specific humoral immune response was examined by serum dilution. Sera reaction were analyzed in highly (squares), moderately (triangles) and non-reactive sera (circles) detected by FACS on NY-ESO-1-expressing yeasts. Furthermore, NY-ESO-1-specific antibody titer was specified in an exemplary patient’s serum determined by Westernblot (0.5 μg bacterially produced NY-ESO-1 for each lane); monoclonal anti-NY-ESO-1 antibody served as positive (+) and irrelevant antibody as a negative (–) control (far left panel). The middle panel shows signals gained from a strong NY-ESO-1-reactive serum (P57) and a negative control serum (HD: healthy donor) at dilution up to 1:20000.
metastatic disease with confirmed osseous lesions (n=31). Antibody reaction increasing in strength and frequency relative to the NY-ESO-1 protein was observed in accordance with disease progression. In detail, a significantly higher fraction (p=0.0098, Fisher’s exact test) of samples from those with metastatic (32%) than those with organ-confined PCA (11%) were serumpositive (Figure 1C, Table I). Interestingly, 8% of all sera from BPH patients revealed positive serum responses. NY-ESO-1 antibody titres of strong (1:10,000-20,000), moderate (1:5,000) and non-responders were analyzed by flow cytometry and Western blot with serial sera dilutions (Figure 1D).

NY-ESO-1 protein expression in PCA and BPH (IHC). Surprisingly, a similar proportion of organ-confined PCA and BPH patients exhibited an immune response to NY-ESO-1. We therefore examined the expression of NY-ESO-1 protein in PCA and BPH tissue. Immunohistochemical staining of tissue sections from seroreactive patients showed a specific NY-ESO-1 cytoplasmatic staining in benign and cancerous prostatic glands in 10/12 PCA and 4/7 BPH samples (Figure 2). NY-ESO-1 expression was limited to epithelial cells of the prostate glands, surrounding stroma cells were negative. No NY-ESO-1 protein expression was detectable in tissue sections from seronegative BPH and PCA patients (data not shown).

Figure 2. Immunohistochemical detection of NY-ESO-1-positive cells in tissue sections from prostate adenocarcinoma patients by use of the anti-NY-ESO-1 IgG. A, D: NY-ESO-1-positive tumor tissue samples; B, E: Examples of NY-ESO-1-negative PCA tissue; C, F: NY-ESO-1-negative BPH tissue; D, G: NY-ESO-1-positive BPH tissue.
Figure 3. Optimization of NY-ESO-1 expression on yeast cell surface. A: Yeast clone EBY100/pYD1-NY-ESO-1 was cultured in a 1 l batch shaking flask and NY-ESO-1 expression levels for each single cell were monitored over time by FACS analysis. A second assay was performed as a fed batch culture. Every day, 80% culture medium was collected and replaced by fresh. B: Best NY-ESO-1 control antigen-expressing yeast samples were stained (anti-pentaHis) and analyzed by FACS. C: Fluorescence microscopy demonstrated NY-ESO-1 cell surface display (anti-NY-ESO-1).

Figure 4. A: NY-ESO-1 peptide cross-presentation after macrocytosis. Uptake of fed, un- or green-labelled dispatched yeast cells to CD14+-derived macrophages over a time period of 36 h were analysed by FACS. Finally, digestion of NY-ESO-1-producing yeast cells by antigen-presenting cells (APCs) was documented by microscopy. B: To evaluate efficiency of phagocytosis, different ratios of APC: yeast were tested: 1:2; 1:5, 1:20. C: Amount of cross-presented NY-ESO-1157-165 peptides depended on the NY-ESO-1-expressing yeast cell number (FACS analysis, anti-HLA-A2/NY-ESO-1157-165). D: CD8+ T-cell activation. Monocyte-derived DCs were incubated for one day with: 1, NY-ESO-1-expressing yeast cells (ratio 1:20); 2, 10 μg/ml bacterially expressed NY-ESO-1/CpG; 3, SSX-2-expressing yeast cells. Cross-presentation of NY-ESO-1157-165 peptide was detected by specific T-cell clone (INF-γ/ELISPOT assay). Unfed DCs and T-cells alone were used as controls (data not shown).
These IHC results are in accordance with IgG responses, which show that patients expressing NY-ESO-1 protein in PCA or BPH usually exhibit a humoral immune response against this CT antigen.

Additionally, 39 PCA and 3 BPH tissues (without information about donor serological NY-ESO-1 activity) were analyzed for NY-ESO-1 expression by IHC using a tumor tissue microarray. In this set, 4/39 PCA samples and none of the three BPH showed NY-ESO-1 expression (Table II, Figure 2).

**Yeast uptake by macrocytosis, NY-ESO-1 cross-presentation and CD8+ T-cell activation.** Next, we directly used our NY-ESO-1-displaying yeast strain as a vaccination platform. We optimized NY-ESO-1 expression levels by fed batch culturing (Figure 3A, B) of yeast to increase the yield and potential of the vaccine. NY-ESO-1-expressing yeasts were stained by a green viable fluorescent dye and fed to macrophages. Nearly 100% uptake of yeast cells by APCs was observed using a feeding ratio of APC:yeast of 1:20 (Figure 4B). After 36 h, most of the phagocytosed yeast cells were digested, indicated by APCs with fluorescence-labelled cytoplasm (Figure 4A). Using an antibody specific for the HLA-A2/NY-ESO-1157-165 complex, we detected that a high levels of the NY-ESO-1157-165 peptide were cross-presented on APCs surface (Figure 4C, shaded curves). No NY-ESO-1 peptides were detected after control yeast feeding (Figure 4C, white curves). The uptake of NY-ESO-1-expressing yeast by APCs induced a strong ability to activate a CD8+ T-cell clone with specificity for NY-ESO-1 peptide157-165, superior to APCs that were fed with bacterially produced NY-ESO-1. APCs fed with control yeast revealed no activation potential (Figure 4D).

**Discussion**

Within our study, we observed serological IgG1 response of PCA patients to the CT antigens SCP-1, CT-7, GAGE-2, SSX-1 and NY-ESO-1. These natural targets of immune responses in cancer patients are appealing structures for immune-based therapies because they are essentially tumor-restricted antigens and there is a lower risk of pre-existing immune tolerance (16). The strongest serological response was seen for the most immunogenic CT antigen known to date, NY-ESO-1 (2). Several other research groups have already identified the NY-ESO-1 antigen as a potential target structure in PCA (11, 17-19). As these sera were tested regardless of the NY ESO-1-expression status in the tumor, the actual frequency of the antibody response in patients with NY-ESO-1-positive tumors is higher, estimated to be in the range of 25-50% in cases with advanced NY ESO 1 positive tumors (2). As described by Fossa et al. (11), we also found a higher rate of humoral immune response against NY-ESO-1 in patients with metastatic PCA compared to those with an early stage of disease. This finding might be based on a rather late expression of NY-ESO-1 protein during PCA progression as described by Fossa et al. (11). Regarding our study, one should keep in mind that PCA was untreated at the time blood samples from patients with organ confined PCA were collected. However, patients with metastatic disease were at the time of blood draw under androgen deprivation therapy and most of them had previously received a curatively intended treatment (e.g. radiation or surgery). These treatments might have facilitated NY-ESO-1 antigen presentation and the initiation of an immune response. In particular, androgen deprivation profoundly acting on viability and cell cycle of PCA cells may have triggered NY-ESO-1 protein expression.

Expression of CT antigens in normal tissues is largely limited to the testis, ovary and placenta. In this context, it was surprising that we found some patients with BPH showing an immune response to NY-ESO-1. Our immunohistochemical studies demonstrated that some non-tumorous epithelial cells of prostate glands with BPH express NY-ESO-1 protein, although undetected coexisting PCA can never be excluded. These results are in accordance with an immunohistochemical study previously published showing weak NY-ESO-1

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**Table I. Summary of serological immune response against analyzed CT antigens.**

<table>
<thead>
<tr>
<th>CT antigen</th>
<th>PCA sera</th>
<th>BPH sera</th>
<th>Healthy donor sera</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
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<tr>
<td>CT-7</td>
<td>2/70</td>
<td>0/14</td>
<td>0/39</td>
</tr>
<tr>
<td>GAGE-1</td>
<td>0/70</td>
<td>0/14</td>
<td>0/39</td>
</tr>
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<td>GAGE-2</td>
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<td>0/39</td>
</tr>
<tr>
<td>GAGE-3</td>
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<td>0/14</td>
<td>0/39</td>
</tr>
<tr>
<td>NY-ESO-1</td>
<td>15/70</td>
<td>2/14</td>
<td>0/39</td>
</tr>
<tr>
<td>LAGE-1a</td>
<td>3/70</td>
<td>0/14</td>
<td>0/39</td>
</tr>
<tr>
<td>LAGE-1b</td>
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<td>0/14</td>
<td>0/39</td>
</tr>
<tr>
<td>SCP1</td>
<td>6/70</td>
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</tr>
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<td>0/14</td>
<td>0/39</td>
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<td>SSX-4</td>
<td>0/70</td>
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<tr>
<td>SSX-5</td>
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**Table II. NY-ESO-1 specific serum response and protein expression.**

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<th>Diagnosis</th>
<th>Seropositive</th>
<th>Percent</th>
<th>Protein expression</th>
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<tr>
<td>BPH</td>
<td>7/83</td>
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<td>4/83</td>
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<tr>
<td>PCA</td>
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<td>11.3</td>
<td>3/38</td>
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<td>Metastatic PCA</td>
<td>10/31</td>
<td>32.3</td>
<td>1/1</td>
</tr>
<tr>
<td>Healthy donors</td>
<td>0/39</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

900
expression in 3 out of 20 BPH samples (20). Although the pathogenesis of BPH is not fully understood, BPH is not a known precursor for PCA. NY-ESO-1 expression in BPH remains obscure and should be further investigated.

Here, we used the powerful method of introducing antigen expressing yeasts into the cytosol of APCs to increase peptide-epitope presentation. To be naturally processed into HLA-peptide complexes, full-length proteins have to be taken up by APCs such as DCs (21). In peripheral tissues, DCs internalize antigen, then migrate to lymphoid organs where they process the sequestered antigens into peptides, load them onto HLA molecules, and, finally, present HLA/peptide complexes on their cell surface (22, 23). When exogenous free proteins are added to DCs in vitro, most of the presentation will occur via HLA class II (23), but proteins may also be processed and presented by the HLA class I pathway if high concentrations of protein are used (24, 25). This phenomenon is called cross-presentation and represents a pre-requisite for the induction and expansion of CD8+ T-cell responses. To date, the major source for recombinant proteins originate from expression and production systems in Escherichia coli, with the potential to produce large quantities under “good manufacturing practice-conditions”. Cross-presentation, however, is usually inefficient for soluble bacterial protein, but can be rendered highly efficient when formulations with adjuvants (26-30). We believe that our antigen delivering systems with adjuvant skills are superior concerning the induction of humoral and cellular immunity. As yet, recombinant yeast-expressed proteins have rarely been used in anticancer immunotherapies. It is known that yeast, in particular Saccharomyces cerevisiae engineered to produce recombinant antigens, provoke a cell-mediated immune response in vitro and in vivo (31). The strong adjuvant potential of S. cerevisiae expressed-antigen can be attributed to a contamination by cellwall components, especially β-1,3-D-glucan and mannan (32, 33). These interact primarily with toll-like receptors (TLRs) 2 and 4, thus demonstrating the ability of yeast to utilize TLRs for the recruitment and activation of the immune system by way of specific ligand receptor interaction (34). There is now evidence for the assumption that yeast-derived vaccines may reduce the need for additional adjuvants to induce a strong, integrated immune response during vaccination.

The system presented has the benefit to rapidly detect immune responses in patients’ sera and, simultaneously, to offer a platform for the development of highly potent vaccines. An innovative expression system was recently presented, indicating the wide range of operation and flexibility by yeast-derived vaccines. In this study, yeast coated with different antigens was tested to stimulate effective T-cell cross-priming (35). The saprophytic yeast S. cerevisiae has become increasingly important over recent years in immunology, gastroenterology and dermatology. Apart from the efficient immune responses induced by yeast cells carrying recombinant proteins, easy handling and safety application need also to be considered.

Yeast is a eukaryote with ‘generally regarded as safe’-status, and seems ideally suited for applications such as the manufacturing of whole-cell vaccines (36).

In conclusion, display of recombinant antigens on yeast surface is attractive for the induction of strong immune response since it combines efficient antigen delivery with DC activation. Mannosylating vaccine candidates by means of fungal systems seem to provide a potent immunostimulatory platform for the development of vaccines and is worth further study.

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References


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