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Recombinant *Saccharomyces cerevisiae* (yeast-CEA) as a potent activator of murine dendritic cells☆

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Summary Recombinant *Saccharomyces cerevisiae* (yeast) represents a unique and attractive vehicle to deliver antigens in vaccine immunotherapy protocols for cancer or infectious disease, in that it has been shown to be extremely safe and can be administered multiple times to hosts. In the studies reported here, we describe the effects of treatment with recombinant yeast on murine immature dendritic cells (DCs). Yeast expressing human carcinoembryonic antigen (CEA) as a model antigen was studied. Injection of mice subcutaneously with yeast-CEA resulted in rapid increases in MHC class II$^+$ cells and total antigen-presenting cells in draining lymph nodes. Post-treatment with yeast-CEA, DCs rapidly elevated both MHC class I and class II, numerous costimulatory molecules and other DC maturation markers, and secreted a range of Type I inflammatory cytokines. Gene expression arrays also revealed the rapid up-regulation of numerous cytokine and chemokine mRNAs, as well as genes involved in signal transduction and antigen uptake. Functional studies demonstrated enhanced allospecific reactivity of DCs following treatment with yeast-CEA or control yeast. Additionally, treatment of DCs with yeast-CEA resulted in specific activation of CEA-specific CD8$^+$ T cells in an MHC-restricted manner in vitro. Lastly, vaccination of CEA-transgenic mice with yeast-CEA elicited antigen-specific CD4$^+$ and CD8$^+$ immune responses in vivo. Thus, these studies taken together form a scientific rationale for the use of recombinant yeast in vaccination protocols for cancer or infectious diseases.

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Introduction

Immunotherapy represents an exciting new area of research devoted to the discovery of new methods of treatment for cancer patients. As opposed to more traditional forms of cancer therapy, the specificity and lack of toxicity of immune responses makes immunotherapeutic approaches extremely attractive [1]. Previous studies have investigated various types of vaccine strategies, including the use of viral vectors [2] as well as DNA-based vaccines [3]. Although viral vectors offer the ability to efficiently deliver antigen into the cytosol, and therefore into the conventional major histocompatibility complex (MHC) class I processing pathway, concerns regarding safety and existing immunity to many potential viral vaccine vector strains may limit the application of such vectors [4]. Similarly, there remain significant safety concerns regarding the use of DNA vaccines with respect to possible host—genome recombination and mutation [4]. Recent work, however, has focused on the nonpathogenic yeast species, *Saccharomyces cerevisiae*, which possesses numerous characteristics that make it a desirable candidate for use in immunotherapy. Clinical studies to date using heat-killed recombinant yeast have shown minimal toxicity, with a maximum tolerated dose yet to be established [1,5—7]. Recombinant *S. cerevisiae* can also be easily engineered to express antigens for infectious diseases or cancer [1,4,8]. Additionally, recombinant yeast is extremely stable, easily transported and stored, and generally easy to administer [4]. In vitro studies have also reported increased production of interleukin (IL)-12, interferon-gamma (IFN-γ), and granulocyte-macrophage colony-stimulating factor (GM-CSF) following *S. cerevisiae* administration, which may obviate the need for coadministration with immune adjuvants [1]. These characteristic advantages have been the basis for preclinical and clinical investigations into using *S. cerevisiae* as a vaccine vehicle.

To effectively generate antigen-specific T-cell responses, a vaccine must be able to deliver whole protein antigens to both MHC class I and class II antigen-processing pathways of professional antigen-presenting cells (APCs) [4]. In a study conducted by Owais et al. and later confirmed by Stubbs et al., recombinant *S. cerevisiae* was shown to be phagocytosed and internalized by DCs, which results in release of the recombinant protein in the phagolysosome. Subsequently, the released protein gains access to the cytosol, leading to efficient delivery of exogenous antigen to both MHC class I and class II through cross-presentation, and stimulation of both humoral and cell-mediated immune responses in vivo [8,9]. The ability of recombinant *S. cerevisiae* to effectively elicit immune responses in vivo prompted further investigation into the interactions between yeast and dendritic cells (DCs) in vitro [8]. In one study using murine DCs, uptake of whole recombinant *S. cerevisiae* was shown to increase surface expression of several costimulatory molecules and to increase production of IL-12 [8]. Murine DCs treated with recombinant *S. cerevisiae* designed to express chicken ovalbumin (OVA) were shown to efficiently prime MHC class I- and class II-restricted antigen-specific T-cell responses [8]. To our knowledge, this [8] was the only study to investigate the effects of whole recombinant yeast on murine DCs in vitro. We therefore sought to extend these findings and to elucidate potential mechanisms for the induction of immune responses observed in vivo, using murine DCs and recombinant *S. cerevisiae* designed to express carcinoembryonic antigen (designated yeast-CEA) as a model antigen. CEA is clinically relevant as a target for immunotherapy since it is expressed on 95% of colorectal, gastric, and pancreatic cancers, as well as on 70% of non-small cell lung tumors [10,11].

We show here for the first time that (a) subcutaneous (s.c.) injection of yeast-CEA results in significant increases in total cell number, number of class II$^+$ cells, and total APCs at the draining lymph node in vivo; (b) yeast-CEA treatment of DCs significantly increases expression of MHC class I and class II, CD11c, CD40, CD80 (B7-1), CD86 (B7-2), CD54 (ICAM-1), and CD58 (LFA-3) as measured by percent-positive cells or mean fluorescence intensity (MFI); (c) yeast-CEA treatment of DCs significantly increases production of IL-12, tumor necrosis factor-alpha (TNF-α), IFN-γ, monocyte chemotactic protein-1 (MCP-1), IL-6, and IL-10; (d) DCs treated with yeast-CEA have altered RNA expression levels of numerous genes involved in cytokine and chemokine production, signal transduction, and antigen uptake and presentation; (e) DCs treated with yeast-CEA greatly enhance proliferation and IFN-γ production of allogeneic T cells; (f) DCs treated with yeast-CEA (and not control yeast) can effectively stimulate CEA-specific CD8$^+$ T cells in vitro, and do so in an MHC class I-dependent manner; and (g) vaccination with yeast-CEA can break tolerance and elicit antigen-specific CD4$^+$ and CD8$^+$ T-cell responses in a transgenic mouse model.

Materials and methods

Mice

For in vivo lymph node studies, 6- to 8-week-old female C57BL/6 (H-2^b) mice were obtained from the National Cancer Institute, Frederick Cancer Research and Development Facility (Frederick, MD). For lymphocyte proliferation and cytotoxic T lymphocyte assays, a breeding pair of C57BL/6 mice that were homozygous for expression of the human CEA gene, designated transgenic (CEA-Tg) mice, was generously provided by Dr. John Shively (City of Hope Medical Center). Transgenic mice were generated as previously described [12]. Homozygosity for CEA expression was tested and verified using PCR analysis on mouse tail DNA [13]. Mice were housed and maintained in microisolator cages under pathogen-free conditions.

Yeast constructs

Recombinant *S. cerevisiae* constructs without antigen (control yeast) or designed to express CEA (yeast-CEA) were engineered by methods similar to those previously described [11] (GlobeImmune, Inc., Louisville, CO).

Poxvirus constructs

Recombinant vaccinia (rV) and recombinant fowlpox (rF) viruses containing murine B7-1, ICAM-1, and LFA-3 genes in combination with human CEA (CEA/TRICOM) have been previously described [14,15]. The rF virus containing the

gene for murine GM-CSF (rF-GM-CSF) has also been previously described [16]. Therion Biologics (Cambridge, MA) kindly provided all of the orthopoxviruses as part of a Collaborative Research and Development Agreement with the National Cancer Institute/National Institutes of Health.

Injections and regional lymph node analyses

Female C57BL/6 mice were injected s.c. on day 0 with PBS or 1 yeast unit (YU) of yeast-CEA (1 YU = 1×10^7 yeast particles) on the medial aspect of the right thigh. On days 2 and 4 post-injection, mice were sacrificed and right inguinal lymph nodes were isolated. Cells were mechanically dispersed and a single-cell suspension was created. Cells were washed three times in cold PBS/BSA, counted by Auto T4 Cellometer Reader (Nexcelom Bioscience LLC, Lawrence, MA), and resuspended at a concentration of $0.5-1.0 \times 10^6$. Cell-surface staining utilized 2-color immunofluorescence. Staining was performed with primary fluorescein isothiocyanate (FITC)-labeled antibodies CD11c, CD3, CD8, DX5, and IA^b, as well as primary phycoerythrin (PE)-labeled antibodies directed against H2-K^b, CD4, CD19, CD11b, and CD80 (B7-1). All antibodies were purchased from PharMingen (Mountain View, CA). Cell fluorescence was analyzed and compared with that of appropriate isotype controls (PharMingen) with a FACScan cytometer using Lysis II software (Becton Dickinson, Mountain View, CA).

Preparation of DCs

DCs were harvested from C57BL/6 mice as previously described [14]. Briefly, bone marrow was flushed from the long bones of the limbs and passed over a Ficoll-Hypaque gradient. Bone marrow cells were subsequently depleted of lymphocytes and Ia^+ cells using a cocktail of magnetic beads coated with monoclonal antibodies specific for CD4, CD8, and MHC class II antigens (MiniMACS; Miltenyi Biotec, Auburn, CA). Cells were plated in 6-well culture plates (10^6 cells/mL, 5 mL/well) in complete medium supplemented with 10 ng/mL GM-CSF and 10 ng/mL IL-4 (R&D Systems, Minneapolis, MN). Cells were replated with fresh cytokine-supplemented medium on day 2 and cultured for 5 days. Cells were then harvested from wells, washed twice in LPA medium, counted by Auto T4 Cellometer Reader (Nexcelom Bioscience LLC), and resuspended at 1.5×10^6 cells/mL. For in vitro studies, cells were placed in a new 6-well plate (1.5 mL/well) and either left untreated or incubated with yeast-CEA or control yeast at a ratio of 1:1 at 37 °C in 5% CO_2 for 48 h.

Flow cytometry analysis

Cell-surface staining of DCs for analysis of phenotypic expression was performed as previously described [17]. Briefly, staining was performed with primary FITC-labeled antibodies Pan-NK, CD11b, CD4, CD19, and CD8, and primary PE-labeled antibodies IA^b, CD58 (LFA-3), CD86 (B7-2), CD11c, and CD45 (B220). Biotin-labeled antibodies CD80 (B7-1), CD54 (ICAM-1), CD40, H2-K^b, and H2-D^b were subsequently labeled with streptavidin-Cy-chrome™. As a control in selected experiments, DCs were matured with lipopolysaccharide (LPS; 50 μg/well). Cell fluorescence was analyzed and compared with that of appropriate isotype-matched controls with a FACScan cytometer using Lysis II software (Becton Dickinson). All antibodies were purchased from PharMingen.

Evaluation of cytokine production

DCs plated in a 6-well plate were incubated with 1 YU of yeast-CEA on day 5 of culture, as described above. Supernatant was harvested from the wells after 48 h and analyzed for production of TNF-α, IFN-γ, MCP-1, IL-2, IL-4, IL-5, IL-6, IL-10, and IL-12 using protocols provided by BD™ Cytometric Bead Array (CBA) Mouse Inflammation and Mouse Th1/Th2 Cytokine Kits (PharMingen, San Diego, CA). Limit of detection was 4 pg/mL. Cytokine production was subsequently analyzed with a FACScan cytometer using BD™ CBA software. To confirm the level of IL-12 production, an ELISA was also performed (R&D Systems) using the protocol provided.

RNA isolation

DCs were cultured in GM-CSF and IL-4 for 5 days, as described above. On day 5, DCs were incubated with yeast-CEA at a ratio of 1:1 at 37 °C in 5% CO_2. After 8 h, cells were harvested from the wells and total RNA was extracted and purified from 5×10^7 cells with the RNeasy Midi Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions.

cDNA oligoarray

cDNA oligoarray, which focuses on 260 genes involved in DC activation and maturation (SuperArray, Bioscience Corp., Frederick, MD), was used to analyze gene expression changes pre- and post-treatment with yeast-CEA. Post-treatment, genes were considered to be up-regulated or down-regulated if their normalized intensity ratio was ≥ 2 or ≤ 0.5 (2-fold cutoff) and ≥ 3 or ≤ 0.33 (3-fold cutoff) respectively. Hierarchical clustering was applied to genes with a normalized intensity ratio of ≥ 3 or ≤ 0.33, as previously described [18].

Mixed lymphocyte reaction (H-2^d vs. H-2^b) and IFN-γ production

The mixed lymphocyte reaction was used to assess the stimulatory function of DCs for allogeneic (H-2^d) and syngeneic (H-2^b) naïve T cells. T cells were isolated from BALB/c (H-2^d) or C57BL/6 (H-2^b) mice, as previously described [15,17]. Briefly, stimulator cells consisted of DCs from C57BL/6 (H-2^b) mice that were either untreated or incubated with control yeast or yeast-CEA for 48 h. Complete medium was added and cells were incubated at 37 °C for 18 h. Cells were then irradiated to achieve 20 Gy and washed. T cells (5×10^4/well) were cocultured with graded numbers of stimulator cells (5×10^3 to 6.25×10^2) in complete medium in flat-bottomed, 96-well culture plates. Cells were then incubated at 37 °C in 5% CO_2 for 4 days, labeled for the final 12–18 h of incubation with 1 μCi/well [^{3}H] thymidine

(New England Nuclear, Wilmington, DE), and harvested with a Tomtec cell harvester (Wallac, Inc., Gaithersburg, MD). The incorporated radioactivity was measured by liquid scintillation counting (Wallac 1205 Betaplate; Wallac, Inc.). Results of the triplicate wells were averaged and reported as mean counts/min with 95% confidence intervals. For analysis of IFN-γ production, supernatants from wells of the flat-bottomed, 96-well plate were harvested 48 h after allogeneic or syngeneic naïve T cells were exposed to stimulator DCs. The level of IFN-γ produced by naïve T cells was measured using BD^{TM} Cytometric Bead Array (CBA) Mouse Inflammation Kit (PharMingen), as described above.

Stimulation of CEA-specific CD8$^+$ T cells

DCs were incubated with either control yeast or yeast-CEA at a ratio of 1:1 for 48 h, as described above. DCs were then irradiated to achieve 20 Gy, washed, and set at a concentration of 2.5×10^5 cells/mL. Irradiated DCs were then cocultured with CAPm8 (H2-D^b-restricted CEA peptide-specific CD8$^+$ T-cell line) at a ratio of 4:1 in a flat-bottomed, 24-well culture plate at 37 °C in 5% CO_2 for 24 h. Supernatants were then harvested from the wells and analyzed for IFN-γ production by BD^{TM} Cytometric Bead Array (CBA) Mouse Inflammation Kit (PharMingen), as described above. For MHC class I blocking studies, anti-H2-D^b or appropriate isotype control antibody was added at 10 μg/mL for the duration of the experiment.

Lymphocyte proliferation assays

To evaluate T-cell immune responses to CEA following vaccination, splenic T cells were analyzed for cell proliferation in response to CEA protein. Splenocytes were harvested and dispersed into single-cell suspensions in 10% FCS/RPMI1640, followed by lysis of red blood cells. Lymphocytes were subsequently separated by centrifugation through a Ficoll-Hypaque gradient. CD4$^+$ or CD8$^+$ cells were then isolated by negative selection and determined to be >90% pure (Miltenyi Biotec). Purified T cells (2×10^5 cells/well) were cultured for 5 days in 96-well flat-bottomed plates with naïve syngeneic splenocytes irradiated with 2000 rads as antigen-presenting cells (APCs) (5×10^5 cells/well), and with CEA protein in 10% FCS/RPMI1640. Cells were incubated at 37 °C in 5% CO_2 for 4 days, labeled for the final 24 h of incubation with 1 μCi/well [^{3}H] thymidine (New England Nuclear), and harvested with a Tomtec cell harvester (Wallac, Inc.). The incorporated radioactivity was measured by liquid scintillation counting (Wallac 1205 Betaplate; Wallac, Inc.). Results of the triplicate wells were averaged and reported as mean counts/min with 95% confidence intervals.

In vivo CEA-specific CD8$^+$ T-cell response

To determine CEA-specific CD8$^+$ T-cell responses following vaccination, spleens were harvested, dispersed into single-cell suspensions, and stimulated with 1 μg/mL of H2-D^b-restricted CEA peptide CEA$_{572-579}$ (GIQNSVSA) (CPC Scientific, San Jose, CA) [19]. After 6 days of incubation, bulk splenic cells were separated by centrifugation through a Ficoll-Hypaque gradient. The recovered lymphocytes were then incubated for 5 h with ^{51}Cr-labeled target cells (EL-4, 5×10^3 cells/well) pulsed with the CEA$_{572-579}$ peptide or vesicular stomatitis virus nucleoprotein VSV-NP$_{52-59}$ (RGYVYQGL) peptide as a control (CPC Scientific). Following the incubation period, radioactivity in supernatants was measured using a gamma-counter (Cobra Autogamma, Packard Instruments, Downers Grove, IL). The percentage of tumor lysis was calculated as follows: % tumor lysis = [(experimental cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm)] × 100.

Vaccination regimens

Naïve CEA-Tg mice were vaccinated with either 1 YU yeast-CEA or recombinant poxviral vaccines. Yeast-CEA mice were injected subcutaneously on days 0 and 7 with 1 YU yeast-CEA. Mice vaccinated with poxviral vectors received 10^8 pfu rV-CEA/TRICOM admixed with 10^7 pfu rF-GM-CSF on day 0 and boosted with 10^8 pfu rF-CEA/TRICOM admixed with 10^7 pfu rF-GM-CSF on day 7. Spleens were harvested 2 weeks post-treatment (day 21) and used in CEA-specific immune assays.

Statistical analysis

Statistical significance was calculated using ANOVA, with repeated measures using Statview 4.1 (Abacus Concepts, Inc., Berkeley, CA). In graphic representations of data, *y*-axis error bars indicate the S.D. for each point on the graph. Significant differences in distribution of flow cytometry analysis data were determined using the Kolmogorov—Smirnov test.

Results

Cellular changes in draining lymph nodes of mice treated with recombinant yeast-CEA

The effects of a whole recombinant yeast vaccine on immune-cell populations at the draining lymph node have not been investigated. In this study, C57BL/6 mice were given a single s.c. injection on the medial aspect of the right thigh of either PBS or 1 YU of recombinant yeast-CEA. On days 2 and 4 post-vaccination, draining inguinal lymph nodes were harvested and analyzed for changes in total cell number and in number of immune-cell populations (Table 1). Two days post-vaccination, the total number of cells at the draining lymph node doubled in mice receiving yeast-CEA, compared to mice receiving PBS. This increase in lymph node cellularity was maintained for at least 4 days post-vaccination. To determine the immune-cell population(s) responsible for these cellular changes, we examined 4 distinct lymph node cell populations: class II$^+$ cells, T cells, B cells, and natural killer (NK) cells (Table 1). There was a 2.3-fold increase in class II$^+$ cells 2 days post-vaccination with yeast-CEA, and a 1.5-fold increase in T cells at the draining lymph node, as defined by CD3$^+$ staining (Table 1). A more in-depth analysis of the T-cell population revealed a slight bias toward CD4$^+$ T cells (3.4-fold increase) vs. CD8$^+$ T cells (2-fold increase). There were also significant

Table 1 Effect of yeast vaccine on lymph node cell populations

Cells	Treatment	Post-treatment			
		Day 2		Day 4	
Total/node	PBS		$7.5 \times l0^6$		$8.3 \times l0^6$
	Yeast-CEA		**$1.5 \times l0^{-7}$**		**$1.3 \times l0^7$**
IA^{b+}	PBS	24%	$1.8 \times l0^6$	29%	$2.4 \times l0^6$
	Yeast-CEA	27%	**$4.1 \times l0^6$**	26%	**3.4×10^6**
$CD3^+$	PBS	78%	$5.8 \times l0^6$	76%	$6.3 \times l0^6$
	Yeast-CEA	57%	**$8.6 \times l0^6$**	68%	**$8.8 \times l0^6$**
$CD4^+$	PBS	41%	$1.7 \times l0^6$	47%	$3.9 \times l0^6$
	Yeast-CEA	38%	**$5.7 \times l0^6$**	39%	**$5.1 \times l0^6$**
$CD8^+$	PBS	23%	$1.7 \times l0^6$	23%	$1.9 \times l0^6$
	Yeast-CEA	23%	**3.4×10^6**	22%	**$2.9 \times l0^6$**
$CD19^+$	PBS	22%	$1.6 \times l0^6$	19%	$1.6 \times l0^6$
	Yeast-CEA	26%	**3.9×10^6**	18%	**2.3×10^6**
DX5+	PBS	2%	$1.5 \times l0^5$	2%	$1.7 \times l0^5$
	Yeast-CEA	2%	**$3.0 \times l0^5$**	2%	**$2.6 \times l0^5$**
APC ($CD11c^+IA^{b+}$)	PBS	1.6%	$1.2 \times l0^5$	2%	1.76×10^5
	Yeast-CEA	2%	**3.15×10^5**	2%	**2.47×10^5**

C57B6 mice ($n = 5$/group) were given 1 YU/mouse s.c. in the right thigh on day 0. Control mice received PBS only. Mice were sacrificed 2 and 4 days post-yeast administration. Total cells from the draining inguinal lymph nodes were counted after lysis of red blood cells and stained for MHC-II$^+$ cell populations (IA^{b+}, $CD19^+$), T-cells ($CD3^+$, $CD4^+$, $CD8^+$), and NK cells ($DX5^+$). APC populations were quantified by $IA^{b+}/CD11c^+$-stained cells. Cells were analyzed by flow cytometry. Bold numbers indicate significant difference ($P < 0.05$).

increases in B cells (2.4-fold) and NK cells (2-fold) 2 days post-vaccination with yeast-CEA. While trends were similar, increases in overall cellularity and individual immune-cell populations were not as substantial at day 4 as they were at day 2 (Table 1). For instance, there was only a 1.4-fold increase in class II$^+$ cells on day 4 compared to a 2.3-fold increase on day 2. This observation was consistent for T cells, B cells, and NK cells as well, indicating that the immune system started to return to baseline 4 days after yeast-CEA vaccination.

A single s.c. injection of yeast-CEA also increased the APC population ($IA^{b+}/CD11c^+$ cells) at the draining lymph node (Table 1). Approximately 1.5% of lymph node cells from untreated mice expressed the phenotype consistent with that of APCs. Two days post-vaccination, mice receiving yeast-CEA showed approximately a 3-fold increase ($P < 0.05$) in the total number of APCs at the draining lymph node, compared to control mice. This increase in the APC population was maintained for 4 days post-vaccination. However, consistent with trends previously observed in other immune-cell populations, the level of APCs began to return to baseline by day 4 post-vaccination (1.4-fold increase on day 4 vs. 2.6-fold increase on day 2). Injection of control yeast produced results similar to those seen with yeast-CEA (Table 1), demonstrating that the yeast vehicle was responsible for the lymph node changes observed. This experiment was repeated two times with similar results. Data depicted in Table 1 is representative of one experiment and the statistical test for trend is representative of two experiments.

Enhanced expression of cell surface markers and increased cytokine production following treatment with yeast-CEA

The sustained increase in the APC population at the draining lymph node in vivo following yeast-CEA vaccination (Table 1) provided the rationale for a more in-depth investigation, specifically examining the effects of recombinant yeast on DC maturation and activation in vitro. A prior in vitro study had shown that uptake of recombinant yeast by DCs increased surface expression (presented as fold increase in mean fluorescent channel [MFC]) of MHC class II, CD40, CD80, CD86, and CD54, as well as production of IL-12 [8,20]. We sought to extend these findings by analyzing the effects of recombinant yeast-CEA on the expression of 10 cell-surface markers indicative of DC maturation and activation in terms of two different parameters — percent-positive cells and MFI — and on the production of 9 inflammatory cytokines. For these studies, immature DCs were incubated with yeast-CEA for 48 h at a ratio of 1:1, as described in Section 'Materials and Methods'. Following the 48-h incubation period, DCs and supernatants were harvested and analyzed for changes in phenotypic expression and cytokine production. Similar results in phenotypic changes and levels

of cytokine production were seen in immature DCs exposed to control yeast and yeast-CEA (data not shown). For brevity, only results employing yeast-CEA are shown here.

As seen in Table 2A, DCs treated with yeast-CEA showed significant increases (75—89%, $P < 0.001$) in class II (IA^b) cell-surface expression. The MFI of these cells, an additional indicator of cellular maturation, also increased. Similarly, while the percentage of class I^+ cells (defined by H2-K^b/D^b) increased only 1% following treatment with yeast-CEA, there was a statistically significant increase ($P < 0.001$) in MFI from 923 to 1274. The up-regulation of MHC class I and class II expression following treatment with yeast-CEA was accompanied by significant increases in expression of CD11c and the costimulatory molecules CD40, CD86 (B7-2), CD80 (B7-1), CD54 (ICAM-1), and CD58 (LFA-3) (Table 2A). By comparison, DCs treated with LPS, a classic activator of DCs in vitro [2,3], also showed increases in the same surface markers (Table 2A). However, the percent cell expression and/or MFI of MHC class I and class II, CD11c, CD40, B7-2, B7-1, ICAM-1, and LFA-3 were greater for cells treated with yeast-CEA compared to those treated with LPS. Finally, to ensure the purity of the DC population, we examined changes in expression of $CD4^+$ and $CD8^+$ (T cells), $CD19^+$ (B cells), and Pan NK (NK cells). All DC populations were negative for these 4 cell-surface markers pre- and post-treatment with yeast-CEA (data not shown).

We next examined the effects of yeast-CEA exposure on DC cytokine production, an additional marker of DC activation (Table 2B). As mentioned above, DCs were also treated with LPS, which served as a positive control. Following exposure to yeast-CEA for 48 h, DCs approximately doubled production of IL-12. This level of IL-12 production by DCs treated with yeast-CEA was subsequently confirmed by ELISA. There were also increases in production of IFN-γ (5-fold), IL-10 (4.5-fold), and MCP-1 (1.2-fold), and substantial increases in TNF-α (84-fold) and IL-6 (23-fold) by DCs treated with yeast-CEA. Levels of 3 cytokines — IL-5, IL-4, and IL-2 — were undetectable pre- and post-exposure to yeast-CEA (Table 2B). The same experiments using DC:yeast particle ratios ranging from 1:20 to 1:0.1 yielded similar results (data not shown).

Oligoarray analysis reveals numerous additional genes differentially expressed in DCs following treatment with recombinant yeast-CEA

cDNA oligoarray analyses were employed to better define changes in gene expression patterns of DCs treated with recombinant yeast-CEA. To recognize early changes in gene expression, total RNA from DCs treated with yeast-CEA was isolated after an 8-h incubation period and compared with RNA from untreated DCs. Using an Oligo GEArray®, quantitative analysis of different levels of expression of 260 genes involved in DC activation and maturation revealed a total of 33 genes that were up-regulated by at least 2-fold and only one gene that was down-regulated at least 2-fold. Additional analysis focused on the 15 genes that exhibited a change in expression of at least 3-fold after treatment with yeast-CEA (Table 3); genes were grouped according to their function. Of the 14 genes up-regulated at least 3-fold at 8 h, 57% (8 genes) encoded proteins that participate in the pro-

Table 2 Effect of recombinant yeast-CEA on immature DCs

DC treatment	DC panel [%positive cells (MFI)]									
	IA^b	H2-K^b/D^b	CD11b	CD11c	CD40	B7-2	B7-1	ICAM-1	LFA-3	CD45
A. Phenotype										
Media	75 (1109)	96 (923)	24 (158)	37 (209)	47 (259)	50 (794)	60 (455)	92 (870)	84 (482)	37 (105)
LPS	**90 (616)**	97 (205)	27 (43)	**34 (88)**	**67 (133)**	**61 (694)**	**79 (202)**	**95 (1292)**	**86 (366)**	**73 (68)**
Yeast-CEA	**89 (1846)**	**97 (1274)**	22 (160)	**50 (303)**	**68 (387)**	**70 (1103)**	**83 (708)**	**99 (1524)**	**95 (653)**	33 (135)

DC treatment	Cytokine (pg/mL)									
	IL-12	TNF-α	IFN-γ	IL-10	IL-6	IL-5	IL-4	IL-2	MCP-1
B. Cytokine									
Media	41	60	20	50	80	<4	<4	<4	14,200
LPS	**98**	**6691**	**2494**	**473**	**15,000**	<4	<4	<4	**16,072**
Yeast-CEA	**79**	**5020**	**102**	**225**	**1,869**	<4	<4	<4	**16,454**

Exposure of immature dendritic cells to recombinant yeast-CEA results in maturation. DCs were derived from C57B6 mouse bone marrow treated with GM-CSF and IL-4. On day 5, DCs were incubated with yeast-CEA at a ratio of 1:1. As a control, DCs were matured with LPS. After 48 h, cells were analyzed by flow cytometry. Supernatant fluids were analyzed by cytometric bead array. Limit of detection was 4 pg/mL. Panel (A) Bold numbers indicate a significant change in either %positive cells or cell-surface expression (MFI) compared with no treatment. Panel (B) Bold numbers indicate a significant change compared with no treatment, as determined by the Kolmogorov—Smirnov test.

Table 3

Functional group	Gene	Description	Fold-change after 8 h
Cytokine/chemokine	IL-12b	IL-12b	4.7
	CXCL10	Chemokine ligand 10	4.5
	IL-6	IL-6	4.4
	CXCL2	Chemokine ligand-2	4.2
	CCL4	Chemokine ligand-4	4
	IFN-g	IFN-gamma	3.5
	IL-1b	IL-1beta	3.5
	CSF2	GM-CSF	3.4
Signal transduction	IFIT1	IFN-induced protein	8.2
	ISG15	ISG15 ubiquitin-like modifier	4
	IFI204	IFN-activated gene 204	3.3
	IFIT3	IFN-induced protein	3
Antigen presentation	CD80	CD80 (B7-1)	3.3
Antigen uptake	MX2	Myxovirus resistance 2	5.6
	PFN1	Profilin 1	−3.2

Genes differentially expressed in yeast-CEA-treated DCs vs. untreated DCs. DCs were derived from C57B6 mouse bone marrow treated with GM-CSF and IL-4. On day 5, DCs were incubated with yeast-CEA at a ratio of 1:1. After 8 h, RNA was harvested and a cDNA oligoarray was performed. Genes were considered differentially expressed if their levels of expression differed at least 3-fold. Depicted is a hierarchical cluster of differentially expressed genes in yeast-CEA-treated vs. untreated DCs.

duction and release of cytokines and chemokines, including IL-12, IL-6, and IFN-γ. This result accords with observations at the protein level shown previously (Table 2B). Of the 14 genes up-regulated at least 3-fold, 29% (4 genes) are involved in signal transduction, including IFIT1, whose expression increased the most (8.2-fold) following treatment with yeast-CEA. IFIT1 encodes an IFN-induced protein that contributes to the induction of an immune response. Another gene up-regulated at least 3-fold, CD80 (B7-1), encodes for a protein involved in antigen presentation. This increase in B7-1 gene expression is also consistent with our previous observations at the protein level, as seen from the phenotypic data presented in Table 2A. The myxovirus resistance 2 gene, which encodes for a protein in the GTPase family, showed a 5.6-fold increase in expression following treatment with yeast-CEA. This gene is in the category of genes involved in antigen uptake and therefore, like many GTPases, likely participates in the intracellular trafficking of proteins and receptors to and from the cell surface [21]. The single gene that was down-regulated at least 3-fold after treatment with yeast-CEA, profilin 1, is also grouped in the antigen uptake category and is involved in the stabilization of actin polymerization [22].

To further characterize the ability of yeast-CEA to activate signal transduction pathways, subsequent studies focused on the Toll-like receptor (TLR) signaling pathway, as it has been implicated in the recognition of various yeast species and yeast cell-wall derivatives. To our knowledge, activation of murine DCs by whole recombinant *S. cerevisiae* through the TLR pathway has not been investigated. Studies were therefore conducted to determine if stimulation of DCs by yeast-CEA, as evidenced by (a) enhanced phenotypic expression (Table 2A), (b) increased inflammatory cytokine production (Table 2B), and (c) up-regulation of immunoregulatory genes (Table 3), involves the TLR signaling pathway. Specifically, DCs were treated with yeast-CEA in the presence or absence of 20 μg/mL blocking antibodies for TLR2 or TLR4. In the absence of either blocking antibody, DCs incubated with yeast-CEA produced 3500 pg/mL TNF-α, similar to that shown in Table 2B. DCs incubated with yeast-CEA in the presence of anti-TLR2 or anti-TLR4 expressed 3400 and 3300 pg/mL TNF-α, respectively. These data indicate that other TLR pathways could be involved in DC activation by yeast or that yeast could be activating DCs in a TLR-independent manner.

Enhanced allostimulatory activity by DCs treated with recombinant yeast

The studies described above demonstrate that recombinant yeast effectively matures and activates DCs in vitro and in vivo. To determine if these effects would translate into enhanced function, the stimulatory capacity of untreated and yeast-treated DCs was assessed by an allospecific mixed lymphocyte reaction. Immature DCs from C57BL/6 (H-2^b) mice were treated with recombinant yeast-CEA or control yeast at a ratio of 1:1 for 48 h, as previously described. To serve as a positive control, DCs were once again treated with LPS (Fig. 1A and B, gray bar). Untreated and treated DCs were then coincubated for 48 h with naïve allogeneic T cells from BALB/c (H-2^d) mice and subsequently assessed for changes in proliferation and IFN-γ production. As seen in Fig. 1A, DCs treated with yeast-CEA (black bar) or control yeast (hatched bar) significantly increased proliferation of allogeneic T cells ($P < 0.05$). Both yeast-treated DC populations induced a 2-fold increase in proliferation compared to untreated DCs (open bar). As an additional measure of enhanced DC function following treatment with recombinant yeast, we examined the level of IFN-γ production by

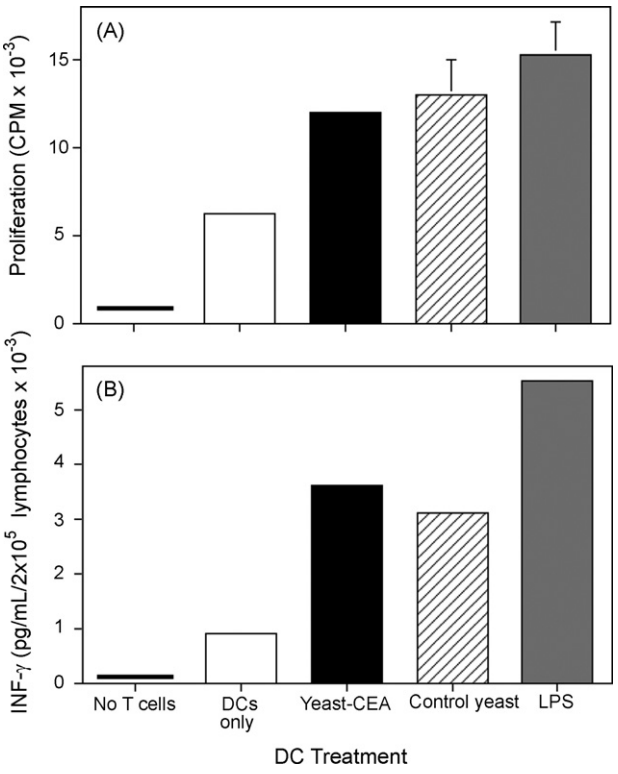

Figure 1 Yeast-CEA enhanced allostimulatory (H-2^d vs. H-2^b) activity by DCs. DCs were derived from C57BL/6 mouse bone marrow treated with GM-CSF and IL-4. DCs were incubated on day 5 with either yeast-CEA or control yeast at a ratio of 1:1 for 48 h, then cocultured with BALB/c allogeneic (H-2^d) T cells for 5 days. For proliferation (A), ^{3}H-thymidine was added during the final 18 h. For IFN-γ production (B), supernatants were analyzed after 5 days by cytometric bead array. Solid line: proliferation of DCs without added T cells; open bars: proliferation of allogeneic T cells cultured with untreated DCs; black bars: DCs treated with yeast-CEA; hatched bars: DCs treated with control yeast; gray bars: DCs treated with LPS.

naïve allogeneic T cells. As seen in Fig. 1B, DCs treated with yeast-CEA or control yeast increased IFN-γ production by T cells > 3-fold compared to untreated DCs. As expected in an allogeneic mixed lymphocyte reaction, there were no significant differences in stimulatory capacity between DCs treated with yeast-CEA or those treated with control yeast.

DCs treated with yeast-CEA effectively stimulate CEA-specific CD8$^+$ T cells in a class I-dependent manner

We next sought to determine if yeast-CEA-treated DCs could stimulate CEA-specific T cells. As previously described, immature DCs were left untreated or treated with either yeast-CEA or control yeast for 48 h. These 3 DC populations were then coincubated with CEA-specific T cells [23] for 24 h, after which supernatants were harvested and analyzed for IFN-γ production. First, to ensure that IFN-γ production by the DC populations could not account for the results, supernatants were also harvested from wells in which no T cells were added. As seen in Fig. 2, levels of IFN-γ production by the DC populations alone were undetectable. The addition of CEA-specific T cells (Fig. 2), whether stimulated by untreated DCs (open bar) or DCs treated with control yeast (hatched bar), produced similar levels of IFN-γ. However, CEA-specific CD8$^+$ T cells stimulated by DCs treated with yeast-CEA (black bar) showed a substantial increase in IFN-γ production relative to untreated DCs or DCs treated with control yeast.

To ensure authentic presentation of the relevant CEA peptide by MHC class I expressed on the surface of yeast-CEA-treated DCs, an MHC class I-blocking antibody (anti-H2-D^b) was added for the duration of the coincubation (Fig. 2). Anti-H2-D^b greatly diminished the amount of IFN-γ produced by CEA-specific T cells incubated with yeast-CEA-treated DCs, reducing IFN-γ production to a level similar to those of untreated DCs and DCs treated with control yeast (Fig. 2). In contrast, no such reduction was seen when an isotype-control antibody was substituted for the MHC class I-blocking antibody (Fig. 2). Taken together, these data demonstrate that only DCs treated with recombinant yeast-CEA are capable of effectively stimulating CEA-specific CD8$^+$ T cells, and that these stimulatory effects are dependent on interactions with antigen-loaded MHC class I.

Vaccination with yeast-CEA produces CEA-specific immune responses in vivo

The studies described above show that yeast-CEA activates and matures DCs, which can subsequently stimulate antigen-specific T cells in vitro. Therefore, we next sought to determine if vaccination with yeast-CEA could elicit CEA-specific immune responses in vivo. For these experiments, we utilized a transgenic mouse model in which CEA is expressed as a self antigen in fetal tissues and various parts of the intestinal tract [24]. These CEA-Tg mice exhibit CEA expression levels analogous to those seen in patients presenting with CEA$^+$ carcinomas. Mice were vaccinated s.c. with 1 YU of yeast-CEA, control yeast, or PBS on days 0 and 7 and sacrificed 14 days later. Spleens were then harvested and CD4$^+$ proliferation and CD8$^+$ cell killing assays performed. As shown in Fig. 3A, vaccination with control yeast (closed triangles) or PBS (open squares) produced negligible CD4$^+$ proliferation at all concentrations of CEA. In contrast, vaccination with yeast-CEA (closed circles) resulted in a 8-fold increase CD4$^+$ proliferation at 50 μg/mL CEA compared to that of control yeast or PBS. Higher levels of proliferation were also observed at 25 μg/mL CEA (6-fold increase) and 12.5 μg/mL CEA (3-fold increase) following yeast-CEA vaccination compared to controls (Fig. 3A). Similarly, vaccination with yeast-CEA produced greater levels of CD8$^+$-mediated cell lysis (between 10% and 20% lysis) compared to that observed when mice were injected with control yeast or PBS (<5% lysis) for all effector:target (E:T) ratios (Fig. 3C). For comparison, CEA-Tg mice were also vaccinated with a well-defined CEA-based vaccine, which utilizes poxviruses as delivery vehicles for tumor-associated antigens in combination with TRIad of T cell COstimulatory Molecules (designated TRICOM; B7-1, ICAM-1, and LFA-3)

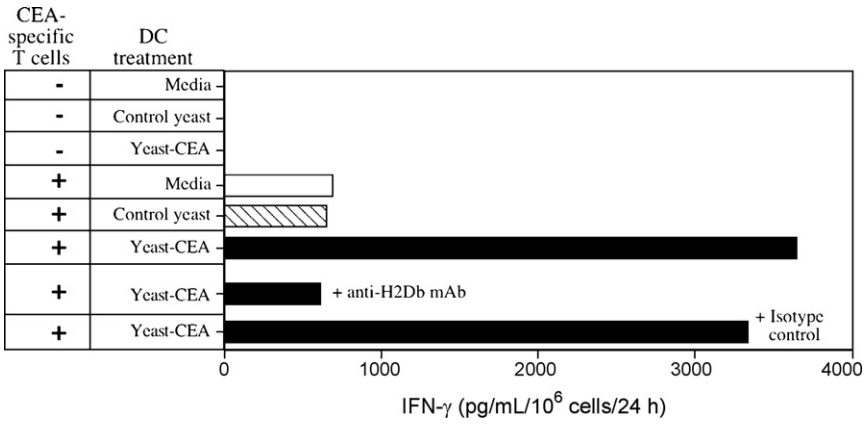

Figure 2 DCs treated with yeast-CEA stimulate CEA-specific T cells. DCs were derived from C57BL/6 mouse bone marrow treated with GM-CSF and IL-4. DCs were incubated on day 5 with either yeast-CEA or control yeast at a ratio of 1:1 for 48 h, then cocultured with CEA-specific T cells at a ratio of 4:1 for 24 h. Supernatants were then analyzed for IFN-γ by cytometric bead array. For MHC blocking studies, anti-H2-D^b monoclonal antibody or isotype control antibody was added at 10 μg/mL for the duration of the experiment.

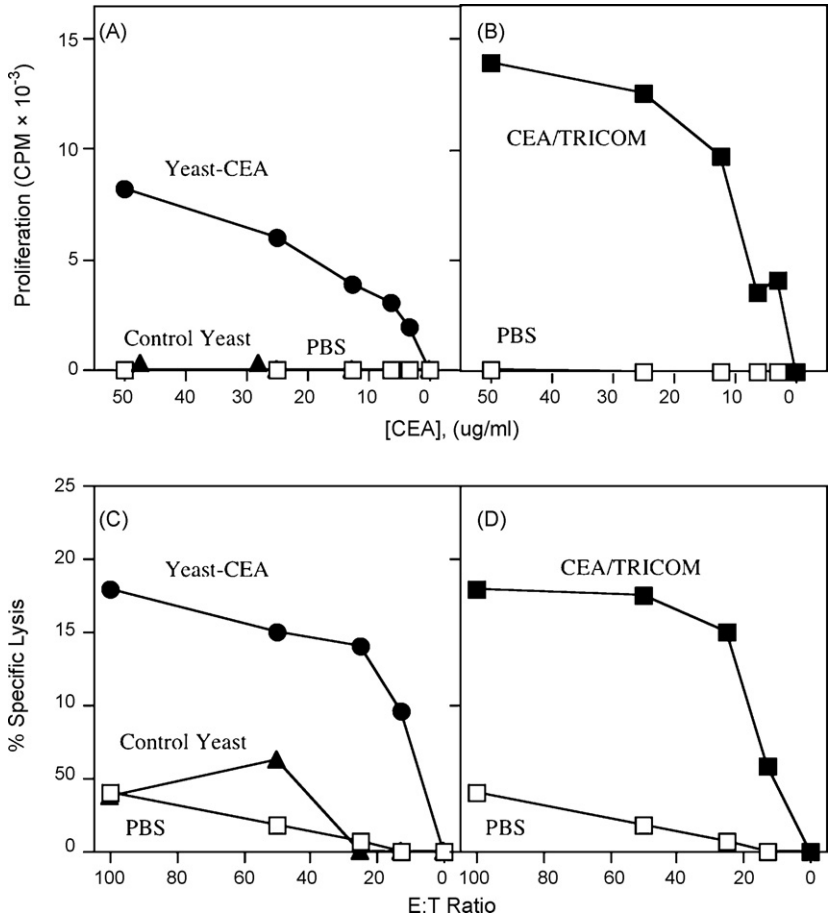

Figure 3 Vaccination with yeast-CEA induces antigen-specific T-cell responses. CEA-Tg mice were vaccinated with 1 YU yeast-CEA on days 0 and 7. For comparison, CEA-Tg mice were vaccinated with rV-CEA/TRICOM + rF-GM-CSF on day 0, and boosted with rF-CEA/TRICOM + rF-GM-CSF on day 7. On day 21, mice were sacrificed, spleens were harvested, and splenocytes were used for assays. (A and B) $CD4^+$ cell proliferation. Purified $CD4^+$ T cells were cultured with irradiated APCs and CEA protein for 5 days. ^{3}H-thymidine (1 μCi/well) was added to the wells for the last 24 h, and proliferation was assayed by measuring incorporated radioactivity. (C and D) $CD8^+$ CTL activity after vaccination. Splenocytes were stimulated with $CEA_{572–579}$ peptide for 6 days before assays. Lymphocytes were incubated for 5 h with ^{51}Cr-labeled target EL-4 cells pulsed with CEA or VSV-NP control peptide. Radioactivity in the supernatant was measured and specific lysis calculated. Shown is %specific lysis after subtraction of lysis noted against VSV-NP peptide.

[25]. Similar to yeast vaccines, TRICOM-based vaccines have been shown to elicit antigen-specific immune responses by enhancing the ability of DCs to activate both naïve and effector T cells in vitro and in vivo [17,26]. Vaccination with CEA-TRICOM (closed squares) resulted in slightly higher levels of $CD4^+$ proliferation (Fig. 3B) compared to that seen in mice vaccinated with yeast-CEA (Fig. 3A). However, the level of $CD8^+$-specific lysis induced following vaccination with CEA-TRICOM (Fig. 3D) was equivalent to that seen with yeast-CEA injection (Fig. 3C). Taken together, these data show that vaccination with yeast-CEA elicits CEA-specific $CD4^+$ and $CD8^+$ immune responses in a ''self'' antigen system in vivo.

Discussion

Recombinant *S. cerevisiae* has numerous characteristics that make it a desirable candidate for use as a vaccine platform for immunotherapy. Paramount among these is the fact that repeated administration of heat-killed recombinant *S. cerevisiae* has been shown to be well-tolerated, with no report of serious adverse events in preclinical or clinical studies [20,27,28]. Furthermore, recombinant *S. cerevisiae* can be easily engineered to express clinically relevant antigens, is easily transported and stored, and can be cultured rapidly in large quantities [1,4]. An added benefit of *S. cerevisiae* is its inherent adjuvant properties, which could potentially abrogate the need for coadministration with expensive immune adjuvants [1,8].

Several recent studies have investigated the use of recombinant *S. cerevisiae* as a vaccine vehicle. It has been shown that, while nonpathogenic, recombinant *S. cerevisiae* can elicit potent immune responses and stimulate both humoral and cell-mediated immunity [1,4,8,20]. Through cross-presentation, recombinant *S. cerevisiae* can deliver exogenous antigen to MHC class I and class II presentation pathways, with subsequent activation of antigen-specific $CD4^+$ and $CD8^+$ T cells [1,8]. Knowing that recombinant yeast can induce robust immune responses, we sought to investigate its local effects not only on $CD4^+$ and $CD8^+$ T cells, but also on several other immune-cell populations, including MHC class II^+ cells, B cells, NK cells, and total APCs. To that end, mice were injected with recombinant *S. cerevisiae* designed to express CEA, and draining lymph nodes were harvested and examined for changes in cellularity 2 and 4 days post-vaccination. While no preferential increase in any particular immune-cell population was observed at these times, the absolute number of all cell populations significantly increased (Table 1), indicating effective recruitment of immune-mediated cells to the draining lymph node following yeast-CEA vaccination. While trends were similar, increases in overall cellularity and individual immune-cell populations were not as marked at day 4 as they were at day 2, possibly indicating that the immune system starts to return to baseline 4 days post-vaccination. This observation may help to pinpoint optimal timing and scheduling of a yeast-based vaccine.

To better understand how these immune responses are induced in vivo [1,4,8,20], we undertook several studies to examine the interactions between recombinant yeast and DCs in vitro. DCs are widely recognized as the key to initiating any immune response [1] and are capable of priming naïve T-cell responses [29]. They have also been shown to be the only APCs capable of efficiently processing exogenous antigen for presentation to naïve precursor $CD8^+$ T cells via the MHC class I pathway [4,30]. It has previously been shown that recombinant yeast activates and matures DCs, defined by increased surface expression of MHC and costimulatory molecules [4]. Stubbs et al. observed increased surface expression of class II, CD40, CD80, CD86, and CD54 on DCs following treatment with recombinant *S. cerevisiae* [8]. The same study also showed that interactions between recombinant yeast and DCs lead to increased IL-12 production, an additional marker of DC activation [8]. In a parallel study, Sheng et al. showed that mannan, an extract from yeast cell walls, can also enhance surface expression of CD40, CD80, and CD86 on DCs and increase production of several inflammatory cytokines involved in Th1 and Th2 immune responses [31].

We sought to extend the findings of Stubbs et al. [8] by analyzing the effects of recombinant yeast-CEA on 5 additional DC surface markers (10 in all) in terms of percent-positive cells and MFI, and on the production of 9 inflammatory cytokines. DCs treated with yeast-CEA showed significantly increased expression of MHC class I and class II, as well as CD11c, CD40, CD80 (B7-1), CD86 (B7-2), CD54 (ICAM-1), and CD58 (LFA-3) (Table 2A), which accords with previously published results [8,31]. We also found that yeast-CEA significantly increased production of IL-12, TNF-α, IFN-γ, MCP-1, IL-6, and IL-10 by DCs (Table 2B), which is consistent with prior reports [8,20,31]. In these studies, LPS was used as a positive control for DC activation. The phenotypic studies reported here showed greater increases by yeast-CEA in percent-positive cells and/or MFI in all markers examined compared to LPS (Table 2A). Similarly, DCs treated with LPS increased production of the same inflammatory cytokines as yeast-CEA-treated DCs, albeit to a greater degree (Table 2B). These varying results are not surprising since yeast and LPS have been shown to activate and mature DCs through different mechanisms. Huang et al. [32] examined the effects of LPS and *Candida albicans* on human DCs and reported discordance between the genes up-regulated by the two stimuli. Furthermore, it is noteworthy that in the cytokine studies reported here, TNF-α and not IL-12, the traditional cytokine marker for DC activation [8,33,34], showed the greatest increase in production following treatment with yeast-CEA. For instance, Stubbs et al. [8] reported IL-12 levels close to 5000 pg/mL following exposure to yeast. Therefore, we sought to confirm the level of IL-12 production reported in Table 2B with an ELISA, which subsequently revealed similar results. While not completely clear, these differences in IL-12 production could be due to the methods in which the DCs are prepared and/or the state of maturation of DCs used in the assays. In the studies reported here, DCs were obtained from highly purified bone marrow depleted of $CD3^+$, IA^{b+}, and $CD19^+$ cells. Lastly, because there was such a substantial increase in TNF-α production following exposure to yeast-CEA and some reports have shown increased cell death due to high levels of TNF-α [35,36], follow-up annexin and propium iodide studies were performed but failed to show any acceleration of apoptosis or necrosis by DCs following treatment with yeast-CEA.

To our knowledge, only one prior study has examined the effects of yeast on DC gene expression levels [32]. In that study, oligonucleotide microarrays were utilized to measure gene expression profiles of human DCs in response to the pathogenic yeast *C. albicans*. At early time points, human DCs showed increased expression of immune cytokines, chemokines, and receptors, as well as induction of signaling genes and transcription factors. That study [32] employed a genus and species of yeast different from the *S. cerevisiae* studied here. In the study reported here, we demonstrate that following exposure to yeast-CEA, murine DCs up-regulate a number of genes involved in inducing an immune response. We found that most of the early-response genes up-regulated encoded for proteins involved in the production and release of cytokines, chemokines, and their receptors (Table 3). Up-regulation of these genes likely contributes to the recruitment of monocytes, macrophages, and other immune-mediated cells to regional lymph nodes in order to initiate an immune response. Furthermore, increases in the expression of genes participating in signal transduction pathways (especially IFN-related genes) following exposure to yeast-CEA (Table 3) may help prepare DCs to receive regulatory signals for appropriate modulation of the immune response.

We have shown here that yeast-CEA vaccination enriches CD11c/MHC-II$^+$ (IA^b) cells (consistent with DCs) at the draining lymph node in vivo (Table 1) and that yeast-CEA strongly activates DCs in vitro (Tables 2 and 3). We next investigated whether these effects would translate into enhanced DC function. While it has been shown that yeast cell-wall extracts can enhance proliferation of allogeneic T cells [31], the capacity of DCs treated with whole recombinant yeast to stimulate allogeneic T cells had not been previously reported. We therefore treated DCs from C57BL/6 (H-2^b) mice with yeast-CEA or control yeast and exposed them to T cells extracted from BALB/c (H-2^d) mice in an allospecific mixed lymphocyte reaction (Fig. 1A). DCs treated with recombinant yeast significantly enhanced the proliferative capacity of allogeneic T cells relative to untreated controls. We also showed that such T cells produce significantly higher levels of IFN-γ when incubated with yeast-treated DCs relative to untreated controls (Fig. 1B). Of note, DCs treated with yeast-CEA induced comparable levels of T-cell proliferation and IFN-γ production as DCs treated with LPS. These results are in accordance with those of previous studies, which found enhanced stimulatory capacity of DCs treated with mannan, a yeast cell-wall extract [31]. Because these studies were performed with allogeneic T cells as opposed to antigen-specific T cells, no significant differences in the stimulatory capacity of DCs treated with yeast-CEA or control yeast were observed (Fig. 1A and B).

Recombinant yeast has been shown to efficiently prime MHC class I- and class II-restricted, antigen-specific T-cell responses [8]. In one prior study, DCs pulsed with recombinant yeast expressing OVA effectively stimulated naïve OVA-specific T cells [8]. We sought to determine if DCs treated with yeast-CEA could effectively stimulate CEA-specific CD8$^+$ T cells (Fig. 2). Our results show that only DCs treated with yeast-CEA significantly enhanced production of IFN-γ by CEA-specific T cells, indicating an antigen-specific T-cell response. Furthermore, the observation that the addition of an MHC class I-blocking antibody drastically diminished the amount of IFN-γ produced by CEA-specific T cells helped to confirm that CEA peptide was efficiently presented by the MHC class I receptor expressed on DCs (Fig. 2).

While previous studies have reported induction of antigen-specific immune responses in vitro and in vivo following injection with yeast [1,8,20,31,37], to our knowledge, the ability of a recombinant yeast vaccination to break tolerance and stimulate antigen specific T cells in a ''self'' antigen system has not been investigated. We showed here that vaccination with yeast-CEA significantly increased the level of CEA-specific CD4$^+$ T cell proliferation as well as enhanced CEA-specific CD8$^+$ cell lysis (Fig. 3A and C). Interestingly, the level of antigen-specific T-cell stimulation following vaccination with yeast-CEA was comparable to that of CEA-TRICOM (Fig. 3B and D), a well-established CEA-based vaccine currently being utilized in numerous clinical trials for cancer immunotherapy [25,38–40]. While further similarities and differences between yeast-CEA and CEA-TRICOM are currently being investigated, both share the critical ability to activate and mature dendritic cells as well as stimulate antigen-specific immune responses. However, the safety profile and ease of production of recombinant yeast may make it a more desirable candidate for use in the clinic.

As mentioned previously, the data reported here regarding phenotypic maturation, inflammatory cytokine production, and stimulation of immune responses are in accord with prior murine studies. Notably, our results also correlate with previous studies that investigated the interactions between recombinant yeast and human DCs. In a paper published in 2006, Barron et al. showed that yeast directly induced phenotypic maturation of monocyte-derived DCs (MDDCs) and enriched blood myeloid DCs by increasing expression of MHC I and II, ICAM, CD40, and CD86 [37]. Furthermore, yeast-pulsed MDDCs and blood myeloid DCs produced inflammatory cytokines, including IL-12, IL-10, and TNF-α, and stimulated allo-reactive T-cell proliferation. Human DCs pulsed with yeast recombinant for HIV-1 Gag protein were also capable of stimulating in vitro expansion of Gag-specific CD8$^+$ memory T cells [37]. By showing that our results using murine models closely mirror those reported using human cells, these data, taken together, form the rational basis for the use of yeast-based vaccines in the clinical setting.

In summary, the data presented here show that vaccination with yeast-CEA results in effective recruitment of immune-mediated cells to the draining lymph node, a necessary precursor to a potent immune response. In vitro investigations demonstrated that treatment with yeast-CEA efficiently matures and activates DCs. Furthermore, these studies also revealed many of the genes that are altered as part of the DC activation and maturation process. Subsequent functionality studies showed that yeast-CEA-treated DCs exhibit an enhanced ability to stimulate antigen-specific T cells in vitro. Finally, vaccination with yeast-CEA resulted in effective stimulation of both CEA-specific CD4$^+$ and CD8$^+$ T cells in a transgenic mouse model. These results provide support for the development of recombinant yeast constructs for immunotherapeutic approaches to cancer and infectious diseases.

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