Vaccination with a Recombinant *Saccharomyces cerevisiae* Expressing a Tumor Antigen Breaks Immune Tolerance and Elicits Therapeutic Antitumor Responses

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Abstract

Purpose: *Saccharomyces cerevisiae*, a nonpathogenic yeast, has been used previously as a vehicle to elicit immune responses to foreign antigens, and tumor-associated antigens, and has been shown to reduce tumor burden in mice. Studies were designed to determine if vaccination of human carcinoembryonic antigen (CEA)-transgenic (CEA-Tg) mice (where CEA is a self-antigen) with a recombinant *S. cerevisiae* construct expressing human CEA (yeast-CEA) elicits CEA-specific T-cell responses and antitumor activity.

Experimental Design: CEA-Tg mice were vaccinated with yeast-CEA, and CD4^{+} and CD8^{+} T-cell responses were assessed after one and multiple administrations or vaccinations at multiple sites per administration. Antitumor activity was determined by tumor growth and overall survival in both pulmonary metastasis and s.c. pancreatic tumor models.

Results: These studies demonstrate that recombinant yeast can break tolerance and that (*a*) yeast-CEA constructs elicit both CEA-specific CD4^{+} and CD8^{+} T-cell responses; (*b*) repeated yeast-CEA administration causes increased antigen-specific T-cell responses after each vaccination; (*c*) vaccination with yeast-CEA at multiple sites induces a greater T-cell response than the same dose given at a single site; and (*d*) tumor-bearing mice vaccinated with yeast-CEA show a reduction in tumor burden and increased overall survival compared to mock-treated or control yeast-vaccinated mice in both pulmonary metastasis and s.c. pancreatic tumor models.

Conclusions: Vaccination with a heat-killed recombinant yeast expressing the tumor-associated antigen CEA induces CEA-specific immune responses, reduces tumor burden, and extends overall survival in CEA-Tg mice. These studies thus form the rationale for the incorporation of recombinant yeast-CEA and other recombinant yeast constructs in cancer immunotherapy protocols.

One of the reasons for interest in recombinant *Saccharomyces cerevisiae* as a vaccine vehicle is its lack of toxicity. Besides being inherently nonpathogenic, this particular species of yeast can be heat-killed before administration and has been shown to be safe in humans in several clinical trials, with maximum tolerated dose not reached (1–3). *S. cerevisiae* can be easily engineered to express one or more antigens in large quantities, can be propagated and purified rapidly, and is very stable (4). In addition, recombinant yeast has been shown to induce a robust host immune response to non-self-antigens (1, 4–6). These characteristics make *S. cerevisiae* a potential component for cancer immunotherapy protocols.

It has been shown that *S. cerevisiae* and other yeast species initiate immune responses by inducing maturation of dendritic cells. In addition to the expected presentation of yeast-expressed antigen via MHC class II, antigen is delivered to MHC class I pathways by cross-presentation (6–10). Because of this ability to induce robust immune responses, several studies have been conducted using *S. cerevisiae* as a vaccine vehicle. Studies with recombinant *S. cerevisiae* expressing several different antigens have shown that vaccination with this construct induces antigen-specific T-cell responses both *in vitro* and *in vivo* (7, 11–13).

Recently, recombinant *S. cerevisiae* constructs expressing tumor-associated antigens (TAA) have been engineered for cancer immunotherapy (1, 4, 7). In tumor prevention studies, vaccination with *S. cerevisiae* expressing TAA has been shown to protect against tumor challenge (1, 7). Additionally, tumor therapy studies have shown that when tumor-bearing mice are vaccinated with *S. cerevisiae* constructs expressing the appropriate point-mutated Ras protein, tumor growth is slowed (11, 14).

Because of the potential of recombinant *S. cerevisiae* for use in cancer therapy, we sought to determine whether a

recombinant *S. cerevisiae* construct expressing carcinoembryonic antigen (CEA) would induce CEA-specific T-cell responses and show antitumor activity against CEA⁺ tumors in CEA-transgenic (CEA-Tg) mice. CEA is a human self-TAA expressed on a large percentage of human carcinomas, including carcinomas of the colon, rectum, stomach, breast, and lung. Because of this, it has frequently been used as a target for immunotherapy (15). In these studies, we used a CEA-Tg mouse model where CEA is expressed as a self-antigen in fetal tissues and various parts of the gut (16), more accurately mimicking its expression in humans. CEA-Tg mice have been shown previously to be tolerant to CEA (17). To our knowledge, this is the first study conducted where yeast vehicles are employed to break tolerance in mice transgenic for the TAA found in the vaccine.

These studies show for the first time that (*a*) vaccination of CEA-Tg mice with yeast-CEA constructs induces CEA-specific CD4⁺ and CD8⁺ T-cell responses, (*b*) multiple yeast-CEA vaccinations can be given with an increase in T-cell responses seen after each administration, (*c*) single-site vaccination of tumor-bearing mice with yeast-CEA significantly decreases tumor volume and increases overall survival, and (*d*) vaccinating in multiple injection sites produces greater increases in T-cell responses and greater decreases in tumor volume than single-site vaccination.

Materials and Methods

Mice. These studies used 6- to 8-week-old female mice. A breeding pair of C57BL/6 mice that were homozygous for expression of the human CEA gene, designated CEA-Tg mice, was generously provided by Dr. John Shively (The Beckman Research Institute of the City of Hope, City of Hope National Medical Center, Duarte, CA). The mice were originally generated by microinjecting a 32.6-kb *Aat*II restriction fragment containing the entire human CEA genomic region into a pronucleus of C57BL/6 zygotes (18). The DNA also contained the regulatory sequences that resulted in tissue-specific expression of CEA protein predominantly in the gastrointestinal tract of the CEA-Tg mice. Homozygosity for CEA expression was tested and verified by screening of progeny mice for CEA expression using PCR analysis on mouse tail DNA (15). All mice were housed and maintained in microisolator cages under specific pathogen-free conditions and in accordance with Association for Assessment and Accreditation of Laboratory Animal Care guidelines. All experimental studies were carried out under approval of the Intramural Animal Care and Use Committee.

Yeast constructs. A recombinant *S. cerevisiae* construct expressing full-length CEA was generated by methods similar to those described previously (14). Control yeast (also termed YVEC for vector-transfected yeast) was constructed as described previously (ref. 7; GlobeImmune), except that a constitutive rather than copper-inducible promoter was employed to drive antigen expression. To express CEA, *S. cerevisiae* was engineered to express full-length glycosylated CEA protein under the control of the yeast constitutive translation elongation factor 1α (TEF2) promoter. Yeast high copy 2 μmol/L expression plasmid pGI-100 was used as the backbone vector as described previously (14). Forward primer 5′-CGGAATTCATGGAGTCTCCCTCGGCCCC-3′ and reverse primer 5′-ATAAGAATGCGGCCGCTAAACTAGTGATGGTGATGGT-GATGTATCAGAGCAACCCCAACC-3′ were used to amplify the full-length CEA cDNA and inserted into plasmid pGI-172 to generate plasmid pGI-162, which was individually transfected into W303α *S. cerevisiae* yeast to create yeast-CEA. Expression of the full-length CEA protein was confirmed by immunoblot analysis of lysates from heat-inactivated yeast-CEA using monoclonal antibodies against human CEA

(Fitzgerald Industries). The results revealed a ~71-kDa polypeptide plus an additional ~130-kDa protein. The 130-kDa polypeptide apparently harbors complex N-linked glycosylation and the GPI lipid anchor, whereas the 70 kDa polypeptide apparently represents a core glycosylated, non-GPI-anchored polypeptide.

Yeast constructs were produced and heat-killed for these studies as described previously (11). Mice were injected with the indicated number of yeast unit (YU; 1 YU = 10⁷ yeast particles) of control yeast or yeast-CEA s.c. in the right flank unless otherwise noted.

Poxvirus constructs. Recombinant vaccinia and recombinant fowlpox viruses containing murine B7-1, ICAM-1, and LFA-3 genes in combination with human CEA (CEA/TRICOM) have been described previously (19). The recombinant fowlpox virus containing the gene for murine GM-CSF has also been described previously (20). Therion Biologics kindly provided all of the orthopoxviruses as part of a Collaborative Research and Development Agreement with the National Cancer Institute/NIH.

Tumor cells. Murine colon carcinoma MC38 cells (H-2ᵇ) expressing human CEA (designated MC38-CEA⁺) were generated by retroviral transduction with CEA cDNA (21). For cytotoxicity assays, the target tumor cell line EL-4 (H-2ᵇ, thymoma) was obtained from American Type Culture Collection. The murine ductal adenocarcinoma cell line Panc02 was generously provided by Dr. Michael A. Hollingsworth (University of Nebraska Medical Center). The parental Panc02 cell line was established through the induction of pancreatic tumors with 3-methylcholanthrene and serial s.c. transplantation in C57BL/6 mice (22). Panc02 cells with stable expression of human CEA (designated Panc02.CEA) were generated by retroviral transduction with human CEA cDNA using methods described previously (21). Panc02.CEA cells were cultured in McCoy's 5A medium supplemented with 1 mmol/L sodium pyruvate, 1× nonessential amino acids, 2 mmol/L L-glutamine, 10 mmol/L HEPES, 300 μg/mL G418 sulfate, and 10% heat-inactivated fetal bovine serum. Unless otherwise indicated, all media and their components were purchased from Mediatech.

Lymphocyte proliferation assays. To evaluate T-cell immune responses to CEA, splenic T cells were tested for cell proliferation in response to CEA protein. Splenic cells were dispersed into single-cell suspensions in 10% FCS/RPMI 1640 followed by removal of RBC. Lymphocytes were then separated by centrifugation through a Ficoll-Hypaque gradient. Cells at the interface were collected and washed in 10% FCS/RPMI 1640. CD4⁺ or CD8⁺ cells were isolated by negative selection and found to be >90% pure (Miltenyi Biotec). Purified T cells (2 × 10⁵ per well) were cultured for 5 days (restimulation) in 96-well flat-bottomed plates with naive syngeneic splenocytes irradiated with 2,000 rads as antigen-presenting cells (5 × 10⁵ per well) and with CEA protein in 10% FCS/RPMI 1640. T cells and antigen-presenting cells were cultured in medium only as a control. [³H]thymidine (1 μCi/well) was added to the wells for the last 24 h and harvested using a Tomtec cell harvester (Wallac). The incorporated radioactivity was measured using a liquid scintillation counter (Wallac 1205 Betaplate; Wallac).

CEA-specific CD8⁺T-cell immune response. To evaluate CEA-specific CD8⁺ T-cell immune responses, spleens were pooled and dispersed into single-cell suspensions and stimulated with 1 μg/mL H-2Dᵇ-restricted CEA peptide CEA₅₇₂₋₅₇₉ (GIQNSVSA; CPC Scientific; ref. 23). Six days later, bulk splenocytes were separated by centrifugation through a Ficoll-Hypaque gradient. For the assay of tumor-killing activity, the recovered lymphocytes and ⁵¹Cr-labeled target cells (EL-4; 5 × 10³ per well) pulsed with the CEA₅₇₂₋₅₇₉ peptide or vesicular stomatitis virus nucleoprotein VSV-NP₅₂₋₅₉ (RGYVYQGL) control peptide (CPC Scientific) were incubated for 5 h in 96-well U-bottomed plates, and radioactivity in supernatants was measured using a gamma counter (Corba Autogamma; Packard Instruments). The percentage of tumor lysis was calculated as follows: % tumor lysis = [(experimental counts/min - spontaneous counts/min) / (maximum counts/min - spontaneous counts/min)] × 100.

Tumor therapy studies. For tumor therapy studies involving the MC38-CEA⁺ cell line, 6-week-old female CEA-Tg mice were injected
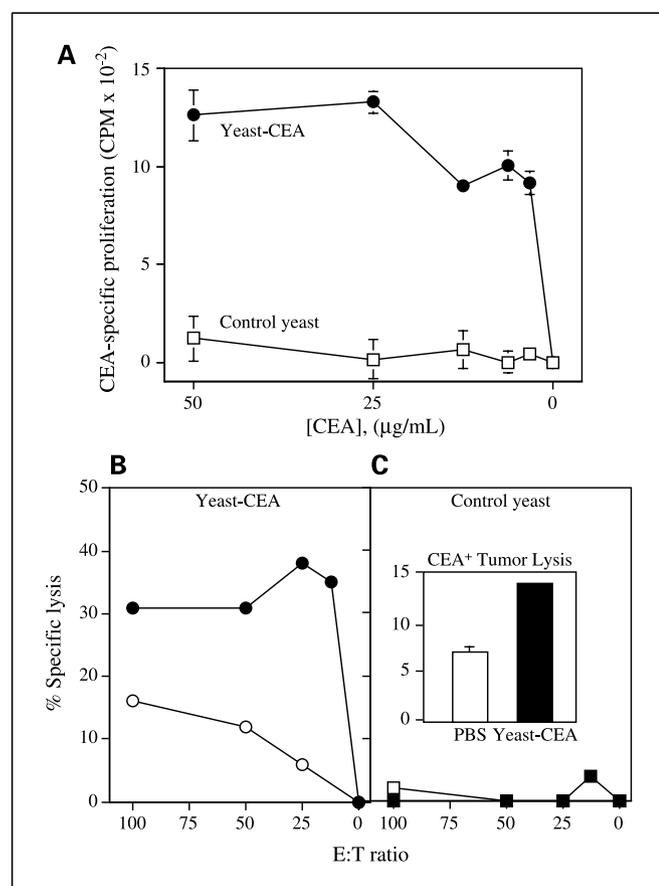
Fig. 1. Vaccination with yeast-CEA induces antigen-specific T-cell responses. CEA-Tg mice were vaccinated with 0.1 YU control yeast or yeast-CEA on days 0 and 7. On day 21, mice were sacrificed, spleens were harvested, and splenocytes were used for assays. *A,* CD4$^+$ cell proliferation. Purified CD4$^+$ T cells were cultured with irradiated antigen-presenting cells 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. *Open squares,* control yeast; *filled circles,* yeast-CEA. *B,* CD8$^+$ CTL activity after vaccination with yeast-CEA. 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 was calculated. SD is based on the mean of triplicate wells. *Open circles,* CTL activity directed against EL-4 cells pulsed with VSV-NP peptide; *filled circles,* CTL activity directed against EL-4 cells pulsed with CEA peptide. *C,* CD8$^+$ CTL activity after vaccination with control yeast. *Open squares,* CTL activity directed against EL-4 cells pulsed with VSV-NP peptide; *filled squares,* CTL activity directed against EL-4 cells pulsed with CEA peptide. *C, inset,* CD8$^+$ CTL activity against tumor cells after vaccination with yeast-CEA. Splenocytes were stimulated with CEA$_{572-579}$ peptide as above. Lymphocytes were incubated for 5 h with ^{51}Cr-labeled target MC38-CEA$^+$ (*filled column*) at an effector/target ratio of 100:1. As a negative control, splenocytes from mice receiving PBS (vehicle control) were stimulated with CEA peptide as above and CTL activity was directed against MC38-CEA$^+$ tumor cells (*white column*).

i.v. in the tail with 1×10^6 MC38-CEA$^+$ cells in a volume of 100 µL. Four days following tumor implantation, mice receiving single-site vaccination were injected s.c. in the right flank with PBS, control yeast, or yeast-CEA at 7-day intervals (as indicated in the figure legends). Mice vaccinated in four sites were injected s.c. in both inner thighs and above each shoulder blade to target the inguinal, axillary, and subclavicular lymph node beds. For studies involving the Panc02.CEA cell line, mice were injected s.c. on the lower back with 1×10^6 Panc02.CEA cells in a volume of 100 µL. In the first of two studies evaluating the antitumor efficacy of the yeast-CEA vaccine against Panc02.CEA tumors, mice received s.c. injections of either PBS or 1 YU yeast-CEA in the left inner thigh 7 days post-tumor challenge followed by six weekly booster

vaccinations (1 YU/vaccination) at the same site. The second study was designed to evaluate escalating doses of yeast-CEA by administering 1 YU per site at one, two, four, or six vaccination sites chosen to target bilateral regional lymph node beds, including the axillary, subclavicular, inguinal, and mesenteric lymph nodes. As in the first study, mice received a primary vaccination followed by six booster vaccinations. Tumors were measured twice weekly by digital caliper in two dimensions, and volumes were calculated as described previously (19). In all experiments, mice were sacrificed when they exhibited signs of respiratory distress and/or appeared moribund or cachectic.

Regulatory T-cell assay. To determine the effects of yeast-CEA on regulatory T cells, spleens were pooled from untreated CEA-Tg mice or mice that were vaccinated in one site with 1 YU every 7 days for 4 weeks. Splenocyte single-cell suspensions were prepared in 10% FCS/RPMI 1640, RBC were removed, and lymphocytes were isolated on a Ficoll-Hypaque gradient. The lymphocytes were collected and washed in 10% FCS/RPMI 1640. A mouse regulatory T-cell staining kit (eBioscience) was used to identify cells staining CD4$^+$CD25$^+$FoxP3$^+$ as regulatory T cells according to the manufacturer's instructions. Cells were washed and fluorescence was measured with a FACScan cytometer (Becton Dickinson). The data were analyzed using Lysis II software (Becton Dickinson).

Memory T-cell staining. To investigate if yeast-CEA vaccinated animals develop central memory T cells, spleens from mice vaccinated in one site every 7 days for 4 weeks with 1 YU at one site were harvested, splenocyte single-cell suspensions were prepared, and lymphocytes were collected as above. Lymphocytes were washed in PBS/5% bovine serum albumin and preincubated with anti-mouse CD16/CD32 (2.4G2) monoclonal antibody (BD Biosciences) on ice for 15 min to block FcR. Cells were incubated for 30 min at 4°C in the following antibodies: FITC rat anti-mouse CCR7 (Abcam), PECy5 rat anti-mouse CD8 (BD Biosciences), or isotype controls. The cells were washed twice with PBS/5% bovine serum albumin and twice with PBS. The stained lymphocytes were resuspended in PBS and fixed using Cytofix Buffer (BD Biosciences). Cell fluorescence was analyzed and compared with that of the appropriate isotype controls (BD Biosciences) with a FACScan cytometer using Lysis II software (Becton Dickinson).

Serum cytokine analysis. Mice were bled and serum isolated 96 h after vaccination. A Th1/Th2 and proinflammatory cytokine panel was used for serum cytokine analysis by Linco Diagnostic Services.

Toxicology. Antibody levels to SM, histone, SCL-70 (DNA topoisomerase I), dsDNA, ssDNA, and circulating immune complexes were determined in a qualitative or semiquantitative manner (Alpha Diagnostic International) according to the manufacturer's instructions.

Statistical analysis. Statistical significance was calculated using ANOVA, with repeated measures using Statview 4.1 (Abacus Concepts). Results of tests of significance were derived from Student's t test using a two-tailed distribution and reported as P values (calculated at 95% confidence intervals). In graphic representations of data, y axis error bars indicate the SD for each point on the graph. In some cases, the variation is such that the plot symbol obscures the error bars. Evaluation of survival patterns in mice bearing lung metastases was done by the Kaplan-Meier method and ranked according to the Mantel-Cox log-rank test using Statview 4.1. Evaluation of trend in tumor volumes with multiple vaccination sites was done by linear least-squares analysis.

Results

Vaccination with yeast-CEA induces antigen-specific T-cell responses. Previous studies have shown that recombinant *S. cerevisiae* constructs can elicit immune and antitumor responses in mice (1, 4, 7, 14). However, to our knowledge, no studies have been reported showing these effects where the recombinant antigen is "self" in a transgenic mouse model.

Therefore, we sought to determine whether vaccination with yeast-CEA could generate CEA-specific immune responses in CEA-Tg mice. Mice were vaccinated with 0.1 YU control yeast or yeast-CEA injected s.c. on days 0 and 7. Mice were sacrificed 14 days later, spleens were harvested, and CD4^{+} proliferation and CD8^{+} cell-killing assays were done. As shown in Fig. 1A, vaccination with control yeast induced minimal proliferation at all concentrations of CEA, whereas vaccination with yeast-CEA induced significantly higher CD4^{+} proliferation at all concentrations of CEA. For example, at 50 μg/mL CEA, there was a 6-fold increase in proliferation ($P = 0.002$); at 25 μg/mL CEA, $P = 0.001$; at 12.5 μg/mL CEA, $P = 0.003$. Vaccination with yeast-CEA also induced high levels of CEA-specific CD8^{+} cell killing (Fig. 1B). CD8^{+} T cells from mice vaccinated with yeast-CEA showed 30% to 40% lysis of target cells pulsed with CEA peptide (*filled circles*) and only 5% to 15% lysis of target cells pulsed with the control VSV-NP peptide (*open circles*; $P = 0.01$
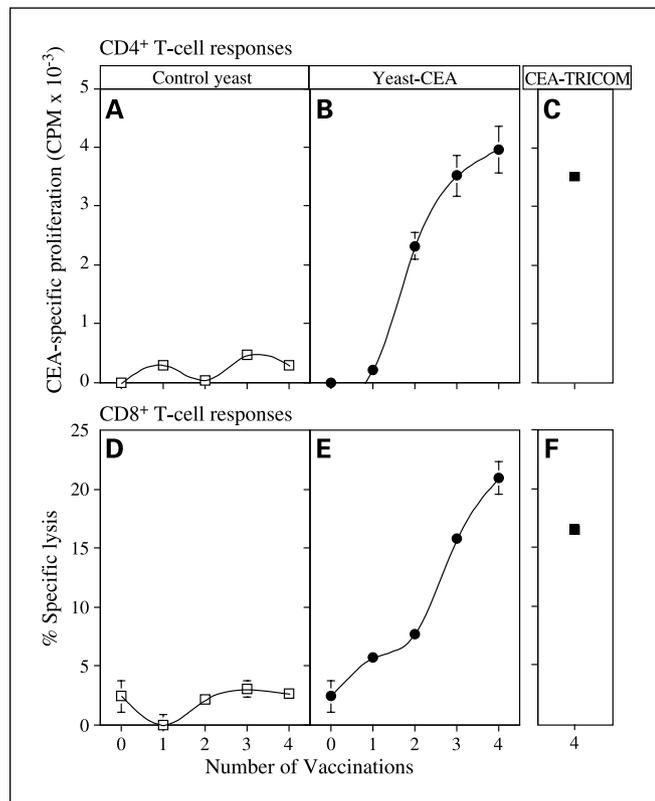

Fig. 3. CD8^{+} CTL responses after dose escalation of yeast-CEA. CEA-Tg mice were vaccinated with 0, 0.01, 0.1, 1, or 10 YU yeast-CEA twice at 7-day intervals. Fourteen days after the last vaccination, mice were sacrificed, spleens were harvested, and splenocytes were stimulated with CEA peptide for 6 d. 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. *Open circles*, EL-4 cells pulsed with VSV-NP peptide; *filled circles*, EL-4 cells pulsed with CEA peptide.

Fig. 2. Multiple vaccinations with yeast-CEA continuously increase T-cell response. CEA-Tg mice were vaccinated with 0.1 YU control yeast or yeast-CEA one, two, three, or four times at 7-day intervals. Fourteen days after the last vaccination, mice were sacrificed, spleens were harvested, and splenocytes were used for assays. For comparison, mice were vaccinated with CEA-TRICOM. *Open squares*, control yeast, *filled circles*, yeast-CEA, *filled squares*, CEA-TRICOM. *A*, CD4^{+} T-cell proliferation after vaccination with control yeast. Purified CD4^{+} T cells were cultured with irradiated antigen-presenting cells and CEA protein for 5 d. [^{3}H]thymidine (1 μCi/well) was added to the wells for the last 24 h, and proliferation was assayed by measuring incorporated radioactivity. *B*, CD4^{+} T-cell proliferation after vaccination with yeast-CEA. *C*, CD4^{+} T-cell proliferation after vaccination with rV/rF-CEA-TRICOM. *D*, CD8^{+} CTL activity after vaccination with control yeast. Splenocytes were stimulated with CEA 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 was calculated. SD is based on the mean of triplicate wells. *E*, CD8^{+} CTL activity after vaccination with yeast-CEA. *F*, CD8^{+} CTL activity after vaccination with rV/rF-CEA-TRICOM vaccines. Data are presented as percent lysis after subtraction of VSV-NP control.
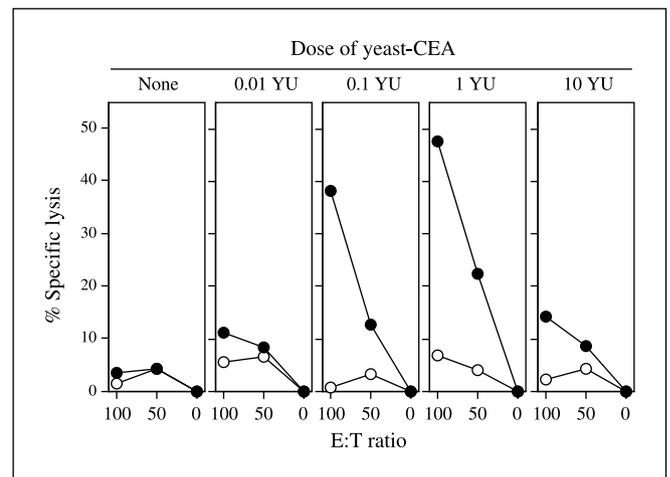
at an effector/target ratio of 100:1). CD8^{+} T cells from mice vaccinated with control yeast lysed <5% of target cells pulsed with either the CEA or VSV-NP peptides (Fig. 1C). In addition, the T cells from CEA-Tg mice vaccinated with yeast-CEA could lyse tumor cells expressing CEA (Fig. 1C, *inset*). CD8^{+} T cells from mice vaccinated with yeast-CEA showed 14% lysis of MC38-CEA^{+} tumor cells (*white columns*), whereas mice injected with PBS (vehicle control) showed only 7% lysis ($P = 0.01$). Taken together, these data show that vaccination with yeast-CEA can break tolerance and induce CEA-specific CD4^{+} and CD8^{+} T-cell responses in CEA-Tg mice and that this effect is specific to the yeast-CEA construct.

Multiple vaccinations with yeast-CEA continuously increase the immune response. Mice were vaccinated one, two, three, or four times at 7-day intervals with 0.1 YU of control yeast or yeast-CEA. Mice were sacrificed 14 days after the last vaccination, spleens were harvested, and splenocytes were used in both CD4^{+} proliferation and CD8^{+} cell-killing assays. CD4^{+} cells from mice vaccinated with control yeast showed minimal proliferation in response to CEA protein irrespective of the number of vaccinations (Fig. 2A). However, in mice vaccinated with yeast-CEA, proliferation of CD4^{+} cells continued to increase as the number of vaccinations increased (Fig. 2B). There was a 10-fold increase in proliferation after the second vaccination ($P = 0.001$) and a 1.5-fold increase after the third vaccination ($P = 0.006$). Although not statistically significant ($P = 0.1$), there was a further increase in proliferation after the fourth vaccination. A similar trend was seen in CD8^{+} cell-killing assays. CD8^{+} cells from mice vaccinated with control yeast showed no/minimal killing even after four vaccinations (Fig. 2D). In mice given yeast-CEA, an increase in cell lysis was seen after each of the first two vaccinations, with a much greater increase in cell lysis after three vaccinations ($P = 0.01$; Fig. 2E) and a significant increase from the third to the fourth vaccination ($P = 0.02$; Fig. 2E). Taken together, these data show that yeast-CEA can be given up to four times, with an increase
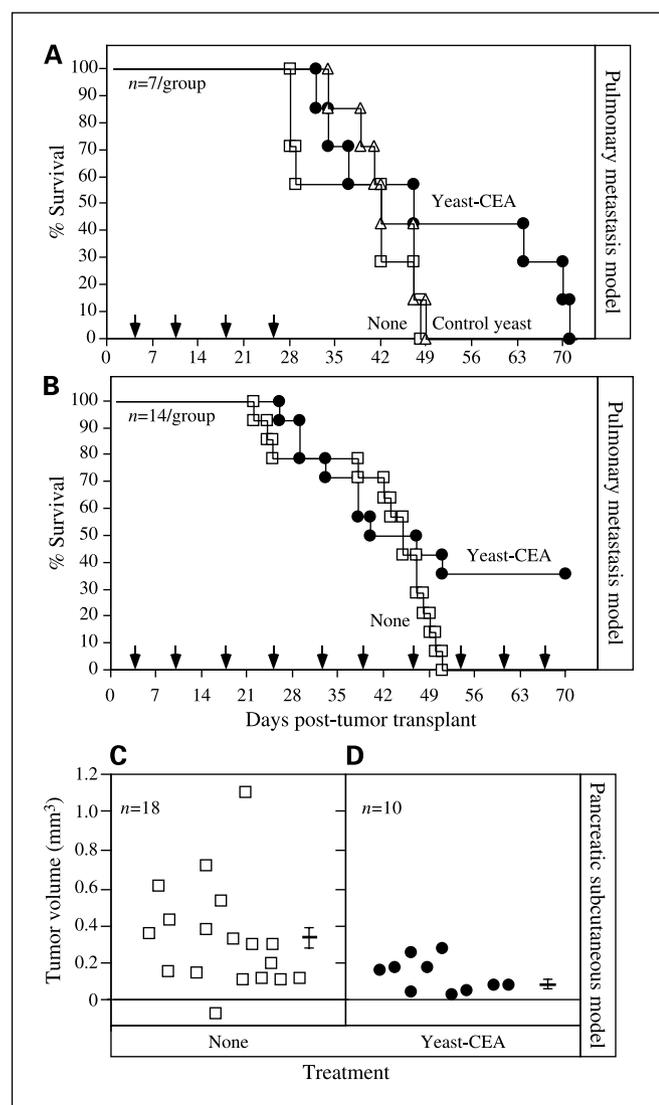
Fig. 4. Vaccination with yeast-CEA reduces tumor growth and increases overall survival in tumor-bearing mice. *A,* survival in an experimental CEA$^+$ lung metastasis model. CEA-Tg mice (*n* = 7 per group) were injected with 1 × 10^6 MC38-CEA$^+$ tumor cells i.v. in the tail on day 0 and mock-treated or injected with 1 YU control yeast or yeast-CEA s.c. on days 4, 11, 18, and 25 (*arrows*). Mice were monitored and survival was recorded. *Open squares,* no treatment; *open triangles,* control yeast; *filled circles,* yeast-CEA. *B,* survival in a lung metastasis model with continuous weekly vaccination (*arrows*). CEA-Tg mice (*n* = 14 per group) were injected with 1 × 10^6 MC38-CEA$^+$ tumor cells i.v. in the tail on day 0 and mock-treated or injected with 1 YU yeast-CEA s.c. starting on day 4 and then weekly for the duration of the experiment. Mice were monitored and survival was recorded. *Open squares,* no treatment; *filled circles,* yeast-CEA. *C* and *D,* vaccination with yeast-CEA in a s.c. pancreatic cancer model. CEA-Tg mice were injected with 1 × 10^6 Panc02.CEA cells s.c. on day 0 and vaccinated with 1 YU yeast-CEA starting on day 7 and then weekly for the duration of the experiment. Tumor volume was measured twice a week and recorded. *C* no treatment (*n* = 18). *D,* yeast-CEA (*n* = 10). *Bars,* average tumor volume ± SD.

in both CD4$^+$ and CD8$^+$ T-cell responses after each vaccination. To compare the relative potency of vaccination with yeast-CEA with another vaccination platform, mice were first vaccinated with a recombinant vaccinia virus expressing CEA and a Triad of Costimulatory Molecules (rV-CEA-TRICOM) and then boosted weekly for three times with a recombinant fowlpox CEA-TRICOM (rF-CEA-TRICOM). Recombinant fowlpox GM-CSF (rF-GM-CSF) was included in this vaccination regimen.

Mice vaccinated with CEA-TRICOM showed comparable levels of CEA-specific CD4$^+$ T-cell proliferation to mice vaccinated four times with yeast-CEA (Fig. 2C). In addition, mice vaccinated with CEA-TRICOM showed similar levels of CEA-specific CD8$^+$ T-cell responses to mice vaccinated four times with yeast-CEA (Fig. 2F).

Effects of yeast-CEA on T-cell responses are dose related. A previous study employing a non-self-antigen has reported a direct correlation between yeast dosage and vaccine efficacy (1). In the experiments described above, 0.1 YU yeast-CEA effectively induced immune responses. To determine the optimal dose of yeast-CEA in this CEA-Tg model, mice were vaccinated with 0, 0.01, 0.1, 1, or 10 YU yeast-CEA on days 0 and 7. Mice were sacrificed 14 days later and spleens were harvested. Splenocytes were incubated with CEA peptide for 6 days before being used in a cell killing assay. As shown in Fig. 3, vaccination with 0.01 YU resulted in only a slight increase in CTL killing versus no treatment. CTL killing increased further with 0.1 and 1 YU, with 1 YU showing the highest amount of killing (0.1 versus 0.01 YU: $P < 0.001$; 1 versus 0.1 YU: $P = 0.01$). Interestingly, vaccinating mice with 10 YU produced a significant decrease in CTL killing compared with the lower doses of 0.1 or 1 YU ($P < 0.001$ versus 1 YU; $P = 0.08$ versus no treatment). These data show that the immune effects of yeast-CEA in this model are dose related and that 1 YU is the optimal dose for eliciting antigen-specific CD8$^+$ T-cell killing. In subsequent studies, we therefore used a dosage of 1 YU yeast-CEA.

To further examine immune responses after vaccination with yeast vectors, studies were conducted on serum cytokines, induction of regulatory T cells, and induction of T cells with a memory phenotype. First, serum was harvested 96 hours after vaccination with 1 YU yeast-CEA or control yeast. Of the 10 cytokines analyzed [IFN-γ, interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, tumor necrosis factor-α, and IL-13], only levels of IL-1β ($P_{\text{trend}} = 0.029$) and IL-6 ($P = 0.0003$) increased, whereas levels of IL-10 decreased ($P_{\text{trend}} = 0.043$) in the serum of mice treated with either yeast-CEA or control yeast compared with the serum of untreated mice (data not shown). Next, studies were conducted to examine the effect of yeast-CEA on the level of regulatory T cells. CEA-Tg mice were vaccinated three times with yeast-CEA and after CD4$^+$/FoxP3$^+$ cells were quantitated from splenocytes 14 days after the last vaccination. Mice vaccinated with yeast-CEA showed 0.57% of the CD4$^+$ T-cell population were FoxP3$^+$, whereas mice that received PBS (vehicle control) showed 0.46% of the CD4$^+$ T-cell population were FoxP3$^+$. This suggests that vaccination with yeast-CEA does not mediate a exaggerated induction of regulatory T cells in CEA-Tg mice. Finally, the phenotype of CD8$^+$ T cells was examined for surface markers associated with memory T cells. CEA-Tg mice vaccinated with yeast-CEA showed 0.76% of the whole spleen population stained CD8$^+$/CCR7$^+$, whereas mice receiving PBS showed 0.67% of the whole spleen population stained CD8$^+$/CCR7$^+$.

Vaccination with yeast-CEA decreases tumor growth and increases survival in tumor-bearing mice. Because the studies described above showed that vaccinating CEA-Tg mice with yeast-CEA induced both CD4$^+$ and CD8$^+$ T-cell responses, studies were then conducted to determine whether these effects would translate to antitumor efficacy. We analyzed yeast-CEA vaccination in two different CEA$^+$ tumor therapy models. In the
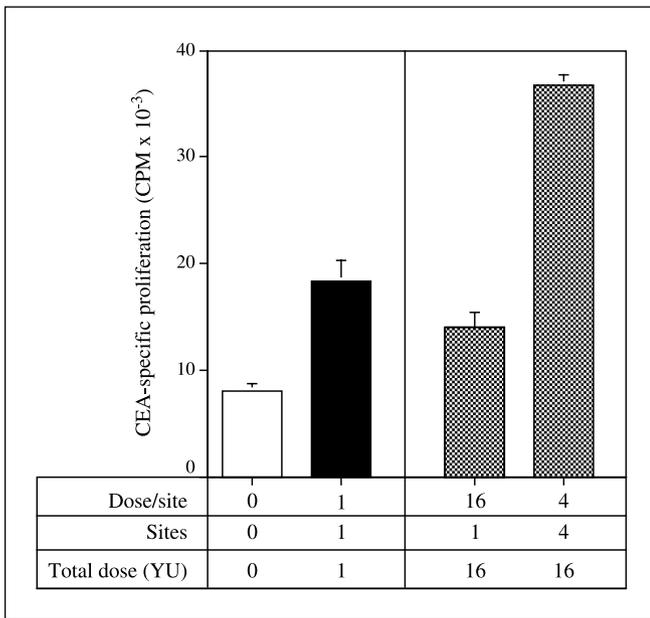
Fig. 5. CD4$^+$ T-cell responses increase when vaccine is distributed to multiple sites. CEA-Tg mice were vaccinated with a total of 1 or 16 YU yeast-CEA s.c. in one or four sites on days 0 and 7. Fourteen days later, mice were sacrificed, spleens were harvested, and CD4$^+$ T cells were purified. Cells were cultured with irradiated antigen-presenting cells 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. SD is based on the mean of triplicate wells. *White column,* no treatment; *black column,* 1 YU in one site; *gray columns,* 16 total YU.

first model, an experimental pulmonary metastasis model, mice were injected with 1×10^6 MC38-CEA$^+$ cells i.v. in the tail. Four days post-tumor transplant, mice (n = 7 per group) were treated with 1 YU control yeast or yeast-CEA weekly for a total of four vaccinations, and their survival was observed and recorded (Fig. 4A). Mice receiving no treatment (*open squares*) and mice vaccinated with control yeast (*open triangles*) all died by day 49

post-tumor transplant. However, mice vaccinated with yeast-CEA (*filled circles*) survived 63 days post-tumor transplant. As there were only seven mice per group, this difference was not significant (P = 0.186 versus mice receiving no treatment or control yeast), but the trend suggested that vaccination with yeast-CEA increased survival. To extend these findings in the same model (Fig. 4B), the number of mice per group was increased to 14 and mice were now vaccinated weekly for the duration of the experiment. Mice receiving no treatment all died by day 50 (Fig. 4B) as in the previous experiment (Fig. 4A). However, by day 70, 35% of mice receiving yeast-CEA were still alive. This effect on survival was significant compared with the no treatment group (P = 0.039). These data show that vaccination with yeast-CEA significantly extends survival in a CEA$^+$ experimental pulmonary metastasis model when continued to be administered weekly.

We next conducted experiments using a murine pancreatic cancer cell line transfected with the CEA gene as described in Materials and Methods. Panc02.CEA cells (1×10^6) were implanted s.c. into CEA-Tg mice on day 0. Mice were vaccinated on day 7 post-tumor transplant and were vaccinated weekly with yeast-CEA for the duration of the experiment; tumor volumes were measured. For mice receiving no treatment (Fig. 4C), average tumor volume at day 35 post-tumor transplant was 0.32 mm^3; for mice receiving yeast-CEA (Fig. 4D), average tumor size at the same time point was significantly smaller (0.13 mm^3; P = 0.03). Reduced tumor volume in this model also correlated with increased survival, as mice receiving yeast-CEA survived significantly longer than those receiving no treatment (P = 0.0008). Taken together (Fig. 4A-D), these data show that vaccination with yeast-CEA slows tumor growth and extends survival in CEA-Tg tumor-bearing mice.

Multiple-site vaccination is more effective than single-site vaccination. In the experiments described above, mice were vaccinated in a single site. However, previous studies using whole tumor cell vaccines have suggested that vaccinating in

Fig. 6. Vaccination in multiple sites increases antitumor efficacy. CEA-Tg mice were implanted with 1×10^6 Panc02.CEA cells s.c. on day 0 and vaccinated in zero, one, two, four, or six sites with 1 YU yeast-CEA/site starting on day 7 and then weekly for the duration of the experiment. Tumor volume was measured twice a week and recorded. *A,* no treatment (n = 10). *B,* 1 YU in one site (n = 9). *C,* 1 YU in two sites (n = 10). *D,* 1 YU in four sites (n = 10). *E,* 1 YU in six sites (n = 10). *Bars,* average tumor volume; $\pm$ SD. *Open squares,* no treatment; *filled circles,* yeast-CEA.
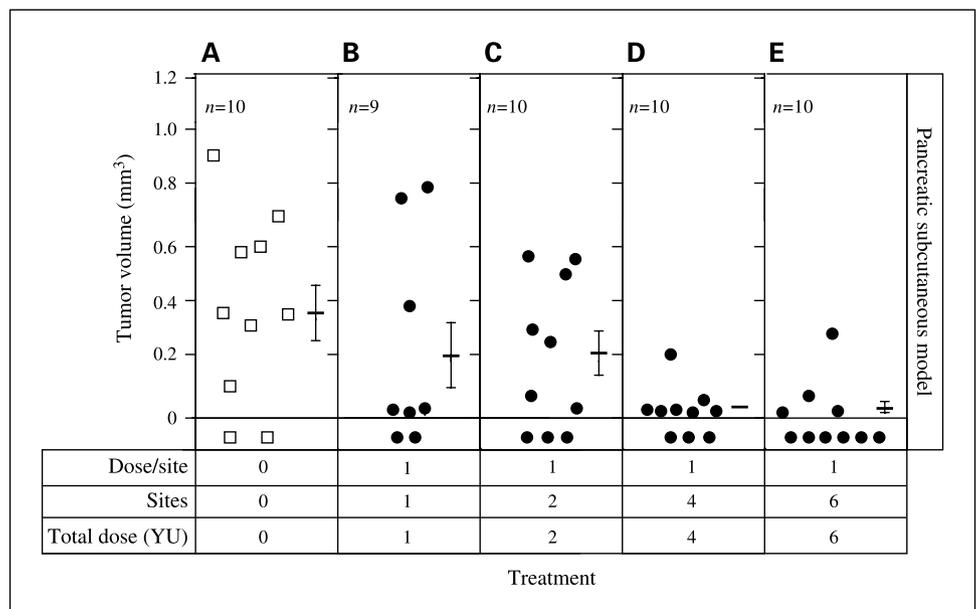
Table 1. Clinical, blood, serologic, and autoimmune variables of CEA-Tg mice vaccinated with yeast-CEA

Test	Vaccinated with yeast-CEA (*n* = 5)*	Age-matched control group (*n* = 5)
Weight (g) [†]	24.39 ± 1.46	23.5 ± 2.59
Blood assays [‡]		
WBC count (k/µL)	9.8	10
RBC count (mol/L/µL)	8.4	8.95
Hemoglobin (g/dL)	12.7	13.5
Mean corpuscular volume (fl)	45.2	45.2
Platelets (k/µL)	1,075	1,083
Lymphocytes (%)	84.1	87.3
Monocytes (%)	4.1	4.1
Eosinophils (%)	1.5	1.4
Basophils (%)	1.6	1.0
Serum assays[§]		
Blood urea nitrogen (mg/dL)	17	15
Creatinine (mg/dL)	949	889
Total protein (g/dL)	4.5	4.5
Albumin (g/dL)	3.3	3.3
Creatine kinase (units/L)	0.3	0.3
Alanine aminotransferase (IU/L)	32	37
Alkaline phosphatase (IU/L)	105	107
Aspartate aminotransferase (IU/L)	104	131
Autoimmune assays[‖]		
Smith antigen	Negative	Negative
Histone antibody	+/-	+/-
DNA topoisomerase I (SCL-70 antibody)	Negative	Negative
dsDNA antibody	Negative	Negative
ssDNA antibody	Negative	Negative
Circulating immune complex	+/-	+/-

*Twelve-week-old CEA-Tg mice were vaccinated with 1 YU/site over four sites (4 YU total). Mice were boosted three times at 7-day intervals. Age-matched mice were CEA-Tg.
[†] Weights were taken 8 wk following the final boost vaccination.
[‡] Blood was drawn 2 wk following the final boost vaccination and pooled before testing.
[§] Sera were drawn 3 wk following the final boost vaccination and pooled before testing.
[‖] Results are semiquantitative and are expressed as +/- or 0 to 4+.

multiple sites, thereby targeting multiple draining lymph nodes, could induce a more effective immune response (24, 25). Mice vaccinated on days 0 and 7 in four sites received vaccine s.c. in both inner thighs and above each shoulder blade to target the inguinal, axillary, and subclavicular lymph node beds. Fourteen days later, mice were sacrificed, spleens were harvested, and splenocytes were analyzed in a CEA-specific CD4^{+} T-cell proliferation assay. Mice injected with 1 YU in a single site (as in previous studies described above) showed a significant increase in CD4^{+} T-cell proliferation versus mice receiving no treatment (Fig. 5, *left*; *P* = 0.001). CD4^{+} T-cell proliferation was then tested in mice vaccinated with 16 total YU (Fig. 5, *right*), either in a single site (*left column*) or distributed over four sites, with 4 YU/site (*right column*). CD4^{+} T-cell proliferation increased significantly in mice vaccinated with 16 YU distributed over multiple sites compared with mice receiving either 1 YU (*P* = 0.0001) or 16 YU (*P* < 0.0001) in a single site. These data show that whereas vaccinating in a single site effectively induces CD4^{+} T-cell responses, spreading the same dose out over multiple sites improves the magnitude of this response.

With this knowledge at hand, studies were conducted to determine whether vaccinating in multiple sites would further increase antitumor efficacy. Mice were implanted with Panc02.CEA cells s.c. on day 0 and vaccinated weekly beginning 7 days post-tumor transplant with 1 YU yeast-CEA in each of zero, one, two, four, or six sites for the duration of the experiment (Fig. 6). Average tumor volumes on day 32 post-tumor transplant were recorded as follows: 0.38 mm^3 for mice receiving no treatment (Fig. 6A), 0.22 mm^3 for mice vaccinated in a single site (Fig. 6B), and 0.23 mm^3 for mice vaccinated in two sites (Fig. 6C). Tumor volume decreased further as the number of vaccination sites increased. Average tumor volume was 0.035 mm^3 for mice vaccinated in four sites (Fig. 6D) and 0.042 mm^3 for mice vaccinated in six sites (Fig. 6E). The number of vaccination sites also correlated with the number of tumor-free mice and with survival, as mice receiving vaccine in four or six sites survived longer than those receiving vaccine in zero, one, or two sites (*P* = 0.04). At day 80 post-tumor transplant, 70% of mice vaccinated at four to six sites (*n* = 20) were alive compared with 55% of mice vaccinated at one to two sites (*n* = 20) and 40% of control mice (*n* = 20). Collectively (Figs. 5 and 6), these data indicate that multiple-site vaccination induces more potent immune response and antitumor efficacy than single-site vaccination.

Vaccination with yeast-CEA does not induce toxicity or autoimmunity. Before the yeast-CEA construct can be used in clinical trials, it must be tested for potential toxicities and autoimmune reactions in an appropriate preclinical model. To this end (Table 1), we vaccinated CEA-Tg mice a total of four times in 7-day intervals with 1 YU yeast-CEA in each of four sites (4 YU total). Eight weeks after the final vaccination, no

difference was observed in the average weight of mice vaccinated with yeast-CEA and age-matched control mice receiving no treatment (Table 1). No abnormal clinical signs associated with yeast-CEA vaccination were seen in mice throughout the observation period. Two weeks after the final vaccination, blood was drawn from the mice, pooled, and a complete blood count was done. All results were within normal limits, with no significant differences between vaccinated and nonvaccinated mice (Table 1). To further examine toxicity, sera were collected from mice 3 weeks after the final vaccination and eight serologic variables were measured. As with the complete blood count, all results were within normal limits, with no significant differences between vaccinated and nonvaccinated mice (Table 1). Because these studies were conducted in CEA-Tg mice, there was a possibility that vaccination with yeast-CEA would induce autoimmunity. To test this, we examined sera samples for levels of antinuclear antibodies specific for nRNP, histone, topoisomerase-1 (SCL-70), dsDNA, ssDNA, or circulating immune complexes. There were no detectable levels of antibody in sera samples from mice receiving yeast-CEA or from age-matched control mice (Table 1). Histone and circulating immune complex levels were similar in both vaccinated and control groups (+/-; Table 1); it should be noted, however, that these levels were only slightly above the lower detection limits of the assays and that normal naive C57BL/6 mice have been shown to experience spontaneous age-related increases in antinuclear antibodies such as histone and circulating immune complex. Thus, although CEA-Tg mice receiving yeast-CEA mounted a therapeutic immune response (Figs. 5 and 6), they showed no evidence of autoimmunity (Table 1).

Discussion

S. cerevisiae, a nonpathogenic yeast, has recently gained interest as a vaccine vehicle for the treatment of cancer and infectious diseases. The construct is safe, as the yeast is heat killed before administration. It can be easily engineered to express antigens in large quantities and can be cultured rapidly. In addition, *S. cerevisiae* can induce a robust host immune response, delivering antigen to both MHC class I and II pathways by cross-priming (1, 4–6, 26). All of these qualities make *S. cerevisiae* an attractive vehicle for cancer immunotherapy. In this study, we sought to determine for the first time whether vaccination with a yeast construct expressing a TAA could break tolerance and induce antigen-specific T-cell and antitumor responses. The data presented here show that vaccination with yeast-CEA not only elicits CEA-specific CD4$^+$ and CD8$^+$ T-cell responses but also decreases tumor volume and increases overall survival in tumor-bearing mice. For comparison, CEA-Tg mice were also vaccinated with a well-defined CEA-based vaccine, which uses poxviruses as delivery vehicles for TAAs in combination with TRICOM (B7-1, ICAM-1, and LFA-3; ref. 19). Similar to yeast vaccines, TRICOM-based vaccines have been shown to elicit antigen-specific immune responses by enhancing the ability of dendritic cells to activate both naive and effector T cells *in vitro* and *in vivo*. Vaccination with CEA-TRICOM resulted in similar levels of CD4$^+$ proliferation (Fig. 2C) compared with that seen in mice vaccinated with yeast-CEA (Fig. 2B). In addition, the level of CD8$^+$-specific

lysis induced following vaccination with CEA-TRICOM (Fig. 2F) was equivalent to that seen with yeast-CEA injection (Fig. 2E). This observation is similar to that reported in CEA-Tg mice by Bernstein et al. (26). 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*.

It has been shown that yeast species such as *S. cerevisiae* initiate immune responses by inducing maturation of dendritic cells (6–10, 26). In this process, the yeast is phagocytized by immature dendritic cells and their proteins are degraded into peptides and presented on the cell surface via MHC class I and II receptors. The dendritic cells mature and migrate to lymphoid organs, where they prime T-cell responses to yeast antigens (1, 7, 27). Studies with recombinant *S. cerevisiae* expressing the HIV-1 Gag protein showed that when blood myeloid dendritic cells were exposed to the recombinant yeast, the dendritic cells stimulated the expansion of Gag-specific CD8$^+$ memory T cells *in vitro* (12). In a separate study, CD8$^+$ T cells from mice vaccinated with a yeast construct expressing a hepatitis C virus (HCV) NS3-core fusion protein killed target cells expressing HCV NS3 (11). Additionally, CTLs from mice vaccinated with recombinant *S. cerevisiae* expressing the HIV-1$_{SF2}$-gp160 envelope protein (13) killed target cells expressing gp160-SF2. HIV-1-gp120-specific helper T cells were also induced after vaccination, showing that yeast vehicles can deliver antigens to both MHC class I and II pathways (7). The study reported here extends these data, as vaccination with yeast-CEA elicited robust antigen-specific CD4$^+$ T-cell proliferation (Figs. 1, 2, and 5) and CD8$^+$ T-cell lysis (Figs. 1-3) of target cells; results were made even more significant by the demonstrated breaking of immune tolerance to a self-antigen. These T-cell responses were functionally significant as evidenced by therapeutic control of tumor proliferation and improved survival in these CEA-Tg mice.

Because therapeutic control of preexisting cancers is likely to require repeated administration of yeast-CEA to effectively activate tumor-specific immune responses, especially to self-antigens, we explored whether host immune responses to the first yeast-CEA vaccination would decrease or neutralize the efficacy of further boosts. Lu et al. reported previously that vaccinating 10 times with a *S. cerevisiae* construct expressing Ras showed increased antitumor efficacy over that seen when six vaccinations were used (55% versus 28% reduction in tumor volume, respectively; ref. 28). Additionally, splenocytes from mice vaccinated up to three times with a yeast construct expressing a HCV NS3-core fusion protein showed cell killing that increased after each vaccination (11). To our knowledge, however, no one has studied the effect of multiple vaccinations on the induction of T-cell responses in a transgenic mouse model. The studies reported here show that CD4$^+$ and CD8$^+$ T-cell responses increase after each of four vaccinations (Fig. 2), extending the previous observations suggesting that neutralization of the yeast construct by potential immune responses would not reduce the effectiveness of continued administrations.

Recently, several studies have examined the effect of recombinant *S. cerevisiae* vehicles expressing TAAs in tumor-bearing mice in nonself systems (1, 4, 7). In several prevention studies, mice vaccinated with a yeast construct expressing a TAA were protected against tumor challenge, whereas mock-treated

mice developed tumor (1, 7). In a spontaneous lung carcinoma model, tumor volume was reduced 28% to 55% in mice vaccinated with yeast constructs expressing mutated Ras (14). These data, taken together, show that *S. cerevisiae* constructs can be used for antigen-specific tumor therapy as well as tumor prevention.

An interesting study by Bos et al. describes a potential mechanism for tolerance in the CEA-Tg mouse. In this study, they suggest that expression of CEA on medullary thymic epithelial cells of CEA-Tg mice is involved in restricting CEA-specific $CD4^+$ T cells (29). Whereas they found that $CD4^+$ T cells were essential for tumor eradication in mice vaccinated with a poxvirus expressing CEA, our previous studies show that both CD4 and CD8 cells have a role in antitumor immunity in MC38-CEA tumors in CEA-Tg mice (30). These observations are notable in that the yeast-CEA vaccine used here induces both CD4 (Figs. 1, 2, and 5) and CD8 (Figs. 1-3) responses in CEA-Tg mice and mediates antitumor activity (Figs. 4 and 6), thus providing further evidence that tolerance was broken in this system.

We examined here antitumor efficacy in two different tumor models where CEA is a self-antigen. The first was a lung metastasis model where colon carcinoma cells were given i.v. in the tail, forming lung metastases. In this model, mice receiving yeast-CEA had a distinct survival advantage over mice receiving no treatment or control yeast (Fig. 4A). When the number of mice per group was increased and vaccinations were given weekly for the duration of the experiment, survival in yeast-CEA-vaccinated mice was statistically significantly extended compared with mice receiving no treatment (Fig. 4B). To further evaluate antitumor efficacy, we used an s.c. CEA^+ pancreatic tumor model to measure tumor volume; mice vaccinated with yeast-CEA had a significantly lower tumor volume at day 35 post-tumor transplant than mice receiving no treatment (Fig. 4C), confirming the antitumor effect of vaccination with yeast-CEA seen in the lung metastasis model. Reduced tumor volume in this model also correlated with increased survival (data not shown).

Here, it was observed that there was a bell-shaped response curve associated with the dose of yeast-CEA with the optimal dose for induction of T-cell responses (Fig. 3). This dose relationship has also been observed in another preclinical model targeting a self-tumor antigen (28). There, Lu et al. examined recombinant yeast delivering rat epidermal growth factor receptor as the tumor antigen as an intranasal vaccine for intracranial rat glioma. Three dose levels of yeast-epidermal growth factor receptor were examined: 0.7, 4, and 8 YU. It was observed that all dose levels improved survival over that of PBS treatment; the most striking survival benefit was seen at the 0.7 YU dose (45 over 28 days for PBS), whereas rats administered 4 and 8 YU had a survival of 42 and 38 days, respectively. It is not clear why there is an optimal dose level for induction of immune responses in the observations of Lu et al. and our own studies here (Figs. 2 and 5), although one could hypothesize that in a self-antigen system T cells might be more susceptible to antigen induced T-cell anergy. Future studies will examine this possibility.

Data from previous studies employing other types of vaccine vehicles suggest that vaccination at the sites of multiple draining lymph nodes elicits a more effective antitumor response than vaccination at a single site (24, 25). In one

study, mice were vaccinated with a whole tumor cell vaccine expressing granulocyte-macrophage colony-stimulating factor before challenge with a squamous cell carcinoma cell line. Three of nine mice vaccinated in multiple sites developed tumors by day 15 post-tumor challenge, whereas four of five mice receiving a single-site vaccination developed tumors (25). One hypothesis for this effect is that a greater number of precursor T cells are exposed to antigen when mice are vaccinated in multiple sites than when they are vaccinated in a single site, resulting in a subsequent increase in antigen-specific effector T cells (31). Data presented here further suggest that there is a maximum effective dose of yeast-CEA that can be given at one site, as $CD8^+$ T-cell lysis was significantly decreased in mice vaccinated with 10 YU versus mice vaccinated with 1 YU (Fig. 3). Because of this, we conducted immune and antitumor experiments comparing vaccination in four sites versus a single site, targeting the inguinal, subclavicular, and axial lymph node beds. Our data show that vaccination in four sites induces a greater antigen-specific T-cell response than the same total dose given in a single site (Fig. 5). Vaccination in multiple sites had the same effect in tumor-bearing CEA-Tg mice, as vaccination in four or six sites resulted in lower tumor volume than vaccination in a single site (Fig. 6).

In several clinical trials, recombinant *S. cerevisiae* vehicles have been found to be safe in both cancer and infectious disease settings. In a phase Ib trial, a yeast construct expressing the HCV NS3-core fusion protein was administered to patients with chronic HCV. Interim analysis from this trial suggests that the yeast construct is safe for use in humans, as no therapy-related serious adverse events or dose-limiting toxicities have been reported (2). Additionally, at the time of the report, 12 of 29 patients (41%) had generated cellular immune responses to HCV (2). In a cancer setting, a phase I clinical trial with a *S. cerevisiae* construct expressing mutated Ras was conducted in patients with Ras^+ cancers. Again, no treatment-related serious adverse events were observed, indicating a platform-wide safety profile for administration of heat-killed recombinant yeast to treat chronic diseases. In addition, 19 of 21 vaccinated patients showed >2-fold antigen-specific responses (3). As a result, a placebo-controlled adjuvant phase II trial in patients with mutated Ras^+ fully resected pancreatic cancer is under way.

The transgenic mice used in this study have been shown to overexpress CEA in many fetal tissues and in various parts of the gut, including the stomach, small intestine, cecum, and colon (16). Because CEA has a similar expression pattern in transgenic mice and in humans, these mice are a useful model for studying potential autoimmune phenomena. We performed 24 tests on age-matched mice receiving either no treatment or yeast-CEA. For the vaccinated mice, weight, complete blood count, serum enzyme levels, and autoimmune assays were all within the normal range and were similar to the age-matched controls, indicating no toxicity or autoimmunity related to yeast-CEA (Table 1). These data thus have implications for potential use of the yeast-CEA vehicle in humans. A potential translational path to test these findings would be to vaccinate patients who have CEA-positive carcinomas with yeast-CEA and measure CEA-specific immune responses. Based on the findings presented here, the patients could be vaccinated in multiple sites, targeting different lymph node beds to maximize the immune response to the yeast-CEA vector. We could envision future studies that

would focus on patients with CEA-positive non-small cell lung carcinoma. Patients could receive yeast-CEA in combination with standard of care chemotherapy, and CEA-specific immune responses as well as time to progression could be monitored.

The data reported here show that vaccination with yeast-CEA can break tolerance and induce CEA-specific CD4^{+} and CD8^{+} T-cell responses, effectively reduces tumor burden, and extends overall survival in tumor-bearing mice without adverse effects. These results thus form the rationale for the potential use of yeast-CEA in immunotherapy protocols for carcinoma patients with CEA[+] tumors.

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