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Exploitation of differential homeostatic proliferation of T-cell subsets following chemotherapy to enhance the efficacy of vaccine-mediated antitumor responses

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Abstract

The 5-year survival rate for stage IB-III non-small-cell lung cancer (NSCLC) remains 15%. Surgical resection followed by adjuvant chemotherapy with cisplatin and vinorelbine is one standard-of-care. We sought to determine in a preclinical model whether (a) the combination of cisplatin and vinorelbine could positively modulate components of the immune system independent of antitumor activity, and (b) there were synergistic effects of this drug combination and vaccine immunotherapy. We examined the effect of cisplatin/vinorelbine on gene expression, cell-surface phenotype, and CTL-mediated cytotoxicity of murine lung carcinoma cells in vitro; we also assessed the effects of cisplatin/vinorelbine on immune subsets and function of Tregs in vivo. Finally, we evaluated the potential synergy between chemotherapy and a recombinant yeast-CEA vaccine in a murine model transgenic for CEA with mice bearing lung tumors. These studies demonstrate that exposure of lung tumor cells to the platinum doublet cisplatin/vinorelbine modulates tumor cell phenotype and increases sensitivity to CTL-mediated cytotoxicity. These studies also demonstrate that cisplatin/ vinorelbine (a) induces sub-myeloablative leucopenia that differentially modulates reconstitution of Treg versus effector T-cell subsets and (b) can be employed synergistically with vaccine, exploiting homeostatic peripheral expansion of T cells. Antitumor studies show for the first time that cisplatin/vinorelbine combined with vaccine increases the survival of mice with established NSCLC. These findings provide the rationale for

the potential clinical benefit of the combined use of vaccine with cisplatin/vinorelbine chemotherapy regimens.

Keywords

NSCLC; Non-small-cell lung cancer; Cisplatin; Vinorelbine; Adjuvant chemotherapy; Immunotherapy

Introduction

Lung cancer is the leading cause of cancer mortality in Europe and the United States, resulting in over 1.18 million deaths per year worldwide, with non-small-cell lung cancer (NSCLC) accounting for over 85% of all new diagnoses [1-3]. Surgical resection with curative intent is the standard of care in patients with good performance status who present with operable early-stage, localized disease. Although large randomized trials have established that adjuvant chemotherapy enhances survival in these patients, over 30% of patients with early-stage NSCLC die of recurrent disease within 5 years [4, 5]. Moreover, the 5-year survival rate for all NSCLC stages combined remains 15% [1, 3]. Thus, there is an urgent need for adjuvant therapies that can reduce the risk of relapse and improve survival.

Immunotherapy is an approach under active investigation in both preclinical and clinical settings for a range of human cancers [6-10]. Recent approval of the first therapeutic anti-cancer vaccine for prostate cancer (Provenge[®], Dendreon, USA) by the FDA, together with other clinical findings indicate that vaccine immunotherapy may have potential therapeutic benefit for the treatment of cancer, including NSCLC [8, 9, 11]. Vaccine therapy in the adjuvant setting may thus translate into clinical benefit for patients with early-stage NSCLC, where tumor burden is minimal and performance status is optimal.

Previous studies in both preclinical and clinical settings have demonstrated that cancer vaccines targeting tumor-associated antigens (TAAs) can break immune tolerance and enhance immune responses to self-antigens [12, 13]. Carcinoembryonic antigen (CEA) is a TAA overexpressed in a variety of adenocarcinomas, including NSCLC [14, 15]. We have previously described a heat-killed recombinant *Saccharomyces cerevisiae* vaccine encoding CEA (designated here as yeast-CEA) [12, 16, 17]. These studies demonstrated that yeast-CEA can break tolerance in a CEA transgenic mouse [12]. The yeast vaccine platform has also been shown to be safe and well tolerated in cancer patients and is currently being further evaluated in the clinic [18, 19].

Chemotherapeutic regimens with cisplatin plus vinorelbine are a front-line therapy for NSCLC, including in the adjuvant setting [4, 5, 20-23]. Patients on chemotherapy often develop moderate to severe leucopenia, an adverse event considered detrimental to the generation of effective anti-tumor immunity [24, 25]. However, in preclinical studies, cisplatin chemotherapy was shown to augment antitumor immunity elicited by viral, DNA, and subunit vaccine platforms [26-28]. It was also demonstrated that cisplatin can increase the expression of functional Fas receptor in tumor cells [29-31] and augment CTL-mediated antitumor activity in a mouse model of NSCLC [29]. Further, vinorelbine has been shown to inhibit the growth of tumor cells [32]. Here, we investigated the combined use of standard-of-care cisplatin and vinorelbine with vaccine in an orthotopic murine model of NSCLC. First, we examined the effect of sublethal doses of cisplatin and/or vinorelbine on Lewis lung carcinoma cells. Next, we evaluated in vivo the effects of the platinum doublet on peripheral leukocytes and individual immune-cell subsets, including on the function of

regulatory T cells (Tregs). We then examined the effect of this chemotherapeutic regimen on CD4⁺ and CD8⁺ immune responses elicited by yeast-CEA vaccine. These studies were first conducted in nontumor-bearing mice to rule out the indirect effects of chemotherapy on the immune system that result from a reduction in tumor size. We then evaluated the antitumor effect of cisplatin/vinorelbine chemotherapy combined with vaccine in tumor-bearing mice with established NSCLC tumor burden.

These studies demonstrate for the first time that concurrent exposure of lung tumor cells to cisplatin and vinorelbine (a) modulates expression of survival genes and tumor cell phenotype and (b) increases sensitivity to CTL-mediated killing. These studies also demonstrate that chemotherapy with cisplatin plus vinorelbine (a) differentially modulates homeostatic peripheral expansion of effector and regulatory T-cell subsets and (b) has synergy with vaccine, resulting in enhancement of CEA-specific CD4⁺ and CD8⁺ immune responses. Moreover, antitumor studies show that administration of cisplatin plus vinorelbine in combination with a recombinant yeast vaccine increases the survival of tumor-bearing mice with established NSCLC, an effect demonstrated to be mediated by both CD4 and CD8 T-cell subsets.

Materials and methods

Animals

All in vivo studies were performed on 6- to 8-week-old female mice. For the study of splenocyte numbers and spleen cell populations, C57BL/6 (H-2^b) mice were obtained from the National Cancer Institute, Frederick Cancer Research and Development Facility (Frederick, MD). Tumor therapy studies and CEA-specific responses of T lymphocytes were evaluated in C57BL/6 mice transgenic for CEA (designated CEA-Tg), originally obtained from a breeding pair generously provided by Dr. John Shively (City of Hope, Duarte, CA). Transgenic mice were homozygous for the expression of the CEA gene and were generated as previously described [33]. Mice were housed under pathogen-free conditions and maintained in micro-isolator cages, in accordance with AAALAC guidelines. All animal studies were conducted under approval of the Intramural Animal Care and Use Committee of the National Cancer Institute.

Yeast-CEA vaccine

The vaccine consisted of a heat-killed recombinant *S. cerevisiae* construct expressing CEA (yeast-CEA), as previously described [12, 16, 17]. Mice received 1 yeast unit (YU = 10⁷ yeast particles) of yeast-CEA in each of 4 sites (shoulders and inner thighs; 4 YU total, s.c.).

Chemotherapeutic drugs

For in vivo studies, 1 mg/ml cisplatin (Abraxis Pharmaceuticals, Schaumburg, IL) and 10 mg/ml vinorelbine (Bedford Laboratories, Bedford, OH) were further diluted with sterile PBS. Chemotherapy consisted of i.p. injection of 50 µg of vinorelbine and 150 µg of cisplatin. For in vitro studies, the drugs were further diluted in complete medium, as described below.

Tumor cell line

LL/2 murine lung adenocarcinoma tumor cells were the gift of Dr. Chandan Guha (Albert Einstein College of Medicine, Bronx, NY). LL/2 tumor cells expressing CEA (LL/2-CEA) were generated by retroviral transduction with CEA cDNA, as previously described [34]. Cells were maintained in complete medium composed of DMEM high glucose supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bio-Products, West Sacramento, CA), 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM sodium

pyruvate, 1 mM HEPES buffer, 50 µg/ml gentamicin, 100 IU/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, and 300 µg/ml G418 sulfate. Unless otherwise indicated, all media and their components were obtained from Mediatech (Manassas, VA).

CEA-specific T cells

The H-2D^b-restricted, CEA-specific CD8-CTL line, designated CAP-M8, was generated from splenocytes of CEA-Tg mice vaccinated with rV-CEA. CAP-M8 cells recognize the peptide epitope CEA₅₂₆₋₅₃₃ (EAQNTTYL) and have been previously described [35].

In vitro studies

Tumor sensitivity to chemotherapy—To evaluate the effects of chemotherapy on the growth of tumor cells, LL/2-CEA cells were exposed in vitro (37°C/5% CO₂) to 5 µg/ml cisplatin and 1.5 µg/ml vinorelbine in complete media for 15 min or were left untreated. After treatment, cells were washed twice with PBS and returned to incubation in complete media. Cells were harvested 24, 48, or 72 h after exposure, and the total number of adherent viable cells was determined by trypan blue exclusion.

Apoptosis PCR array—The mouse apoptosis PCR array (RT² Profiler, PAMM-012) was used to analyze the expression of 84 key genes involved in apoptosis, in a 384-well format, according to the manufacturer's instructions (SA Biosciences). LL/2-CEA cells were exposed to cisplatin plus vinorelbine as described above. At 48 h post-treatment, cells were harvested and kept at -80°C until analysis. Data were normalized using multiple housekeeping genes and analyzed by comparing 2^{ΔCt} of the normalized sample, according to manufacturer's instructions.

Phenotypic analysis—To investigate the effects of in vitro exposure of tumor cells to chemotherapy on the cell-surface expression of immune-relevant proteins, adherent LL/2-CEA cells were harvested 48 h after treatment with cisplatin and vinorelbine, as described above. Cells were labeled with anti-CD95 (Jo2)-PE, H-2 K^b-FITC, and H-2D^b-FITC (BD Biosciences, San Jose, CA) for 30 min on ice. Cell-associated immunofluorescence was determined and compared with that of adequate isotype-matched controls using a FACScan flow cytometer equipped with Lysis II software (Becton–Dickinson, Mountain View, CA).

Caspase 3 functional assay—LL/2-CEA tumor cells were exposed to cisplatin/vinorelbine as described above and incubated for 48 h. Cells were washed and subsequently incubated for 18–24 h with varying concentrations of agonistic anti-Fas antibody (Jo2, BD Pharmingen) plus protein G (10 µg/ml; Amersham Pharmacia Biotech, Uppsala, Sweden) to maximize cross-linking of the primary mAb. Control cells were incubated with isotype-matched control antibody (BD Pharmingen). Cells were then fixed and permeabilized before staining for intracellular caspase-3 with a PE-labeled mAb (BD Pharmingen). The level of activated caspase-3 was quantified via flow cytometry as described above and compared with immunoglobulin M isotype control.

Cytotoxicity assay—To evaluate the effect of chemotherapy on the sensitivity of tumor cells to CTL-mediated killing, LL/2-CEA cells were exposed in vitro to cisplatin and/or vinorelbine, as described above. After 24–48 h post-treatment, LL/2-CEA target cells (2 × 10⁶) were harvested and labeled with 50 µCi of ¹¹¹In-labeled oxyquinoline (Medi-Physics Inc., Arlington Heights, IL) for 20 min at 37°C. Cells were incubated in 96-well round-bottomed plates at different effector:target ratios, using the CEA-specific CTL cell line CAP-M8 as effector cells. After 18 h of incubation at 37°C/5% CO₂, supernatants were collected and radioactivity was quantitated 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. Spontaneous ^{111}In release in the absence of effector cells was <15% and subtracted from that induced by CTL-mediated killing. The data were averaged and depicted as percentage of lysis ±SEM.

Concanamycin A treatment—To investigate the mechanism of CTL-mediated cytotoxicity of tumor cells exposed to chemotherapy, CAP-M8 CTLs were pre-incubated for 2 h with 100 nmol/l concanamycin A, a specific inhibitor of perforin-dependent lysis. CTLs were then used as effectors against tumor targets in a standard 18-h cytotoxicity assay in the presence of concanamycin A.

In vivo studies

Complete blood counts—To evaluate the effect of chemotherapy on cell populations present in peripheral blood, standard analysis of complete blood counts was performed before (day 0) or 2, 4, and 7 days after C57BL/6 mice received a single dose of cisplatin and vinorelbine (0.15/0.05 mg i.p.).

ELISA—To assess the effect of chemotherapy on systemic IL-7, animals received cisplatin/vinorelbine as described above. At specific times post-chemotherapy, animals were bled and serum levels of IL-7 were determined by ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). The assay sensitivity was 3.5 pg/ml.

Phenotypic analysis—To investigate the effects of chemotherapy on splenic cell populations, spleens were harvested from C57BL/6 mice 0, 2, 4, or 7 days after receiving a single dose of cisplatin and vinorelbine (0.15/0.05 mg i.p.). The total number of splenocytes in individual spleens was determined after lysis of red blood cells. Phenotypic analysis was performed in splenocytes from individual animals after blocking Fc receptors with anti-CD16/CD32 (2.4 G2) antibody. Cells were then labeled with anti-CD3-FITC, anti-CD4(L3T4)(RMA-5)-FITC, or anti-CD8(Ly-2)(53-6.7)-FITC antibodies (BD Biosciences). Splenocytes were labeled for Tregs ($\text{CD4}^+\text{CD25}^+\text{FoxP3}^+$) using a murine Treg staining kit according to the manufacturer's instructions (bioScience Inc., San Diego, CA). Cellular immunofluorescence was analyzed and compared with that of adequate isotype-matched controls on a FACScan cytometer using Lysis II software (Becton–Dickinson).

Treg functional assay— $\text{CD4}^+\text{CD25}^-$ cells (5×10^4) from control mice were incubated with 1×10^5 antigen-presenting cells (APCs; splenic cells irradiated with 30 Gy) and 1 $\mu\text{g}/\text{ml}$ soluble anti-CD3 cross-linking antibody in the presence or absence of 5×10^4 $\text{CD4}^+\text{CD25}^+\text{FoxP3}^+$ Tregs from either PBS-treated control mice or cisplatin/vinorelbine-treated mice (0.15/0.05 mg i.p. on day 0). Tregs were isolated and assayed 0, 2, and 4 days post-chemotherapy. Assay cultures were harvested and counted after a 72-h incubation. [^3H]-thymidine was added to the culture for the last 12 h of incubation. Control wells containing Tregs, APCs, and anti-CD3 without $\text{CD4}^+\text{CD25}^-$ cells were used to determine background levels of proliferation in culture. Lack of proliferation of $\text{CD4}^+\text{CD25}^+\text{FoxP3}^+$ cells further identified them as Tregs. $\text{CD4}^+\text{CD25}^-$ cells and APCs were incubated with concanavalin A (Sigma–Aldrich, St. Louis, MO) as a positive control for proliferation (data not shown). $\text{CD4}^+\text{CD25}^-$ cells and $\text{CD4}^+\text{CD25}^+\text{FoxP3}^+$ Tregs were isolated from splenocytes using a Treg isolation kit according to manufacturer's instructions (Miltenyi Biotec, Auburn, CA).

Combination therapy studies—To evaluate whether chemotherapy improves the therapeutic potential of vaccine-mediated immunotherapy, CEA-Tg mice were left untreated or primed on day 0 with s.c. yeast-CEA and boosted on days 7, 14, and 21. On day four,

animals received a single i.p. dose of cisplatin/vinorelbine (0.15/0.05 mg) alone or in combination with yeast-CEA vaccine (1 YU/site at four sites). On day 35, animals were killed and spleens were harvested for analysis of CD4⁺ and CD8⁺ T-cell responses.

Lymphocyte proliferation assay—To evaluate CEA-specific T-cell immune responses elicited by combination therapy, we evaluated the cellular proliferation of pooled splenic CD4⁺ T cells in response to protein antigens, as previously described [12]. Briefly, purified CD4⁺ T cells (1.5×10^5 cells/well) were incubated in 96-well flat-bottomed plates with irradiated syngeneic naïve splenocytes (5×10^5 cells/well, 20 Gy) as APCs in the presence of 25 µg/ml human β -galactosidase protein (Prozyme, San Leandro, CA) or 25 µg/ml CEA protein (AspenBio Pharma, Littleton, CO). As a positive control, cells were stimulated with 2.5 µg/ml of the T-cell mitogen concanavalin A (Sigma-Aldrich). T cells and APCs were cultured with T-cell medium [RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 7 mM HEPES, 50 µg/ml gentamicin, 50 mM 2-mercaptoethanol, and 0.1 mM nonessential amino acids] only as a negative control. Cells were cultured for 5 days at 37°C. [³H]-thymidine (1 µCi/well) was added to the wells for the last 18 h, and cells were harvested using a Tomtec cell harvester (Wallac Inc., Gaithersburg, MD). The incorporated radioactivity was measured using a Wallac 1205 Betaplate liquid scintillation counter (Wallac Inc.). The mean cellular proliferation of negative control responses was subtracted from proliferation in response to CEA and β -galactosidase protein antigens. The data were averaged and depicted as $\Delta\text{cpm} \pm \text{SEM}$.

CEA-specific CD8⁺ T-cell frequency—To evaluate the frequency of CEA-specific CTLs in CEA-Tg mice after combination therapy, splenocytes were harvested, red blood cells were lysed, and pooled splenocytes were separated by centrifugation through a Ficoll-Hypaque gradient. Cells at the interface were collected and washed twice with complete T-cell media. After FcR blocking with anti-CD16/CD32 (2.4 G2) monoclonal antibody (20 min on ice), cells were incubated for 30 min on ice with FITC-conjugated CD8 monoclonal antibody and PE-conjugated CEA_{572–579}/H-2D^b tetramer (Beckman Coulter, Fullerton, CA). As a negative control, splenocytes were stained with FITC-conjugated CD8 monoclonal antibody and PE-conjugated HIVgag_{390–398}/H-2D^b tetramer. Immunofluorescence was compared with the appropriate isotype-matched controls and analyzed with CellQuest software using a FACSCalibur cytometer (BD Biosciences). Results were generated from data gathered from 100,000 events. Negative control tetramer samples were <5% positive. Results were depicted as number of CEA-tetramer⁺CD8⁺ cells/million CD8⁺ T cells. Monoclonal antibodies were purchased from BD Biosciences unless otherwise indicated.

Tumor therapy studies—To examine whether chemotherapy of well-established growing tumors improves the efficacy of a recombinant vaccine regimen in an orthotopic model of Lewis lung carcinoma, CEA-Tg mice were inoculated i.v. with 5×10^5 LL/2-CEA⁺ cells on day 0. Animals were left untreated or vaccinated weekly starting on day 11 with yeast-CEA vaccine (1 YU/site s.c. in four sites). On day 8, mice received a single dose of cisplatin/vinorelbine (0.15/0.05 mg i.p.) alone or in combination with yeast-CEA vaccine. Animals were monitored daily for survival.

T-cell depletion studies—CEA-Tg mice were inoculated i.v. with 5×10^5 LL/2-CEA⁺ cells on day 0. On days 4–7 and every week thereafter, animals were depleted of CD4⁺ T cells, CD8⁺ T cells, or both CD4⁺ and CD8⁺ T cells by i.p. administration of a daily dose (100 µg) of anti-CD4 (GK 1.5) and/or anti-CD8 (Lyt 2.2) hybridomas in PBS. These depletion conditions were validated by flow cytometry analysis of peripheral blood using FITC-conjugated monoclonal antibodies reactive to CD4 and CD8 (PharMingen, San Diego, CA); 99% of the relevant cell subset was depleted, where all other subsets remained within

normal levels. Animals received weekly vaccinations starting on day 11 with yeast-CEA vaccine (1 YU/site s.c. in four sites) in combination with a single dose of cisplatin/vinorelbine (0.15/0.05 mg i.p.) on day 8. Animals were monitored daily for survival.

Statistical analysis—Significant differences between treatment groups were determined by ANOVA based on a confidence interval of 95% using Prism 4.0c software (GraphPad Software Inc., La Jolla, CA). Statistical differences between individual data points were analyzed with 95% confidence using unpaired Student's *t*-test with a 2-tailed distribution and reported as *P* values. Significant differences in the distribution of flow cytometry analysis data were determined by the Kolmogorov–Smirnov test, using CellQuest software (BD Biosciences). Evaluation of survival patterns in tumor-bearing mice was performed by the Kaplan–Meier method and ranked according to the Mantel–Cox log-rank test using Prism 4.0c software.

Results

In vitro exposure of tumor cells to chemotherapy modulates phenotype and increases sensitivity to CTL-mediated killing

We first sought to determine whether exposing tumor cells to sublethal doses of cisplatin plus vinorelbine could induce changes in cellular survival and proliferation, cell-surface expression of Fas and MHC class I molecules, and sensitivity to CTL-mediated cytotoxicity. To address these issues, we chose a Lewis lung carcinoma mouse cell line expressing CEA. To identify sublethal doses of chemotherapy, dose–response studies were performed using various concentrations of cisplatin and vinorelbine based on reported clinical unbound peak plasma concentrations and relative ratios between peak plasma concentrations of both drugs [36, 37]. LL/2-CEA cells were exposed in vitro to cisplatin (5 µg/ml) and vinorelbine (1.5 µg/ml) for 15 min or were left untreated. At 24, 48, and 72 h post-treatment, the total number of viable LL/2-CEA cells was determined by trypan blue exclusion; cells were harvested prior to confluency. As shown in Fig. 1a, exposure of LL/2-CEA cells to a sublethal dose of cisplatin and vinorelbine markedly decreased cellular growth, corresponding to a ninefold reduction in growth rate relative to that of untreated cells (*P* = 0.016). However, cells remained viable and were proliferating, albeit to a lower degree.

Next, we sought to examine the effect of the platinum doublet on the expression of pro-apoptotic and anti-apoptotic/survival genes. Tumor cells were left untreated or received cisplatin plus vinorelbine as described above. At 48 h post-treatment, the expression of 84 key genes was analyzed by real-time PCR array profiling. These included members of the bcl-2, caspase, IAP, TRAF, CARD, death domain, death effector domain, and CIDE families, TNF ligands and their receptors, as well as genes involved in the p53 and ATM pathways. Data were normalized using multiple housekeeping genes and analyzed by comparing fold changes of the normalized samples. After chemotherapy exposure, the expression of 10 genes was significantly downregulated relative to untreated cells (supplemental Table I). These included the anti-apoptotic/survival genes Lhx4, CD40Ig, Naip1, TNF, IL-10, Pak7, and Traf1. The pro-apoptotic genes Casp7 and FasL were also significantly downregulated. Exposure of tumor cells to cisplatin plus vinorelbine did not increase the expression of any of the genes studied, including members of the pro-apoptotic caspase family. Altogether, these data indicate that exposure of tumor cells to sub-lethal chemotherapy decreases the expression of pro-apoptotic genes and genes that promote the survival of tumor cells.

We next examined the effect of cisplatin/vinorelbine on cell-surface expression of Fas and MHC class I molecules. LL/2-CEA cells were exposed to the chemotherapeutic agents, as described above, and harvested 48 h after exposure. Exposure of LL/2-CEA cells to

cisplatin/vinorelbine resulted (Fig. 1b) in an increase in the population of Fas⁺ cells from 24.5 to 49% ($P \leq 0.001$ vs. untreated control). Additionally, mean fluorescence intensity (MFI) of Fas⁺ cells increased 49% after treatment. Fas was determined to be functional in these cells by agonistic antibody crosslinking and caspase-3 cleavage assays [29, 38]. As shown in Fig. 1b, Fas crosslinking of LL/2-CEA cells resulted in the activation of caspase 3 in both untreated and treated cells. In addition, exposure of tumor cells to cisplatin plus vinorelbine resulted in a twofold increase of active caspase 3.

Substantial increases in cell-surface expression of MHC class I molecules were also observed in cells exposed to the platinum doublet. Cell-surface expression of H-2 K^b and H-2D^b increased 41 and 26%, respectively; MFIs of H-2 K^b- and H-2D^b-positive cells also increased 85 and 200%, respectively.

To evaluate the functional significance of gene expression and phenotypic changes in tumor cells after exposure to chemotherapy, we examined the sensitivity of target LL/2-CEA cells to CTL-mediated cytotoxicity after exposure to cisplatin alone, vinorelbine alone, or to cisplatin plus vinorelbine. We used the CTL line CAP-M8 originated from mice vaccinated with a CEA-based vaccine that recognizes the H-2D^b-restricted epitope CEA₅₂₆₋₅₃₃ on CEA-expressing carcinoma cells [35]. LL/2-CEA mouse carcinoma cells, positive for H-2D^b and CEA, were exposed to cisplatin and/or vinorelbine for 15 min or were left untreated. Twenty-four to 48 h post-treatment, the sensitivity of LL/2-CEA target cells to CAP-M8-mediated killing was determined in a standard 18-h assay. Exposure of tumor cells to cisplatin alone or vinorelbine alone did not increase CTL-mediated lysis compared to untreated target cells ($P > 0.05$; Fig. 1c). However, simultaneous exposure of tumor cells to cisplatin and vinorelbine resulted in a significant enhancement of CEA₅₂₆₋₅₃₃-specific lysis by CTLs relative to untreated tumor cells ($P < 0.006$; Fig. 1c). Taken together, these data indicate that exposure of tumor cells to sublethal doses of cisplatin plus vinorelbine slows their proliferation, downregulates the expression of survival genes, induces upregulation of Fas and other immune-relevant cell-surface proteins, and increases their sensitivity to Fas-dependent CTL-mediated killing. Further, data indicates that these effects are dependent upon exposure of tumor cells to both chemotherapeutic agents combined.

Perforin/granzyme and Fas/FasL death pathways are considered the main mechanisms mediating the lytic effects of T cells on target tumor cells [39]. Thus, to further examine the contribution of Fas/FasL-mediated cytotoxicity of tumor cells after chemotherapy treatment, we examined the sensitivity of LL/2-CEA tumor cells to lysis by CAP-M8 CTLs in the presence of concanamycin A (CMA) in a standard 18-h assay. CMA inhibits a vacuolar type H⁺-ATPase, thereby abrogating perforin-mediated cytotoxicity, without affecting Fas-mediated killing [40]. Tumor cells received chemotherapy or were left untreated. Lysis of untreated tumor cells was markedly reduced to less than 3% in the presence of CMA ($P < 0.0001$), suggesting that lysis of untreated tumor cells under this experimental setting is solely dependent on perforin/granzyme, without any contribution of Fas/FasL-mediated mechanisms (Fig. 1d). CEA-specific killing of tumor cells 48 h after chemotherapy was significantly increased ($P = 0.0001$) relative to that of untreated tumor targets. In the presence of CMA, lysis of LL/2-CEA cells exposed to chemotherapy was similar to that of untreated tumor targets in the absence of CMA ($P = 0.623$). Altogether, these results suggest that the increased sensitivity of tumor cells to CTL cytotoxicity as a result of chemotherapy is mediated by Fas/FasL cell death pathway.

Chemotherapy induces transient sub-myeloablative leucopenia in vivo

The chemotherapeutic combination of 75–80 mg/m² cisplatin plus 25–30 mg/m² vinorelbine is approved for the clinical treatment of NSCLC [41]. Based on these dosages, we calculated the equivalent doses for a 20- to 25-g mouse to be 0.45 mg of cisplatin and 0.15 mg of

vinorelbine. Due to life-threatening toxicity, a dose of 0.15 mg of cisplatin plus 0.05 mg of vinorelbine was selected for all in vivo studies. Vinorelbine is known to bind extensively to lymphocytes [42]. In addition, leucopenia has been reported in NSCLC patients receiving platinum-based regimens [22, 43]. Therefore, we sought to determine the effects of this cisplatin/vinorelbine doublet on cellular populations in peripheral blood after a single i.p. dose in nontumor-bearing C57BL/6 mice. Complete blood counts were analyzed in individual animals ($n = 3-5$) 0, 2, 4, or 7 days after treatment (Fig. 2a). Two days after chemotherapy, the total number of monocytes and polymorphonuclear neutrophils (polys) was decreased, with a nadir on days 2 and 4 post-treatment, respectively. However, these decreases did not attain statistical significance. No significant changes were observed in the number of eosinophils or basophils. Two days after chemotherapy, the total number of white blood cells (WBC) was reduced by 32% relative to untreated controls ($P = 0.029$). A significant decrease in the number of lymphocytes ($P = 0.016$) was also observed 2 days after treatment, corresponding to 31% reduction relative to untreated animals. However, both WBC and lymphocyte levels were fully recovered on day 4 post-chemotherapy ($P = 0.27$ and $P = 0.50$, respectively). These data indicate that chemotherapy induces significant but transient sub-myeloablative leucopenia followed by a rapid recovery of WBC and lymphocyte counts.

Initial T-cell reconstitution following lymphodepleting therapeutic regimens is modulated by increased levels of systemic IL-7, which drives homeostatic peripheral expansion (HPE) of T cells [44, 45]. Thus, to better capture the physiology of T-cell recovery after sub-myeloablative leucopenia, animals received a single dose of cisplatin plus vinorelbine as previously described. On days 0, 2, and 4 post-treatment, serum levels of IL-7 were determined. As shown in Fig. 2b, 2 days after chemotherapy, there was a significant increase in serum IL-7 levels, corresponding to 5.4-fold difference relative to that of untreated control animals ($P = 0.007$). This increased level of serum IL-7 was maintained at 4 days post-treatment ($P = 0.018$). Overall, these data suggest that lymphocyte reconstitution following sub-myeloablative chemotherapy with cisplatin plus vinorelbine is mediated through IL-7-associated homeostatic expansion of peripheral T-cell pools.

Chemotherapy induces transient pancytopenia in vivo

Next, to better capture the physiology of homeostatic peripheral expansion (HPE) after chemotherapy, we examined individual splenic immune-cell subsets after a single i.p. dose of cisplatin plus vinorelbine in nontumor-bearing C57BL/6 mice. Total splenocytes and individual immune-cell subsets were analyzed in individual animals ($n = 5$) 0, 2, 4, or 7 days after treatment (Fig. 3). There was a significant decrease in the total number of splenocytes 2 days after treatment relative to untreated animals ($P = 0.047$; Fig. 3a). However, splenocyte levels were fully recovered on day 4 post-treatment. On day 2 post-treatment, significant decreases were also observed in the absolute number of NK cells ($P = 0.002$; Fig. 3f), as well as CD3⁺ ($P = 0.047$; Fig. 3b), CD4⁺ ($P = 0.047$; Fig. 3c), and CD8⁺ ($P = 0.007$; Fig. 3d) T-cell subsets. Levels of these immune-cell subsets (with the exception of NK cells) were recovered by day 4 post-treatment. The total number of B cells (Fig. 3e) showed a similar pattern, with a nadir on day 2 post-treatment; however, this decrease did not attain statistical significance ($P = 0.071$). Overall, these data indicate that cisplatin/vinorelbine induces a significant but transient pancytopenia given a rapid recovery of peripheral T-cell subsets.

Cisplatin/vinorelbine chemotherapy induces significant and sustained deletion of Tregs in vivo

We next examined the effect of a single i.p. dose of cisplatin plus vinorelbine on the number of CD4⁺CD25⁺FoxP3⁺ Tregs in individual mice ($n = 5$) at various times post-

chemotherapy. Treatment with cisplatin/vinorelbine resulted in a significant and, more importantly, sustained decrease in the number of Tregs (Fig. 4a). Two days after treatment, the number of Tregs was reduced by 39% relative to untreated controls ($P = 0.0003$). Reduced levels of Tregs were maintained throughout the 7-day study period. On day 7, Treg numbers had decreased by 58%, a significant reduction relative to untreated counterparts ($P < 0.001$). We then examined the functionality of Tregs in mice following treatment with cisplatin/vinorelbine. Regulatory T cells from healthy and chemotherapy-treated mice were selected by microbead separation and used in a standard in vitro Treg assay. Over 90% of selected cells were confirmed to be $CD4^+CD25^+FoxP3^+$. In addition, these cells expressed high levels of GITR and low levels of CTLA-4. To further confirm that the $CD4^+CD25^+FoxP3^+$ cells were Tregs, they were incubated with soluble anti-CD3 and APCs, which did not lead to significant cell proliferation ($P < 0.0001$; Fig. 4b). The proliferation of naïve $CD4^+CD25^-$ responder cells was significantly reduced in the presence of Tregs from untreated mice (day 0, $P < 0.0001$). The proliferation of responder cells in the presence of Tregs from mice receiving chemotherapy was also significantly decreased 2 days post-treatment relative to Tregs from untreated mice ($P = 0.0092$). Treg function recovered at day 4 post-chemotherapy ($P = 0.0003$). Analysis of mean cell number ratios of $CD4^+$ T cells relative to Tregs at different time points post-chemotherapy (Fig. 4c) indicates that treatment with cisplatin/vinorelbine results in a 1.5- and twofold increase in $CD4^+$ /Treg ratio 4 and 7 days post-chemotherapy, respectively. These data, taken together, indicate that cisplatin/vinorelbine induces both a transient modulation of Treg function and also markedly reduces the absolute number of Tregs available for immune suppression, thus increasing the overall balance of $CD4^+$ effector T cells relative to suppressor Tregs.

Vaccination combined with chemotherapy enhances CEA-specific T-cell responses

Next, we sought to determine whether vaccinating into this more permissive environment with less suppressive elements after cisplatin/vinorelbine treatment had an effect on vaccine-mediated antigen-specific T-cell responses in the setting of a self-antigen. CEA-Tg mice were vaccinated s.c. with yeast-CEA on days 0, 7, 14, and 21. Chemotherapy was administered i.p. on day 4. On day 35, CEA-specific $CD4^+$ and $CD8^+$ immune responses were examined. The level of CEA-specific $CD4^+$ responses in animals receiving chemotherapy was also seen in untreated animals (Fig. 5a, $P = 0.433$). Vaccine alone was able to break immune tolerance to CEA and elicit a significant increase in CEA-specific $CD4^+$ responses ($P = 0.027$). However, CEA-specific $CD4^+$ immune responses were highest in mice that received vaccine in combination with chemotherapy versus chemotherapy alone ($P = 0.01$), vaccine alone ($P = 0.039$), or no treatment ($P = 0.017$). Moreover, CEA-tetramer $^+CD8^+$ T cells were clearly enhanced in mice receiving vaccine plus chemotherapy versus chemotherapy alone ($P < 0.01$), vaccine alone ($P < 0.01$), or untreated animals ($P < 0.01$; Fig. 5b). Taken together, these data indicate that chemotherapy with cisplatin/vinorelbine enhances both $CD4^+$ and $CD8^+$ T-cell responses generated by yeast-CEA vaccine.

Chemotherapy plus vaccine increased survival in a mouse model with established lung tumor burden, an effect mediated by both CD4 and CD8 T cells

To determine the antitumor efficacy of vaccine plus chemotherapy, CEA-Tg mice were inoculated with LL/2-CEA carcinoma cells i.v. on day 0, and tumors were allowed to develop for 11 days without treatment. One cohort of animals received a single dose of cisplatin/vinorelbine on day 8. On day 11 and every 7 days thereafter, a separate cohort of mice was vaccinated with yeast-CEA. A subgroup of mice receiving vaccine was also administered chemotherapy on day 8. Control animals were left untreated. As shown in Fig. 6a, chemotherapy alone had no effect on the survival of tumor-bearing mice ($P = 0.695$). These animals had a median survival of 52 days, compared to 53 days in untreated controls.

Vaccine alone failed to enhance either median survival (46 days) or overall survival ($P = 0.180$ vs. no treatment). However, the combination of vaccine plus cisplatin/vinorelbine resulted in a significant increase in overall survival compared to no treatment ($P = 0.046$), chemotherapy alone ($P = 0.027$), or vaccine alone ($P = 0.0038$). Moreover, median survival increased to 74 days in animals receiving chemotherapy plus vaccine. Next, we sought to identify the effector cells mediating the survival benefit after treatment with chemotherapy plus vaccine. After tumor transplant and prior to vaccine administration, mice were depleted of CD4 and/or CD8 T cells. Anti-CD4 and/or anti-CD8 depleting antibodies were given during and after treatment to ensure continued depletion of the relevant T-cell subsets. As seen in Fig. 6b, depletion of CD4 T cells alone ($P = 0.156$) or CD8 T cells alone ($P = 0.086$) had a minor and not significant effect on survival. However, depletion of both CD4 and CD8 T cell subsets significantly reduced ($P = 0.023$) survival relative to non-depleted animals receiving chemotherapy plus vaccine.

Discussion

There is increasing interest in the potential therapeutic benefits of multimodal treatment regimens combining cancer vaccines with standard-of-care chemotherapy. Many cancer vaccine clinical protocols have been conducted in patients who have failed prior chemotherapy. Chemotherapeutic regimens often result in moderate to severe lymphopenia [46]. Thus, until recently, it was generally believed that when used in combination with a cancer vaccine, chemotherapy would invariably have a negative effect on vaccine-mediated immune responses and antitumor activity [47]. However, accumulating evidence suggests that certain chemotherapeutic agents can render tumor-cell death immunogenic [48, 49]. Further, recent studies have indicated that, depending on tumor stage and the specific vaccine and chemotherapeutic agents used, chemotherapy can enhance vaccine-mediated immune responses [38, 50-52]. Recent work also suggests that the immediate period of T-cell reconstitution following chemotherapy-associated lymphopenia offers a unique opportunity to expand effective anti-tumor immunotherapy [45, 53].

In Europe and the United States, cisplatin combined with vinorelbine is a standard-of-care treatment for NSCLC after complete resection, as chemotherapy regimens combining both agents have shown promise in the adjuvant setting [4, 5, 20, 25, 54]. The few reported preclinical studies combining vaccine and cisplatin-based regimens focused on cisplatin as a single agent in combination with various vaccine platforms, such as subunit, viral, or DNA vaccines [26-28]. However, to the best of our knowledge, there are no reported studies on the combination of cisplatin plus vinorelbine regimens with vaccines targeting CEA.

In this study, we sought to determine whether vaccination with yeast-CEA could induce antigen-specific T-cell and antitumor responses when combined with standard-of-care chemotherapy composed of cisplatin and vinorelbine in a murine model of NSCLC. Data presented here show for the first time that vaccination with yeast-CEA combined with cisplatin/vinorelbine induces synergistic CEA-specific immune responses and increases the overall survival of tumor-bearing mice, an effect mediated by both CD4+ and CD8+ T cells (Figs. 5, 6). Several factors may account for these results. Leucopenia, a common side effect of most chemotherapeutic regimens, has been considered detrimental to the generation of vaccine-mediated immune responses. Moderate to severe leucopenia is a common adverse event in cancer patients receiving cisplatin and vinorelbine [22, 24, 25]. Acute response to leucopenia includes activation of homeostatic mechanisms that result in the expansion of lymphocytes in the periphery [45, 55]. This process is driven by increased levels of IL-7, an essential cytokine for T-cell homeostasis and survival of naïve T cells [44]. IL-7 has been shown to mediate a rapid and selective increase of CD4 and CD8 T cells, along with a decrease of Tregs in both preclinical and clinical settings [56, 57]. Further, IL-7 has been

shown to dramatically augment CD8 immune responses to subdominant antigens [58]. Several preclinical and clinical studies have suggested that vaccinating during homeostatic peripheral expansion of lymphocytes, when T cells are over-responsive to antigenic stimulation, promotes the generation of strong CD8⁺ T-cell responses [26, 59, 60]. Moreover, several studies have shown synergistic antitumor responses after combining vaccine with chemotherapy via cross-presentation, where an exogenous antigen is presented through the MHC class I pathway to CD8⁺ T cells present at the apoptotic tumor body generated by cytotoxic agents. In this context, combining vaccine with a chemotherapeutic regimen could be a powerful strategy for conditioning the tumor cells for immune recognition and enhancing the presentation of tumor-specific antigens to CD8⁺ T cells.

In a variety of malignancies, patients have an increased pool of Tregs in the peripheral blood and other immune compartments, and this increased level of Tregs has been associated with cancer-related immunosuppression. Therapies that may reduce Treg levels and/or functionality are promising therapeutic modalities [61]. Previous studies have shown that chemotherapeutic agents such as cyclophosphamide, fludarabine, and docetaxel can differentially affect the number and/or functionality of circulating Tregs [38, 62, 63]. In this study, we demonstrate for the first time that chemotherapy with cisplatin/vinorelbine transiently modulates Treg function (Fig. 4b) and induces a sharp and sustained decline in Treg numbers (Fig. 4a). Moreover, recovery from iatrogenic leucopenia translated into an increased balance of CD4⁺ effector T cells relative to suppressor Tregs (Fig. 4c). Similar trends were observed in the tumor microenvironment.

Our results confirm and expand previous preclinical and clinical reports showing that lymphopenic conditions favor homeostatic peripheral expansion of effector T cells through IL-7 signaling but not Tregs [45, 55]. We hypothesize that the expansion of this effector pool is paralleled with poor peripheral expansion/conversion of Tregs. The combined absence of normal CD4 levels as a pool for Treg conversion and homeostatic IL-2 serum concentration may contribute to a sustained delay in Treg recovery. Secondly, it has been reported that IL-7 decreases FoxP3 expression, a determinant of Treg activity [64]. More importantly, we observed that vaccinating into a more permissive environment with reduced regulatory elements and recovering immune components induced a synergistic increase in both CD4 and CD8 immune responses to CEA (Fig. 5).

The studies reported here also examined the effects of cisplatin plus vinorelbine on lung tumor cells to determine whether chemotherapy could induce immune-relevant changes. Our data indicate that chemotherapy inhibits the growth of tumor cells, decreases the expression of anti-apoptotic/survival genes, and enhances cell-surface expression of immune-relevant proteins for at least 7 days. These changes translated into enhanced sensitivity of tumor cells to CTL-mediated killing, a process driven solely by both cisplatin and vinorelbine combined (Fig. 1). These findings support and expand on previous reports indicating that exposure to cisplatin alone modulates tumor cells' phenotype and sensitivity to T cell-mediated cytotoxicity [27-29]. We hypothesize that the clinical use of cisplatin combined with vinorelbine synergize to down-regulate anti-apoptotic/survival genes, rendering tumor cells less resistant to CTL-mediated killing [65, 66]. Although it has been shown that vinorelbine-induced cell death is independent of Fas [67], we hypothesize that in vivo the combined use of both drugs mediates antitumor activity through both Fas-dependent and Fas-independent mechanisms. A recent report suggested that the increased sensitivity of tumor cells to CTL-mediated lysis after treatment with cisplatin alone is mediated by augmented permeability of tumor cells to granzyme B, allowing CTLs to induce tumor cell death through a bystander mechanism, thus bypassing the requirement for antigen recognition [68]. We have examined this possible mechanism in a standard 4-h cytotoxicity assay. CAP-M8-mediated killing of ¹¹¹In-labeled LL/2 tumor targets was determined 48 h after treatment for 15 min with cisplatin (5 µg/ml)

plus vinorelbine (1.5 µg/ml) in the presence of treated LL/2- or LL/2-CEA cells. Lysis (E:T = 100) of LL/2-treated targets in the presence of treated LL/2-CEA or LL/2 cells was 7.0 or 4.0%, respectively. These results suggest that increased sensitivity of tumor cells to CTL-mediated lysis after chemotherapy with cisplatin plus vinorelbine is a result of increased Fas/FasL-mediated mechanisms with minimal contribution from perforin/granzyme mechanisms (Fig. 1). This discrepancy may be due to differences in the experimental setting, including different cell lines, different cisplatin concentration, concomitant exposure to vinorelbine, and exposure time.

Several preclinical findings suggesting that combination therapy using cisplatin alone plus various cancer vaccine platforms generate better antitumor effects compared with either modality alone [26-28]. Our findings support these observations and expand them to a murine model of NSCLC, where vaccine administered into the permissive immunological environment mediated by iatrogenic HPE generated by cisplatin/vinorelbine resulted in increased anti-tumor immunity and overall survival. Strikingly, in a recent phase II clinical study, Ramlau et al. [51] reported no increase in overall survival in patients with advanced-stage NSCLC who received cisplatin/vinorelbine combined with a viral vaccine. However, in this study, vaccine was administered concurrently with chemotherapy, as opposed to post-chemotherapy, thus not benefiting from HPE-associated mechanisms.

We envision a translational path to clinically test these findings in the adjuvant setting by immunizing patients with early-stage NSCLC after surgical resection. Patients would be vaccinated with tumor vaccines to generate tumor-specific T cells, then undergo standard-of-care chemotherapy with cisplatin/vinorelbine and receive vaccine boosts between chemotherapy cycles. The control group would receive standard-of-care chemotherapy alone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Effect of chemotherapy on growth, cell-surface expression of Fas, and sensitivity to CTL-mediated killing of Lewis lung carcinoma cells. **a** Effect of chemotherapy exposure on LL/2-CEA cells. Cells were exposed in vitro for 15 min to cisplatin (5 $\mu\text{g}/\text{ml}$) and vinorelbine (1.5 $\mu\text{g}/\text{ml}$) (open circles) or were left untreated (closed circles). At various times after exposure, the total number of viable cells was determined by trypan blue exclusion. Results presented as mean $\pm$ SEM from three replicate flasks. **b** Effect of chemotherapy on Fas expression. Forty-eight hours after exposure for 15 min to cisplatin (5 $\mu\text{g}/\text{ml}$) and vinorelbine (1.5 $\mu\text{g}/\text{ml}$), LL/2-CEA cells were harvested and analyzed by flow cytometry for cell-surface expression of Fas and activated caspase 3. Untreated cells were used as controls. Values in upper brackets indicate percentage of positive cells, followed by MFI. **c** Effect of chemotherapy on sensitivity to CTL-mediated killing. Twenty-four to 48 h after 15 min exposure to cisplatin alone (5 $\mu\text{g}/\text{ml}$, closed triangles), vinorelbine alone (1.5 $\mu\text{g}/\text{ml}$, closed squares), or to both drugs combined (open circles), LL/2-CEA cells were harvested and labeled with ^{111}In . The sensitivity of LL/2-CEA target cells to CEA-specific killing was determined. After cells were incubated at different effector:target ratios for 18 h, supernatants were harvested and specific lysis was determined. Results are presented as

mean $\pm$ SEM from three replicate wells. *Asterisks* denote statistical significance ($P < 0.05$) relative to untreated cells. Data are representative of one independent experiment. Studies were repeated twice with similar results. *D*, to investigate the mechanism of CTL-mediated lysis of tumor cells exposed to chemotherapy, LL/2-CEA cells were left untreated (*open bars*), or exposed to cisplatin (5 $\mu\text{g/ml}$) and vinorelbine (1.5 $\mu\text{g/ml}$) for 15 min (*closed bars*), and used as targets of CAP-M8 CTLs (E:T ratio = 50:1) in a standard 18-h cytotoxicity assay in the presence or absence of concanamycin A, a specific inhibitor of perforin-dependent lysis. Where applicable, CTLs were pre-incubated for 2 h with 100 nmol/l concanamycin A. Results are presented as mean lysis $\pm$ SEM from 3 replicate wells

Fig. 2.

Effect of chemotherapy on peripheral blood cell counts and systemic IL-7. C57BL/6 female mice received a single i.p. dose of cisplatin/vinorelbine (0.15/0.05 mg) on day 0. **a** effect of chemotherapy on complete blood counts. At various times after chemotherapy, the total number of circulating white blood cells (*closed squares*), lymphocytes (*open squares*), polymorphonuclear neutrophils (polys) (*open triangles*), monocytes (*open circles*), eosinophils (*open diamonds*), and basophils (*cross*) was determined in peripheral blood of individual animals ($n = 2-5$). Complete blood counts were assessed in 3 independent experiments yielding similar results. Data are representative of one experiment and are presented as mean $\pm$ SEM. **b** effect of chemotherapy on systemic IL-7. At various times after chemotherapy, serum levels of IL-7 were determined in individual animals ($n = 5$). Data are representative of one experiment and are presented as mean $\pm$ SEM. Experiment was repeated with similar results. Each animal was bled once. Asterisks denote statistical significance ($P < 0.05$) relative to untreated animals

Fig. 3.

Effect of chemotherapy on number of different spleen cell populations. At various times after administration of a single i.p. dose of cisplatin/vinorelbine (0.15/0.05 mg) to C57BL/6 mice on day 0, the total number of indicated populations in individual animals ($n = 2-5$) was determined by flow cytometry analysis. Cell populations were analyzed in 3 independent experiments with similar results. Data are representative of one experiment and are presented as mean $\pm$ SD. *Solid lines* represent mice receiving chemotherapy. *Dotted lines* represent mean number of cells in untreated animals on day 0. *Asterisks* denote statistical significance ($P < 0.05$) relative to untreated animals

Fig. 4.

Effect of chemotherapy on number and function of Tregs. C57BL/6 mice received a single i.p. dose of cisplatin/vinorelbine (0.15/0.05 mg) on day 0. **a** Analysis of Treg numbers after chemotherapy. On days 2, 4, and 7, the total number of splenic CD4⁺CD25⁺FoxP3⁺ Tregs in individual animals ($n = 2-5$) was determined by flow cytometry analysis. Results are representative of 3 independent experiments and are presented as mean $\pm$ SD. *Solid lines* represent treated animals. *Dotted lines* represent mean number of cells in untreated animals on day 0. *Asterisks* denote statistical significance ($P < 0.05$) relative to untreated animals. **b** Analysis of suppressive activity of Tregs. Mice were killed 0, 2, or 4 days after chemotherapy. CD4⁺CD25⁻ cells from untreated mice were co-cultured for 72 h with APCs

(splenocytes irradiated 30 Gy) and soluble anti-CD3 in the presence (*closed columns*) or absence (*dashed columns*) of Tregs from either control mice or cisplatin/vinorelbine-treated mice. Control wells containing Tregs, APCs, and anti-CD3 without CD4⁺CD25⁻ cells (*open columns*) were used to determine background levels of proliferation in culture. Results are representative of 2 independent experiments and are presented as mean $\pm$ SD of 3 replicate wells. *Asterisks* denote statistical significance ($P < 0.0001$) relative to proliferation of CD4⁺CD25⁻ cells in the absence of Tregs. **c** *ratio* of mean numbers of CD4⁺ T/Treg cells at various times post-chemotherapy, defined by [CD4⁺ T cells-CD4⁺CD25⁺FoxP₃⁺ Tregs]/[CD4⁺CD25⁺FoxP₃⁺ Tregs] as per flow cytometry analysis

Fig. 5.

CEA-specific T-cell responses post-treatment with the combination of vaccine plus chemotherapy. CEA-Tg mice ($n = 5/\text{group}$) were left untreated or primed on day 0 with s.c. yeast-CEA and boosted with yeast-CEA on days 7, 14, and 21. On day 4, animals received a single i.p. dose of cisplatin/vinorelbine (0.15/0.05 mg) alone or in combination with vaccine. **a** CD4⁺ T-cell responses. On day 35, purified CD4⁺ splenic T cells were tested for reactivity to CEA protein (25 $\mu\text{g}/\text{ml}$) in an in vitro lymphoproliferation assay. Results are depicted as mean CD4 proliferation $\pm$ SEM after subtraction of background CD4 reactivity from untreated controls. **B**, CEA-specific tetramer CD8⁺ T-cell responses in mice treated as in **a**. To evaluate the frequency of CEA-specific CTLs after combination therapy, CEA₅₇₂₋₅₇₉/H-2D^b-specific CD8⁺ T cells were analyzed by flow cytometry in pooled splenocytes on day 35. Results are depicted as number of CEA-tetramer⁺CD8⁺ cells/10⁵ CD8⁺ T cells after subtraction of CEA-tetramer⁺CD8⁺ cells from untreated controls. * $P < 0.01$. All results are representative of 3 independent experiments

Fig. 6.

The combination of chemotherapy with vaccine increased survival in tumor-bearing mice. *Upper panels* denote experimental schema. CEA-Tg mice were inoculated i.v. with LL/2-CEA cells on day 0. **a** effect of combination therapy on survival of tumor-bearing mice. Mice ($n = 8-10$ /group) received cisplatin (0.15 mg i.p.) plus vinorelbine (0.05 mg i.p.) on day 8 (*closed triangles*), or were left untreated (*open squares*). Another cohort of animals received weekly vaccinations with yeast-CEA (1 YU $\times$ 4 sites, sc) starting on day 11 (*open circles*). A sub-cohort of vaccinated animals also received cisplatin/vinorelbine on day 8 (*closed circles*). All animals were monitored daily for survival. Figure *insert* denotes Overall Survival (OS) of the different treatment groups. Study was repeated twice yielding similar results. Data are representative of one experiment and are presented as percent survival. **b** effect of T-cell depletion on survival of tumor-bearing mice receiving combination therapy. Animals ($n = 15$) received combination therapy alone (*closed circles*) or concurrently with CD4⁺ and/or CD8⁺ T cell-depleting antibodies. Animals receiving clones L3T4 (CD4) and/

or Lyt 2.2 (CD8) were administered a single daily dose (100 µg/i.p.) on days 4–7. All animals were monitored daily for survival. Data are presented as percent survival. Statistical significance ($P = 0.023$) was assessed relative to animals receiving combination therapy in the absence of T-cell depletion