Epigenetic Potentiation of NY-ESO-1 Vaccine Therapy in Human Ovarian Cancer

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Abstract
The cancer-testis/cancer germline antigen, NY-ESO-1, is a vaccine target in epithelial ovarian cancer (EOC), but its limited expression is a barrier to vaccine efficacy. As NY-ESO-1 is regulated by DNA methylation, we hypothesized that DNA methyltransferase inhibitors may augment NY-ESO-1 vaccine therapy. In agreement, global DNA hypomethylation in EOC was associated with the presence of circulating antibodies to NY-ESO-1. Preclinical studies using EOC cell lines showed that decitabine treatment enhanced both NY-ESO-1 expression and NY-ESO-1–specific CTL-mediated responses. On the basis of these observations, we performed a phase I dose-escalation trial of decitabine, as an addition to NY-ESO-1 vaccine and doxorubicin liposome chemotherapy, in 12 patients with relapsed EOC. The regimen was safe, with limited and clinically manageable toxicities. Both global and promoter-specific DNA hypomethylation occurred in blood and circulating DNAs, the latter of which may reflect tumor cell responses. Increased NY-ESO-1 serum antibodies and T-cell responses were observed in the majority of patients, and antibody spreading to additional tumor antigens was also observed. Finally, disease stabilization or partial clinical response occurred in six of ten evaluable patients. On the basis of these encouraging results, evaluation of similar combinatorial chemo-immunotherapy regimens in EOC and other tumor types is warranted.

Introduction
The ability of the immune system to recognize and reject tumors is dependent on several factors that include the expression of immunogenic target antigens, the generation of high frequencies of tumor antigen-specific T cells with potent effector function, and the capacity to overcome mechanisms by which tumors escape immune attack. Although several in vitro and in vivo studies demonstrated that the amount and duration of antigen stimulation can influence antigen-specific T-cell responses (1), the majority of cancer immunotherapy studies have focused on the generation of tumor antigen-specific T cells without concomitant manipulation of antigen expression. This is a critical consideration because (i) tumor antigens are generally not expressed at 100% frequency in cancers, (ii) even when expressed, the antigen density may be low, and (iii) expression is often heterogeneous with areas of tumor that are distinctly positive or negative for the tumor antigen. Moreover, the generation of tumor-specific immunity may result in tumor “immunosculpting” that could influence the ability to evade immune eradication (2). In this regard, a number of murine and human studies indicate that the adaptive immune response can edit tumor antigen expression (3). Similar observations have been made in human immunotherapy clinical trials, in which a proportion of patients showed evidence of immunoediting with either loss of antigen expression or MHC class I expression (4, 5).

In cases where epigenetic mechanisms regulate tumor antigen expression or result in immunosculpting, treatment with epigenetic modulatory drugs may reverse the phenotype. For example, edited tumor cells in a murine melanoma model, which have lost several antigens, could be induced to reexpress antigens when treated with the DNA methyltransferase inhibitors (DNMTi) 5-azacytidine (5-aza), increasing their susceptibility to further vaccination (6). The DNMTi 5-aza and 5-aza-2-deoxycytidine (decitabine) have recently entered common clinical practice for the treatment of myeloid malignancies. Clinical approval of these agents for the treatment of myelodysplastic syndrome followed the recognition that low-dose treatments resulted in progressive DNA hypomethylation and clinical responses, combined with significantly reduced systemic toxicity (7). In addition, recent
work showed that low-dose 5-aza, combined with the histone deacetylase inhibitor (HDACi) entinostat, provided clinical benefit for lung cancer (8). To date, clinical trials in epithelial ovarian cancer (EOC) have focused on using DNMTi as a means to restore responsiveness to platinum-based chemotherapy (9, 10).

The molecular mechanisms underlying the clinical benefits of DNMTi are incompletely defined, although it is postulated that DNA hypomethylation and gene reactivation are involved. A number of preclinical studies have taken an unbiased approach to assess the pharmacologic activity of DNMTi, focusing on gene expression. A consistent observation is that a major response to DNMTi is the activation of cancer–testis or cancer germline antigen genes (11, 12). Cancer–testis antigen genes are normally silenced by DNA methylation in somatic cells but are activated in cancer by promoter DNA hypomethylation (13). However, most tumors do not express these genes, and in ones that do, expression is frequently heterogeneous (14). Data suggest that DNA methylation is the basis for this heterogeneity, with tumor regions expressing these genes showing promoter-specific and global DNA hypomethylation (15). These data raise the hypothesis that DNMTi could augment cancer–testis antigen expression in tumors, which may render them more susceptible to cancer–testis antigen vaccine efficacy (14, 16). This notion is supported by the observation that although the cancer–testis antigen NY-ESO-1 is expressed in up to 40% of EOC, spontaneous immune responses are found in only a fraction of patients (17, 18), potentially because the levels of antigen expression are below the threshold for heterogeneous recognition in most patients. DNMTi can induce cancer–testis antigen promoter hypomethylation and gene expression in patients with cancer, and can augment cancer–testis antigen immunity in cell line and animal model systems (19-22). In agreement, a recent study showed that decitabine treatment increased T-cell reactivity to MAGE antigens in patients with Hodgkin’s lymphoma (23). A final important, and often overlooked, point is that DNMTi induces MHC class I, which could have a dramatic impact on antitumor immunity (11, 16, 20).

In the current study, we examined the combinational effect of an epigenetic therapy (decitabine) with a tumor antigen-targeted cancer vaccine [NY-ESO-1 protein and granulocyte macrophage colony-stimulating factor (GM-CSF) emulsified in Montanide]. We tested this combination in patients with EOC at the time of relapse, as an addition to the second-line chemotherapy doxorubicin. Our data show that this novel combination therapy is safe, promotes integrated immunologic responses, and resulted in stable disease or partial response in six of ten evaluable patients. These findings encourage testing of similar chemo-immunotherapeutic approaches for the treatment of EOC and other human malignancies.

Materials and Methods

Preclinical studies

Decitabine and doxorubicin liposome were obtained from Sigma, A2780 and OVCAR3 EOC cell lines were cultured as described (24), and drug treated as described in the Results section and in figure legends. T-cell responses to drug-treated cells were measured as described in Fig. 1D. NY-ESO-1 mRNA expression was determined by real-time PCR (RT-PCR), and long interspersed repeated sequence (LINE-1) and NY-ESO-1 methylation were determined using sodium bisulfite pyrosequencing, as described (15). All clinical samples were obtained under Institutional Review Board (IRB)-approved protocols at Roswell Park Cancer Institute (RPCI).

Inclusion criteria

The study protocol (NCT00887796) was approved by the IRB at RPCI (Protocol #1127008), and all patients gave informed consent. Eligible patients were women with relapsed EOC (including fallopian tube and primary peritoneal cancer), who normally receive doxorubicin as salvage therapy for recurrent disease. Inclusion criteria were Karnofsky performance status of at least 70%, normal complete blood count and kidney–liver function, and no concomitant anti-tumor therapy or immnosuppressive drugs. Exclusion criteria were pregnancy, seropositivity for HIV or hepatitis B surface antigen, brain metastasis, clinically significant autoimmune disease, or New York Heart Association class III–IV heart disease.

Study protocol

This was an open-label study with escalating doses of decitabine, a fixed standard dose of doxorubicin liposome (40 mg/m²), and a fixed dose of vaccine (NY-ESO-1 protein + Montanide + GM-CSF). The treatment schedule consisted of decitabine on day 1, doxorubicin on day 8, and vaccine on day 15. In the first cohort (3 patients), decitabine was administered at a dose of 45 mg/m² × d1; in the second cohort (6 patients), decitabine was used at 90 mg/m² × d5 (days 1–5), for each 28-day cycle. The choice of decitabine dose was based on previous studies of decitabine in EOC (9, 25). For vaccination, 250 μg of NY-ESO-1 protein, admixed with 250 μg of GM-CSF, was emulsified in Montanide and injected s.c. Treatments were repeated every 28 days, for a total of four cycles. After completion of the regimen, patients with stable disease or those responding on therapy were continued on doxorubicin until progression. Patients were evaluated for treatment toxicity, immune responses, and DNA methylation changes.

Investigational agents

NY-ESO-1 protein for immunization was manufactured at the Ludwig Institute for Cancer Research (LICR) as described (26). Incomplete Freund’s adjuvant (Montanide ISA-51), contained mineral oil (Drakeol) and anhydro mannitol octadecanoate and was manufactured by Seppic Inc. Sargramostim was obtained from Bayer. Decitabine was obtained from Eisai Pharmaceuticals under a research agreement. Pegylated liposomal doxorubicin for injection was supplied as a sterile, translucent red liposomal dispersion.

DNA methylation pharmacodynamics

Samples from 2 patients in the 10 mg/m² × d5 decitabine cohort were obtained pretreatment and on days 8 and 15 of
Figure 1. Preclinical studies supporting the use of decitabine to modulate NY-ESO-1 vaccine efficacy. A, LINE-1 is hypomethylated in NY-ESO-1–seropositive EOC patients. LINE-1 methylation was determined by pyrosequencing in EOC samples from patients positive or negative for serum NY-ESO-1 antibodies at the time of diagnosis. NY-ESO-1 seropositivity was defined as a reciprocal titer of more than 100 by ELISA. B, effect of decitabine (DAC) and doxorubicin (Doxil) treatments on NY-ESO-1 promoter methylation in EOC cells. A2780 and OVCAR3 cell lines were treated with the indicated concentrations of decitabine, doxorubicin, or decitabine followed by doxorubicin, as described in B. A NY-ESO-1–specific HLA-A2–restricted CD8⁺ T-cell clone was stimulated for 6 hours with OVCAR3 cells following harvest and wash out of reagents. IFN-γ production and CD107 expression on HLA-A2/NY-ESO-1157–165 tetramer⁺ CD8⁺ T cells was determined by flow cytometry. As a positive control, CD8⁺ T-cell responses were tested against NY-ESO-1157–165 peptide (0.4 µg/mL)-pulsed OVCAR3 cells. Values in quadrants indicate percentages of cells. A, NY-ESO-1 mRNA expression following drug treatment and removal. A2780 cells were treated with the indicated concentrations of decitabine or decitabine + doxorubicin, and cells were extracted at the indicated time points following drug removal and used to determine NY-ESO-1 mRNA expression by RT-PCR.
Humoral responses to NY-ESO-1 and other tumor antigens

NY-ESO-1–specific antibodies were measured by ELISA using serum collected on days 29, 57, 85, and 113, and 6 months after the last vaccination, as described (17, 27). In addition, serum antibody titers for 22 unrelated recombinant proteins were determined by ELISA as described (27). Recombinant proteins included NY-ESO-1/CTAG1B, LAGE-1/CTAG2, MAGEA1, MAGEA3, MAGEA4, MAGEA10, CT7/MAGEC1, CT10/MAGEC2, CT45/RP13-36C9, CT46/HORMAD1, CT47/RP6-166C19, K67/MKI67, KRAS, SCP1/SYCP1, SOX2, SPANX1, SXX1, SXX2, SXX4, p53/TP53, XAGE1B, and DHFR (27, 28). Specificity was determined by comparing seroreactivity among the various antigens tested, including the ubiquitously expressed nontumor antigen, dihydrofolate reductase (DHF). A reciprocal titer was estimated from optical density readings of serially diluted plasma samples as the maximal dilution signifying significantly reacting to antigen (28). Reciprocal titers of more than 100 were considered significant. Negative control sera from healthy individuals, and positive control sera for each antigen from patients with cancer, were included in all assays.

Analysis of NY-ESO-1–specific T cells

Sample processing, freezing, and analysis were performed in the Immune Analysis Facility of the Center for Immunotherapy at RPCI. Assay performance and data reporting conformed to Minimal Information about T Cell Assays (MIATA) guidelines. A pool of synthetic overlapping 20- to 25-mer NY-ESO-1 peptides covering the entire NY-ESO-1 protein sequence was used for in vitro stimulation. Purified CD8+ or CD4+ T cells were presensitized with peptide-pulsed irradiated autologous PBMC depleted of CD4+ and CD8+ T cells, as described (29). Target cells for analysis were activated T-cell antigen-presenting cells (T-APCs) or autologous EBV-B cells. The frequency of NY-ESO-1–specific CD8+ or CD4+ T cells was assessed by ELISPOT or intracellular cytokine staining, as described (30). For ELISPOT assays, responses were considered positive when spot numbers in the presence of target cells significantly exceeded the cutoff value (>50 spots/50,000 cells), and were at least 3 times more than that spot count of unpulsed target cells. The average number of spots against no-peptide-pulsed cells was 45. In a subset of patients with relevant HLA alleles, the frequencies of CD8+ T cells were analyzed using human leukocyte antigen (HLA) multimers HLA-A2/NY-ESO-1157–165, HLA-B35/NY-ESO-1164–172, and HLA-Cw3/NY-ESO-192–100. HLA multimers were manufactured at the Peptide Synthesis Facility of the ICR (Lausanne, Switzerland). Responses were considered significant if tetramer-positive cells were 0.1% or more of CD8+ T cells, and at least 3 times more than the percentage obtained with control tetramer.

Molecular typing of HLA class I and II

HLA typing was performed at the HLA typing laboratory of RPCI as described (31), using primers obtained from Genovision.

Statistical analysis

Patient demographic and disease characteristics were summarized using frequencies and descriptive statistics. The platinum-free interval was defined as the number of months between the last platinum-based chemotherapy treatment and enrollment on study, and patients with an interval less than 6 months were considered resistant (32). Associations between methylation levels of NY-ESO-1 and LINE-1 were quantified using the Pearson test. Differences in LINE-1 methylation in NY-ESO-1 antibody-positive and -negative patients were assessed using the Welch two-tailed t test. Statistical analyses were performed using Graphpad Prism 5.0.

Results

DNA hypomethylation in EOC is associated with spontaneous immune responses to NY-ESO-1

Previously we found that (i) NY-ESO-1 is expressed in less than 50% of patients and is often focal or heterogeneous in the lesions in which it is expressed (17); (ii) NY-ESO-1 inter- and intratumor heterogeneity is associated with promoter-specific and global DNA methylation (15); (iii) decitabine treatment induces NY-ESO-1 hypomethylation and expression in EOC samples; and (iv) loss of NY-ESO-1 expression in EOC occurs during NY-ESO-1 vaccine therapy (33). These data raised the possibility that decitabine may enhance the efficacy of NY-ESO-1 vaccines in EOC. To evaluate this hypothesis, we determined whether DNA methylation status in EOC is predictive of spontaneous immune response to NY-ESO-1, by measuring tumor LINE-1 methylation and circulating NY-ESO-1 antibodies in matched EOC patient samples. The LINE-1 repetitive element comprises approximately 17% of the genome and is regulated by DNA methylation. We previously validated LINE-1 methylation as a biomarker for global DNA methylation status in EOC, and showed that LINE-1 methylation is significantly correlated with NY-ESO-1 promoter methylation in this disease (34). NY-ESO-1–seropositive EOC had significantly reduced tumor LINE-1 methylation compared with seronegative patients (Fig. 1A), consistent with the hypothesis that DNA hypomethylation promotes NY-ESO-1–specific immune responses in EOC.
Combination decitabine + doxorubicin chemotherapy promotes NY-ESO-1 promoter hypomethylation, gene expression, and immune recognition in EOC cell lines

We sought to develop a clinical regimen combining epigenetic therapy with NY-ESO-1 vaccine. Because combination therapy of this type has not been tested before and safety is uncertain, we developed an initial test of a decitabine + NY-ESO-1 vaccine in the setting of recurrent EOC, which is uniformly fatal, as an add-on to a standard second-line chemotherapy, doxorubicin liposome.

We initially used two NY-ESO-1-negative human EOC cell lines, A2780 and OVCAR3, to determine the optimal sequencing of decitabine and doxorubicin treatment. Treatment with decitabine followed by doxorubicin, but not the reverse sequence, resulted in NY-ESO-1 promoter hypomethylation and NY-ESO-1 gene induction (Fig. 1B and C). In addition, this sequence resulted in the recognition of EOC cells by NY-ESO-1–specific CTL clones, consistent with functional presentation of NY-ESO-1 epitopes on EOC cells posttherapy (Fig. 1D). There was also evidence that doxorubicin potentiated CTL responses to equivalent doses of decitabine (Fig. 1D). Also supporting the combination treatment strategy is that decitabine potentiates apoptotic responses to doxorubicin in solid tumor cell lines (35). Although CTL clones produced more than 70% IFN-γ response against peptide-pulsed melanoma cell lines (Supplementary Fig. S1), the response against peptide-pulsed OVCAR3 was comparatively less (Fig. 1D), suggesting that ovarian cancer cell lines are inherently less immunogenic than melanoma. To determine how long-lived NY-ESO-1 induction is following drug treatment, we measured NY-ESO-1 using RT-PCR in A2780 cells up to 1 month after drug treatment (Fig. 1E). We observed continued NY-ESO-1 expression throughout this time, albeit with diminishing expression at later time points (Fig. 1E).

Phase I trial of decitabine + NY-ESO-1 vaccine in patients receiving doxorubicin for recurrent EOC

Twelve patients with relapsed EOC gave written informed consent and were entered into a Phase I clinical trial (NCT00887796). The trial design involved fixed doses of doxorubicin and NY-ESO-1 vaccine, with dose escalation of decitabine (see Materials and Methods). Unlike previous NY-ESO-1 vaccine trials, tumor NY-ESO-1 expression was not used as an inclusion criterion, and 4 of 11 (36%) patients expressed NY-ESO-1 at baseline (Table 1). Patient characteristics are summarized in Supplementary Table S1. The median age at study entry was 59 years, and the median time from diagnosis to study entry was 21 months. Nine of 12 (75%) patients received at least two previous lines of chemotherapy. Eleven of 12 (92%) patients had poorly differentiated tumors. Twelve of 12 (100%) were Federation Internationale des Gynaecologistes et Obstetrists (FIGO) stage III, and 11 of 12 (92%) displayed serous histology. On the basis of Gynecologic Oncology Group clinical response criteria (32), 10 of 11 (91%) patients with available information were considered platinum resistant.

The regimen was well tolerated. The most frequent grade 3/4 adverse event was neutropenia, and only 1 patient experienced febrile neutropenia (Supplementary Table S2). Vaccine injection site reactions were common, but only two were grade 3 events. All adverse events were clinically manageable. We observed three serious adverse events: septicemia, intestinal obstruction, and dehydration were observed at the 45 mg/m² × d1, 90 mg/m² × d1, and 10 mg/m² × 45 decitabine doses, respectively. All three of these adverse events were considered unrelated to the study drugs. Dose-limiting toxicity was observed in 1 patient (grade 4 neutropenia) at the 90 mg/m² dose, requiring a 25% dose reduction of decitabine.

Effects on DNA methylation

We obtained samples from 2 patients in the 10 mg/m² × d5 decitabine cohort to confirm the effect of the regimen on DNA methylation. We collected gDNA from surrogate normal tissues (PBMCs and granulocytes), as well as from circulating DNA present in serum and plasma, which likely is comprised of tumor cell DNA (36). Indeed, LINE-1 methylation in serum has been used as a pharmacodynamic marker for DNMTi in other solid tumor trials (37). We used quantitative bisulfite pyrosequencing to measure LINE-1 and NY-ESO-1 promoter methylation. DNA from PBMCs and granulocytes showed significant hypomethylation of both LINE-1 and NY-ESO-1 promoters posttherapy (Fig. 2A–D). Hypomethylation was pronounced in granulocytes, likely due to the rapid cycling rate and shorter half-life of these cells as compared with PBMCs (Fig. 2A and C). Significant hypomethylation of LINE1 and NY-ESO-1 also occurred in plasma and serum DNA, suggesting tumor cell hypomethylation (Fig. 2A–D). In general, greater hypomethylation occurred at day 8 of each cycle as compared with day 15, consistent with remethylation following decitabine withdrawal, as observed previously (19). The effect on LINE-1 and NY-ESO-1 methylation was highly correlated (Fig. 2E and F).

Humoral responses

Spontaneous and treatment-induced humoral immune responses in patients were investigated first by measuring serum immunoglobulin G against recombinant full-length NY-ESO-1 protein using ELISA (Fig. 3A; Table 1). Four patients (# 01, 02, 03, and 06) were baseline seropositive for anti-NY-ESO-1 antibody and all remained seropositive during the course of therapy. Among the 6 baseline seronegative patients for which information was available (Pts. # 05, 07, 08, 09, 10, 11), five (83%) converted to seropositive, and this occurred rapidly after two to three cycles (Fig. 3A; Table 1). A comparison of maximal NY-ESO-1 antibody titers in each cohort indicated no significant difference between cohorts 2 and 3 (Fig. 3B). Cohort 1 had higher NY-ESO-1 antibody titers than cohorts 2 and 3 (Fig. 3B), likely reflecting their seropositivity at baseline (Fig. 3A; Table 1).

To determine whether the treatment resulted in antigen spreading, we tested humoral immune responses against a panel of 22 recombinant proteins, including cancer–testis antigens (LAGE1, MageA, MageC, SsyX, Xage, Ct45, Ct46, Ct47, Spanx), mutational antigens (TP53), and embryonic/stem cell antigens (SOX2), for recognition by ELISA, as described previously (27). Overall, de novo serum reactivity to antigens not included in the vaccine was observed in 3 of 3 patients analyzed before and after therapy, with titers ranging
Table 1. Summary of NY-ESO-1–specific immune responses

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<th>Patient number</th>
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<th>CD4+ T cell</th>
<th>RT-PCR</th>
<th>IHC</th>
<th>Decitabine dose</th>
<th>No. of therapy cycles</th>
<th>Pre</th>
<th>Post</th>
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Antibody reciprocal titer key: +, 100–1000; ++, 1000–10,000; ++++, >10,000.

Abbreviation: N/A, sample not available.
from 130 to 450 (Fig. 3C). Antibodies against recombinant DHFR, measured as a control, were not detected. These data indicate that the combination regimen leads to a broadened profile of antitumor immune responses in vivo.

**T-cell responses**

Preexisting NY-ESO-1–specific CD8⁺ T cells were detectable by IFN-γ ELISPOT assays in 2 of 10 (20%) patients with EOC analyzed (Fig. 4A; Table 1). These 2 patients were in cohort 1, and both showed further increases in NY-ESO-1–specific CD8⁺ T cells following treatment (Fig. 4A). There was de novo induction of NY-ESO-1–specific CD8⁺ T-cell responses in 3 additional patients (2 in cohort 2 and 1 in cohort 3; Fig. 4A; Table 1). Thus, 5 of 11 (45%) evaluable patients had antigen-specific CD8⁺ T-cell responses following therapy (Fig. 4A; Table 1). Preexisting NY-ESO-1–specific CD4⁺ T-cell responses were more widespread, and were detected in seven of ten (70%) evaluable patients (Fig. 4A; Table 1). These responses persisted during therapy and, in addition, there was de novo induction of CD4⁺ T cells in 2 patients (Pts. #09 and 10).
We determined the epitope clusters of therapy-induced CD8+ T and CD4+ T cells by testing overlapping 20-mer peptides against NY-ESO-1 in ELISPOT assays. Figure 5A illustrates the responses from patients #03 and #06 before and after therapy, and at the time of follow-up (i.e., 6 months after the last treatment). In all patients, therapy-induced CD8+ T-cell epitopes were clustered in the p81–110 and 119–143 regions of the NY-ESO-1 protein (Fig. 5B). Notably, CD4 epitopes showed evidence of epitope broadening following therapy and spanned the 51–180 region (Fig. 5).

To assess whether therapy-induced CD8+ T cells were capable of recognizing naturally processed NY-ESO-1 antigen, we used NY-ESO-1–expressing cancer cell lines as targets. Polyclonal populations of NY-ESO-1–specific CD8+ T cells were enriched by sorting tetramer-reactive cells or IFN-γ–producing cells using IFN-γ capture reagents. Although treatment-induced polyclonal CD8+ T cells recognized partially HLA-matched NY-ESO-1+ melanoma cell lines (MZ-MEL-9, SK-MEL-95, SK-MEL-139, SK-MEL-52), the SK-MEL-29 (B35+ ES0+) line was not recognized (Supplementary Fig. S2). To characterize CD4+ T cells, NY-ESO-1–specific CD4+ T cells were enriched on the basis of CD154 expression upon restimulation with NY-ESO-1 peptide, and tested for Th1/Th2 polarization and protein recognition, as described (38). Th1 and Th2 differentiation was determined on the basis of IFN-γ and interleukin (IL)-4 production upon stimulation with NY-ESO-1–poolede peptides. As shown in Supplementary Fig. S2, Th1 differentiation increased following treatment, and therapy-induced NY-ESO-1–specific CD4+ T cells recognized the full-length NY-ESO-1 protein.

**Clinical responses**

A total of 10 patients were evaluable for clinical response using immune-related response criteria (modified Response Evaluation Criteria in Solid Tumors; ref. 39; Table 2). Notably, 5 patients had stable disease (50%) and 1 patient had a partial response/disease remission (10%). The median duration of stable disease was 6.3 months (range, 3.9–7.8 months), and the partial response duration was 5.8 months. The clinical response in the three different decitabine cohorts appeared distinct (Table 2). In the first cohort (45 mg/m2), 2 of 2 evaluable patients showed stable disease or partial response/remission. Similarly, in the third cohort (10 mg/m2 × d5), 2 of 2 evaluable patients showed stable disease. However, in the second cohort and the highest dose level (90 mg/m2), only 2 of 6 patients (33%) showed stable disease. Finally, it was notable that only one of five patients showing stable disease or partial response expressed NY-ESO-1 pretreatment (Tables 1 and 2).

**Discussion**

Here, we report a novel therapeutic regimen for patients with EOC that is applicable to other human malignancies. The approach utilizes an epigenetic therapy, decitabine, known to induce cancer–testis antigen gene expression, in combination with a vaccine targeted against the activated antigen, in this case NY-ESO-1. We used this novel combination as an add-on to a standard EOC chemotherapy agent, doxorubicin, although this is not a requisite part of the strategy. In addition to
NY-ESO-1, other epigenetically regulated cancer–testis antigens for which vaccines are available could be targeted using this approach. Because cancer–testis antigens are regulated by DNA methylation, histone acetylation, and other epigenetic marks, agents other than decitabine (e.g., HDACi) could also be tested in combination with cancer–testis antigen vaccines (14). In addition to EOC, other cancers, for example, melanoma and lung, are candidates for this approach, as cancer–testis antigens are epigenetically regulated in most human tumors, and cancer–testis antigen vaccines are in an advanced stage of clinical development in these malignancies (14).

A number of preclinical observations supported this approach. NY-ESO-1 expression is regulated by DNA methylation in EOC cells and tumor tissues, and decitabine treatment induces NY-ESO-1 expression in EOC cell lines (15). In addition, heterogeneous intratumor expression of NY-ESO-1, which frequently occurs in EOC and other tumor types, is associated with DNA methylation of the NY-ESO-1 promoter and also the global methylation status of the tumor cells (15). Notably, we found that NY-ESO-1 serum antibodies in patients with EOC at baseline are associated with LINE-1 hypomethylation in matched tumors. This observation provides support to the notion that global DNA methylation status plays a key role in regulating NY-ESO-1 expression and immune recognition in vivo.

Because of an unknown safety profile, we tested the regimen in patients with recurrent EOC, as an add-on to second-line doxorubicin chemotherapy. To determine the proper sequencing of decitabine and doxorubicin, we conducted treatment studies on EOC cell lines, and found that decitabine treatment must precede doxorubicin to hypomethylate and activate NY-ESO-1. This finding is consistent with a requirement for
continued DNA synthesis to incorporate decitabine and to remodel DNA methylation, as doxorubicin treatment likely blocks decitabine incorporation and cell cycling. Importantly, EOC cells treated with the decitabine + doxorubicin combination promoted NY-ESO-1 antigen-restricted CTL recognition of EOC cell lines, and the effect of drug treatment on NY-ESO-1 expression was longlasting.

Two patients enrolled in the trial, at the 10 mg/m² × d5 decitabine dose level, were used to evaluate pharmacodynamic effects on DNA methylation. In both patients, there was a marked reduction of methylation of the LINE-1 and the NY-ESO-1 promoters in blood cells, and hypomethylation of the two regions were remarkably correlated. Because of logistical constraints, we were unable to obtain tumor tissues posttherapy to study DNA methylation or NY-ESO-1 expression. However, we observed robust hypomethylation in circulating DNA obtained from serum and plasma posttherapy. Because circulating DNA in patients with cancer is derived from tumor cells (36), our data suggest that decitabine treatment causes DNA hypomethylation in clinically relevant target cells. These findings are also consistent with the prior demonstration that decitabine treatment leads to hypomethylation of genes detected in plasma DNA, which is hypermethylated in platinum-resistant EOC (9).

NY-ESO-1–specific antibodies were generated in two thirds (67%) of the baseline seronegative patients. Remarkably, de novo induction of humoral immune responses against the unrelated antigens MAGEA4, CT45, CT47, CT55, and SOX2 was also observed, indicative of antigen spreading. Similar evidence of antigen spreading was not observed in our previous NY-ESO-1 vaccine trials in patients with EOC (18, 40). In a previous report, sera from patients vaccinated with the recombinant NY-ESO-1 protein sometimes showed nonspecific reactivity against other recombinant proteins (41), and the possibility was raised that
there could be reactivity against bacterial components or His-tag in the vaccine preparation. We ruled this out in the current study, as no antibody responses were observed against the ubiquitous control protein (DHRF). There are several potential mechanisms by which antigen spreading may have been facilitated with the current regimen. The first is upregulation of additional tumor antigens and/or MHC class I by decitabine, leading to improved recognition by the immune system (16). The second is chemotherapy-induced immunogenic cell death, which can lead to cross-presentation of tumor antigens (42). Finally, the destruction of tumor cells by NY-ESO-1−specific immunity may release additional tumor antigens, leading to the generation of multiple heteroclitic serologic responses (43).

Humoral immune responses posttherapy were accompanied by antigen-specific CD8+ T-cell responses in 50% of the patients. In contrast, with the exception of patients vaccinated with recombinant NY-ESO-1 protein and CpG in Montanide (44), less than 50% of the patients vaccinated with NY-ESO-1 protein in previous trials developed antigen-specific CD8+ T-cell responses (+46). Consistent with previous studies (44), we found that the specificity of the CD8+ T-cell responses was clustered in the central region of the NY-ESO-1 protein. Moreover, therapy-induced CD8+ T-cell lines from patients recognized naturally processed full-length antigen and tumor targets. Although antigen-specific CD8+ T-cell responses were present at baseline in the majority of patients, the regimen led to broadening of epitopes, which persisted during the follow-up period. In addition, the combination therapy led to skewing toward Th1 differentiation of NY-ESO-1−specific CD4+ T cells, as compared with baseline preexisting antigen-specific CD4+ T cells. Together, our results reveal integrated induction of antibody, CD8+, and CD4+ T cells, along with antigen spreading, using this novel regimen.

The major limitation for combining cytotoxic chemotherapy, such as doxorubicin, and immunotherapy is that cytotoxic drugs are generally regarded as immunosuppressive due to toxicity to dividing immune cells. However, the potential for therapeutic synergy of chemotherapy and immunotherapy has been recognized (47). Recent work has shown that doxorubicin enhances the proliferation of tumor antigen-specific CD8+ T cells in tumor-draining lymph nodes, and promotes tumor infiltration by IL-17−secreting γδ T cells and activated IFN-γ−secreting CD8+ T cells (48, 49). Moreover, doxorubicin has the capacity to increase the permeability of tumor cells to granzyme B, thereby rendering them more susceptible to CTL-mediated lysis, even if they do not express the antigen recognized by CTLs (50).

The majority of patients enrolled in this trial had multiple previous lines of chemotherapy and were considered platinum resistant. Thus, the observed cases of stable disease and partial response in this population are notable. Although the current trial was not powered to address response rates and progression-free survival, the clinical results obtained are encouraging. A limitation of our study design is our inability to delineate the individual contributions of doxorubicin, decitabine, and vaccination to the immunologic and clinical results. However, this question can be addressed in the phase II setting.

The present study suggests that conventional chemotherapy, decitabine, and immunotherapy may promote the generation of vaccine-induced immune responses. Although integrated humoral and T-cell responses occurred in a significant proportion of patients with ovarian cancer in our previous NY-ESO-1 vaccine trials (18, 33, 40), this is our first observation of antigen spreading with de novo induction of immune responses against a broad array of unrelated tumor antigens. In addition, we observed immunologically and clinically beneficial responses regardless of the pretreatment tumor NY-ESO-1 expression status, potentially increasing the patient population eligible for vaccine therapy. We conclude that the current strategy may be capable of overcoming several potential mechanisms by which ovarian and other cancers escape immune attack.

**Disclosure of Potential Conflicts of Interest**

E. Griffiths has received a commercial research grant from Supergen, Inc, and has received honoraria from service on the speakers’ bureau from Celgene, Inc, Alexion Pharmaceuticals, and Incyte, Inc. G. Ritter has ownership interest (including patents) as coinventor on primary affiliation assigned NY-ESO-1 patents. S. Gnjatic has ownership interest (including patents) in NY-ESO-1-related patents. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Odunsi, J. Matsuuzaki, S.R. James, T. Tsuji, A. Miller, E. Griffiths, S. Gnjatic, A.R. Karpf

Writing, review, and/or revision of the manuscript: K. Odunsi, J. Matsuuzaki, S.R. James, S.N. Akers, E. Griffiths, G. Ritter, S. Lele, S. Gnjatic, A.R. Karpf

Table 2. Clinical responses

<table>
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</table>

Abbreviations: N/E, not evaluable, safety assessment only; PD, progressive disease; PR, partial response/remission; SD, stable disease.

aThe median duration of SD responses was 6.3 months (range, 3.9–7.8), and the PR duration was 5.8 months.

bLeft study early; no posttreatment CT scan.

cLeft study early; contraindicated corticosteroid use.
References


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