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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 (DNMT) 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. Pre-clinical studies using EOC cell lines showed that decitabine treatment enhanced both NY-ESO-1 expression and NY-ESO-1-specific CTL-mediated responses. Based on these observations, we performed a phase I dose-escalation trial of decitabine, as an addition to NY-ESO-1 vaccine and doxorubicin liposome (doxorubicin) 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 6/10 evaluable patients. Based on these encouraging results, evaluation of similar combinatorial chemo-immunotherapy regimens in EOC and other tumor types is warranted.

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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 (TA) 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 TA-specific T cells without concomitant manipulation of antigen expression. This is a critical consideration because (i) TAs 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 TA. 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 TA expression (3). Similar observations have been made in human immunotherapy clinical trials, where 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 TA 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 re-express antigens when treated with the DNA methyltransferase inhibitors (DNMTi) 5-azacytidine (5-aza; vidaza), increasing their susceptibility to further vaccination (6). The DNMTi 5-aza and 5-aza-2′-deoxycytidine (decitabine; dacogen) 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 pre-clinical studies have taken an unbiased approach to assess the pharmacological activity of DNMTi, focusing on gene expression. A consistent observation is that a major response to DNMTi is the activation of cancer-testis (CT) or cancer-germline antigen genes (11, 12). CT 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 CT antigen expression in tumors, which may render them more susceptible to CT antigen vaccine efficacy (14, 16). This notion is supported by the observation that although the CT 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 immune recognition in most patients. DNMTi can induce CT antigen promoter hypomethylation and gene expression in cancer patients, and

can augment CT 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 induce MHC class I, which could have a dramatic impact on antitumor immunity (11, 16, 20).

In the current study we examined the clinical combination of an epigenetic therapy (decitabine) with a TA-targeted cancer vaccine (NY-ESO-1 protein and GM-CSF emulsified in Montanide). We tested this combination in EOC patients 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 immunological responses, and resulted in stable disease or partial response in 6/10 evaluable patients. These findings encourage testing of similar chemo-immunotherapeutic approaches for the treatment of EOC and other human malignancies.

Materials and Methods

Pre-clinical studies

5-aza-2′-deoxycytidine (decitabine) and doxorubicin liposome (doxorubicin) were obtained from Sigma. A2780 and OVCAR3 EOC cell lines were cultured as described (24), and drug-treated as described in the *Results* and Figure Legends. T cell responses to drug-treated cells were measured as described in Fig. 1D. *NY-ESO-1* mRNA expression was determined by RT-PCR, and *LINE-1* and *NY-ESO-1* methylation were determined using sodium bisulfite pyrosequencing, as described (15). All clinical samples were obtained under IRB-approved protocols at Roswell Park Cancer Institute (RPCI).

Inclusion Criteria

The study protocol (NCT00887796) was approved by the IRB at RPCI (Protocol #I127008), 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 antitumor therapy or immunosuppressive 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 (40mg/m^2), and 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/m^2 × d1; in the second cohort (6 patients), decitabine was used at 90 mg/m^2 × d1; and in the third cohort (3 patients), decitabine was used at 10 mg/m^2 × 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 subcutaneously. Treatments were repeated every 28 days, for a total of 4 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, IFA (Montanide™ ISA-51), contained mineral oil (Drakeol) and anhydro mannitol octadecanoate and was manufactured by Seppic Inc. Sargramostim, (Leukine®) was obtained from Bayer (Seattle, WA). 5-aza-2′-deoxycytidine (Decitabine, Dacogen™) was obtained from Eisai Pharmaceuticals (Bridgewater, NJ), under a research agreement. Pegylated liposomal doxorubicin (Doxil®, Ben Venue Laboratories, Inc., Bedford, Ohio) for injection was supplied as a sterile, translucent, red liposomal dispersion.

DNA methylation pharmacodynamics

Samples from two patients in the 10 mg/m^2 × d5 decitabine cohort were obtained pre-treatment and on days 8 and 15 of each treatment cycle for DNA methylation analyses. Whole blood and serum were collected from patients and processed within 24 hours. Serum was spun down to remove debris and the supernatant was recovered. Plasma, PBMCs, and granulocytes were obtained by fractionating whole blood using continuous density gradient centrifugation and Lymphocyte Separation Medium (LSM). Blood samples were diluted in Hank's buffered salt solution (HBSS), layered on LSM, and centrifuged to obtain the top plasma layer, the middle PBMC layer, and the bottom granulocyte/RBC layer. After recovering the plasma, PBMCs were washed with HBSS, and the cell pellet was resuspended in RPMI medium. The granulocyte/RBC layer was incubated with RBC lysis buffer, and the remaining granulocyte pellet was washed with HBSS and resuspended in RPMI medium. Genomic DNA was extracted from each component using the QIAamp DNA blood mini kit (Qiagen). DNA methylation of the *long interspersed repeated sequence* (*LINE-1*) and *NY-ESO-1* was determined by bisulfite pyrosequencing as described (15).

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/RP13-36C9.1, CT46/HORMAD1, CT47/RP6-166C19.1, K_i-67/MKI67, KRAS, SCP1/SYCP1, SOX2, SPANXA1, SSX1, SSX2, SSX4, p53/TP53, XAGE1B, and DHFR (27, 28). Specificity was determined by comparing seroreactivity among the various antigens tested, including the ubiquitously expressed non-tumor antigen, dihydrofolate reductase (DHFR). A reciprocal titer was estimated from optical density readings of serially diluted plasma samples as the maximal dilution significantly reacting to antigen (28). Reciprocal titers of >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 20–25-mer NY-ESO-1 peptides covering the entire NY-ESO-1 protein sequence were used for *in vitro* stimulation. Purified CD8$^+$ or CD4$^+$ T cells were pre-sensitized with peptide-pulsed irradiated autologous PBMC depleted of CD4$^+$ and CD8$^+$ T cells, as described (29). Target cells for analysis were activated T cell APCs (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 (ICS), 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× greater 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 HLA multimers HLA-A2/NY-ESO-$1_{157–165}$, HLA-B35/NY-ESO-$1_{94–102}$, and HLA-Cw3/NYESO-$1_{92–100}$. HLA multimers were manufactured at the Peptide Synthesis Facility of the LICR, Lausanne, Switzerland. Responses were considered significant if tetramer-positive cells were ≥ 0.1 % of $CD8^+$ T cells, and at least 3× greater 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*.

Statistics

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 six months were considered resistant (32). Associations between methylation levels of *NY-ESO-1* and *LINE-1* were quantified using Pearson's test. Differences in *LINE-1* methylation in NY-ESO-1 antibody-positive and -negative patients were assessed using Welch's 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 <50% of EOC patients and is often focal or heterogeneous in the lesions in which it is expressed (17), ii) NY-ESO-1 inter- and intra-tumor heterogeneity is associated with promoter-specific and global DNA methylation (15), iii) decitabine treatment induces NY-ESO-1 hypomethylation and expression in EOC cells (15), 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 ~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 patients had significantly reduced tumor *LINE-1* methylation compared to 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 (doxorubicin).

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–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 post-therapy (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). Whereas CTL clones produced >70% IFN-γ response against peptide-pulsed melanoma cell lines (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 one month post-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 liposome and NY-ESO-1 vaccine, with dose-escalation of decitabine (see *Methods*). Unlike previous NY-ESO-1 vaccine trials, tumor NY-ESO-1 expression was not used as an inclusion criterion, and 4/11 (36%) patients expressed NY-ESO-1 at baseline (Table 1). Patient characteristics are summarized in Table S1. The median age at study entry was 59 years, and the median time from diagnosis to study entry was 21 months. 9/12 (75%) patients received at least two previous lines of chemotherapy. 11/12 (92%) patients had poorly differentiated tumors, 12/12 (100%) were FIGO stage 3, and 11/12 (92%) displayed serous histology. Based on Gynecologic Oncology Group (GOG) clinical response criteria (32), 10/11 (91%) patients with available information were considered platinum-resistant.

The regimen was well-tolerated. The most frequent grade 3/4 adverse event (AE) was neutropenia, and only one patient experienced febrile neutropenia (Table S2). Vaccine injection site reactions were common, but only two were grade 3 events. All AEs were clinically manageable. We observed three serious AEs: septicemia, intestinal obstruction, and dehydration were observed at the 45 mg/m^2 × d1, 90 mg/m^2 × d1, and 10 mg/m^2 × d5 decitabine doses, respectively. All three of these AEs were considered unrelated to the study drugs. Dose-limiting toxicity was observed in one patient (grade 4 neutropenia) at the 90 mg/m^2 dose, requiring a 25% dose reduction of decitabine.

Effects on DNA methylation

We obtained samples from two patients in the 10 mg/m^2 × 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 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 post therapy (Fig. 2A–D).

Hypomethylation was pronounced in granulocytes, likely due to the rapid cycling rate and shorter half-life of these cells as compared to 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 d8 of each cycle as compared to d15, consistent with re-methylation following decitabine withdrawal, as observed previously (19). The effect on *LINE-1* and *NY-ESO-1* methylation was highly correlated (Fig. 2E–F).

Humoral responses

Spontaneous and treatment-induced humoral immune responses in patients were investigated first by measuring serum IgG 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 six 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 2–3 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 CT antigens (LAGE1, MAGEA, MAGEC, SSX, 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 3/3 patients analyzed pre- and post-therapy, with titers ranging 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

Pre-existing NY-ESO-1-specific $CD8^+$ T cells were detectable by IFN-γ ELISPOT assays in two of ten (20%) EOC patients analyzed (Fig. 4A, Table 1). These two patients were in cohort 1, and both demonstrated further increases in NY-ESO-1-specific $CD8^+$ T cells following treatment (Fig. 4A). In addition, there was *de novo* induction of NY-ESO-1-specific $CD8^+$ T cell responses in three additional patients (two in cohort 2, and one in cohort 3) (Fig. 4A, Table 1). Thus, 5/11 (45%) evaluable patients had antigen-specific $CD8^+$ T cell responses following therapy (Fig. 4A, Table 1). Pre-existing NY-ESO-1-specific $CD4^+$ T cell responses were more widespread, and were detected in 7/10 (70%) evaluable patients (Figure 4A, Table 1). These responses persisted during therapy and, in addition, there was *de novo* induction of $CD4^+$ T cells in two patients (Pts. # 09, 10) (Fig. 4A, Table 1). In a subset of applicable patients for whom pre-validated HLA tetramers were available, the frequencies of $CD8^+$ T cells were further analyzed using HLA tetramers for HLA-A2/NY-ESO-$1_{157–165}$, HLA-B35/NY-ESO-$1_{94–102}$, HLA-B35/NY-ESO-$1_{94–104}$ and HLA-Cw3/NY-ESO-$1_{92–100}$. As shown in Fig. 4B, EOC patient #03 (baseline seropositive) and #06 (baseline seronegative) demonstrated expansion of NY-ESO-1-specific $CD8^+$ T cells by day 57, although the magnitude of the NY-ESO-1-specific cells had waned 6 months following therapy.

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. Fig. 5A illustrates the responses from patient #03 and #06 pre- and post-therapy, and at the time of follow-up (i.e.

six 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 utilized 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. Whereas treatment-induced polyclonal $CD8^+$ T cells recognized partially HLA-matched NY-ESO-1^{+ve} melanoma cell lines (MZ-MEL-9, SK-MEL-95, SK-MEL-139, SK-MEL-52), the SK-MEL-29 ($B35^{-ve}ESO^{-ve}$) line was not recognized (Fig. S2). To characterize $CD4^+$ T cells, NY-ESO-1-specific $CD4^+$ T cells were enriched based on CD154 expression after re-stimulation with NY-ESO-1 peptide, and tested for Th1/Th2 polarization and protein recognition, as described (38). Th1 and Th2 differentiation were determined based on IFN-γ and IL-4 production upon stimulation with NY-ESO-1 pooled peptides. As shown in 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 ten patients were evaluable for clinical response using immune-related response criteria (modified RECIST) (39) (Table 2). Notably, five patients had stable disease (50%) and one patient had a partial response/disease remission (10%). The median duration of stable disease was 6.3 months (range: 3.9 to 7.8 months), and the partial response duration lasted 5.8 months. The clinical response in the three different decitabine cohorts appeared distinct (Table 2). In the first cohort (45 mg/m^2), 2/2 evaluable patients showed stable disease or partial response/remission. Similarly, in the third cohort (10mg/m^2 × d5), 2/2 evaluable patients showed stable disease. However, in the second cohort and the highest dose level (90 mg/m^2), only 2/6 patients (33%) showed stable disease. Finally, it was notable that only 1/5 patients showing stable disease or partial response expressed NY-ESO-1 pre-treatment (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 CT 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 doxorubicin, although this is not a requisite part of the strategy. In addition to NY-ESO-1, other epigenetically regulated CT antigens for which vaccines are available could be targeted using this approach. Because CT 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 CT antigen vaccines (14). In addition to EOC, other cancers, e.g. melanoma and lung, are candidates for this approach, as CT antigens are epigenetically regulated in most human tumors, and CT 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). Additionally, 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 EOC patients 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*.

Due to an unknown safety profile, we tested the regimen in patients with recurrent EOC, as an add-on to second-line doxorubicin chemotherapy. To determine proper sequencing of decitabine and doxorubicin, we conducted treatment studies on EOC cell lines, and found that decitabine treatment must precede doxorubicin in order to hypomethylate and activate *NY-ESO-1*. This 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 long-lasting.

Two patients enrolled in the trial, at the 10 mg/m^2 × 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. Due to logistical constraints, we were unable to obtain tumor tissues post-therapy to study DNA methylation or NY-ESO-1 expression. However, we observed robust hypomethylation in circulating DNA obtained from serum and plasma post-therapy. Because circulating DNAs in cancer patients are 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 are hypermethylated in platinum-resistant EOC (9).

NY-ESO-1-specific antibodies were generated in two-thirds (67%) of the baseline seronegative patients. Remarkably, there was also *de novo* induction of humoral immune responses against the unrelated antigens MAGEA4, CT45, CT47, CT55, and SOX2, indicative of antigen-spreading. Similar evidence of antigen-spreading was not observed in our previous NY-ESO-1 vaccine trials in EOC patients (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 His6-tag in the vaccine preparation. We ruled this out in the current study, as no antibody responses were observed against the ubiquitous control protein (DHFR). There are several potential mechanisms by which antigen-spreading may have been facilitated with the current regimen. The first is up-regulation of additional TAs 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 TAs (42). Finally, the destruction of tumor cells by NY-ESO-1-specific immunity may release additional TAs, leading to the generation of multiple heteroclitic serological responses (43).

Humoral immune responses post-therapy 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 (44–46). Consistent with previous studies (44), we found that the specificity of the CD8$^+$ T cell responses were 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 CD4$^+$ 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 towards 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 TA-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 (PFS), 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 immunological 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 ovarian cancer patients 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 TAs. In addition, we observed immunologically and clinically beneficial responses regardless of the pre-treatment 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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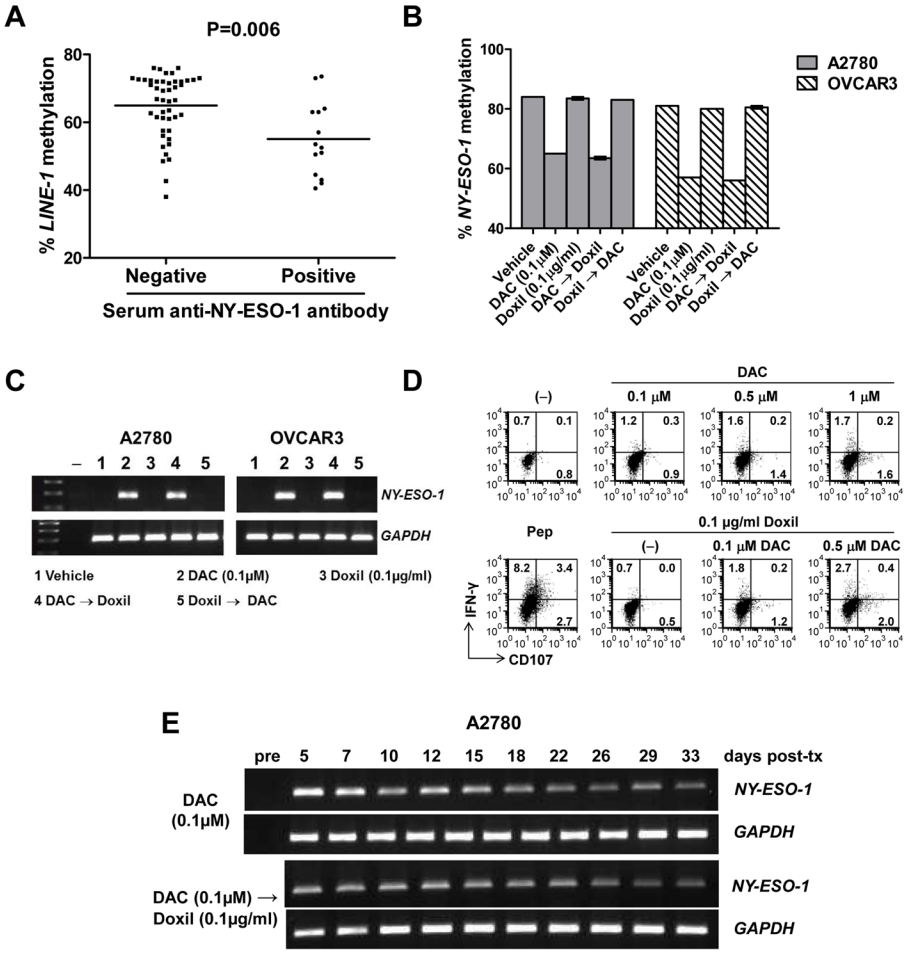

Figure 1. Preclinical studies supporting the use of decitabine to modulate NY-ESO-1 vaccine efficacy

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 >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 PBS (control), 0.1μM decitabine for 48 hours, 0.1μg/ml doxil for 24 hours, or both drugs, in either sequence. gDNA was harvested 72 hours after the initial drug treatment, and used to determine *NY-ESO-1* promoter methylation by pyrosequencing. **C,** Effect of decitabine (DAC) and doxorubicin (doxil) treatment on *NY-ESO-1* mRNA expression in EOC cells. EOC cells were treated as described in (**B**), and RNA was harvested and used to measure *NY-ESO-1* expression by RT-PCR. *GAPDH* was amplified as a positive control. **D,** NY-ESO-1-specific $CD8^+$ T cell response following decitabine and/or doxorubicin (doxil) treatment. OVCAR3 ($HLA\text{-}A2^{+ve}NY\text{-}ESO\text{-}1^{-ve}$) cells were treated with the indicated concentrations of decitabine (DAC), doxil, or decitabine followed by doxil, 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-$1_{157–165}$ $tetramer^+$ $CD8^+$ T cells was determined by flow cytometry. As a positive control, $CD8^+$ T cell responses were tested against NY-ESO-$1_{157–165}$ peptide (0.4μg/ml)-pulsed OVCAR3 cells. Values in quadrants indicate percentages of cells. A value of ≥ 1% was considered positive. **E,** *NY-ESO-1*

expression following drug treatment and removal. A2780 cells were treated with the indicated concentrations of DAC or DAC + Doxil, and cells were extracted at the indicated time points following drug removal and used to determine *NY-ESO-1* expression by RT-PCR.

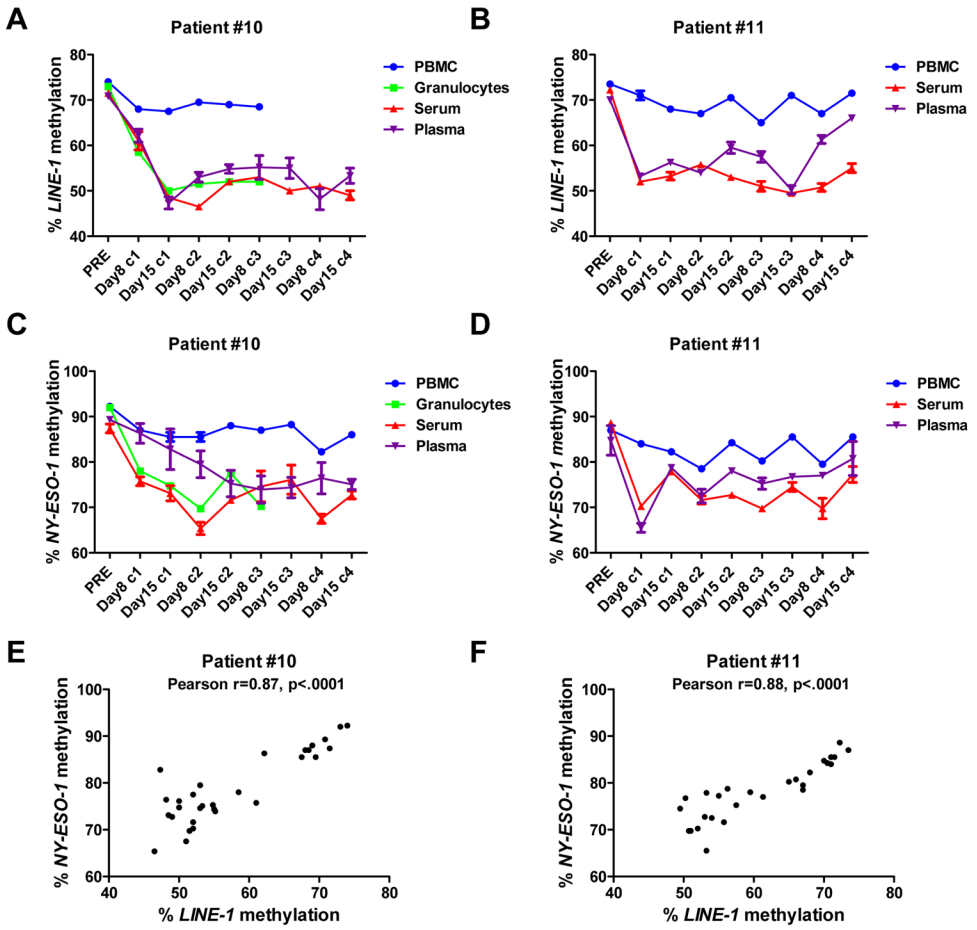

Figure 2. ***In vivo*** **DNA methylation pharmacodynamics**

Two patients in the 10mg/m^2 × d5 decitabine cohort were used to assess DNA methylation pharmacodynamics during therapy. Whole blood was collected pre-treatment and at day 8 and 15 of each of the first four cycles of therapy. PBMCs, granulocytes, serum, and plasma samples were prepared and used for gDNA isolation. *LINE-1* and *NY-ESO-1* promoter-methylation were determined by pyrosequencing. **A–B,** *LINE-1* methylation in samples from patients #10 and #11. **C–D,** *NY-ESO-1* methylation in samples from patients #10 and #11. **E–F,** Association between *LINE-1* methylation and *NY-ESO-1* methylation in samples from patients #10 and #11. C1–C4 refers to the therapy cycle number. Error bars indicated +/− 1SD. Pearson correlation statistics are shown in panels **E–F**.

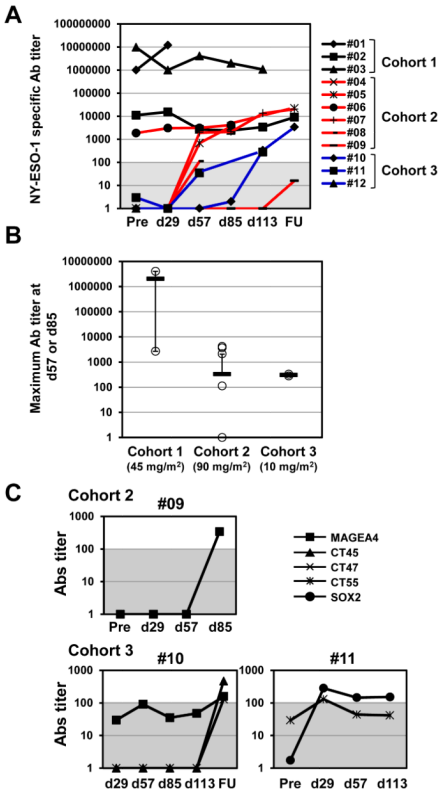

Figure 3. Humoral responses to NY-ESO-1 and other tumor antigens

A, NY-ESO-1 antibody titers in serum were measured by ELISA. EOC patients received decitabine at 45 mg/m^2 × d1 (Cohort 1), 90mg/m^2 × d1 (Cohort 2), or 10mg/m^2 × d5 (Cohort 3), followed by doxorubicin on day 8, and vaccination on day 15, for each 28 day cycle. Patients #01, #02, #03 and #06 were baseline seropositive for NY-ESO-1 antibody. **B,** Comparison of maximal NY-ESO-1 antibody titers in each patient cohort at days 57 or 85. Bars indicate median ± s.d. **C,** Serum reactivity against a panel of 22 unrelated recombinant proteins was tested for recognition by ELISA. A positive response was considered > 100 reciprocal titer.

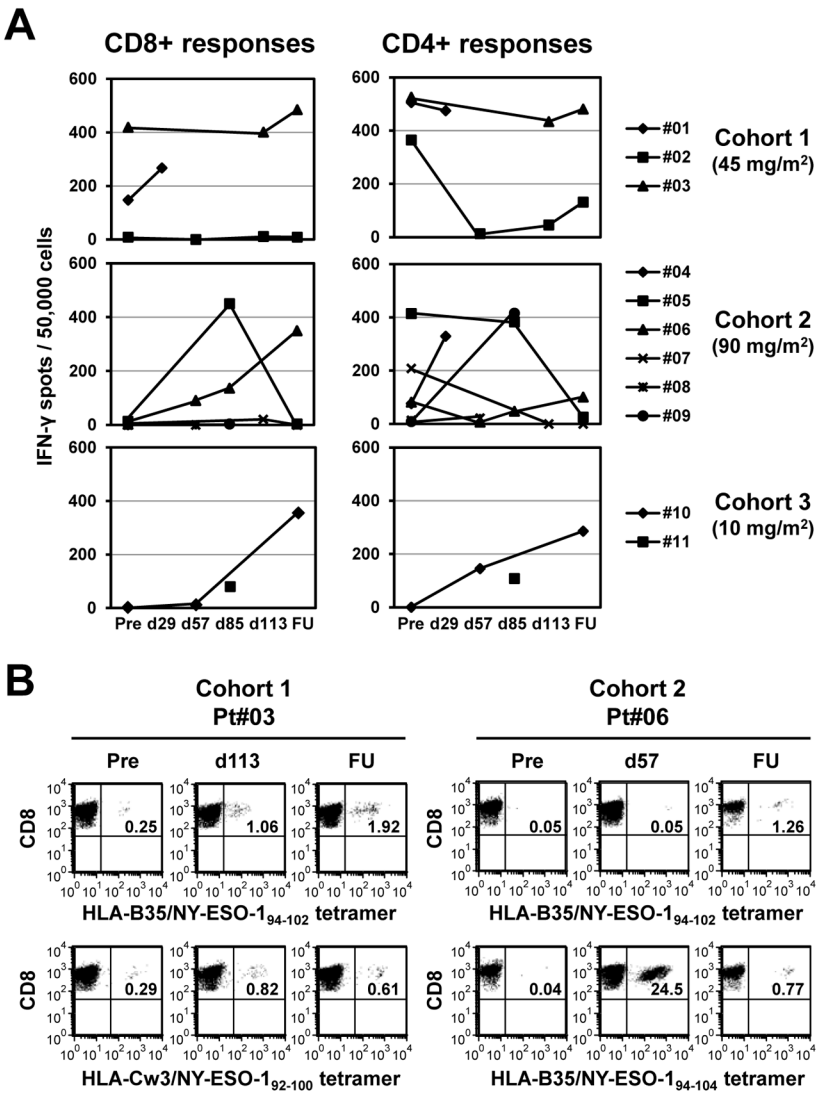

Figure 4. T cell responses to NY-ESO-1

$CD8^+$ and $CD4^+$ T cells isolated from PBMCs were independently stimulated with $CD8^-CD4^-$ cells pulsed with NY-ESO-1 pooled peptides and cultured for 14–15 days. **A,** IFN-γ production from $CD8^+$ or $CD4^+$ T cells was determined by ELISPOT assay against NY-ESO-1 pooled peptide-pulsed PHA-blasted $CD4^+$ T cells. The number of IFN-γ spots indicated reflects subtraction of the number of spot against peptide-unpulsed target. **B,** NY-ESO-1 specific $CD8^+$ T cell frequency was assessed by flow cytometry following staining with the indicated tetramers.

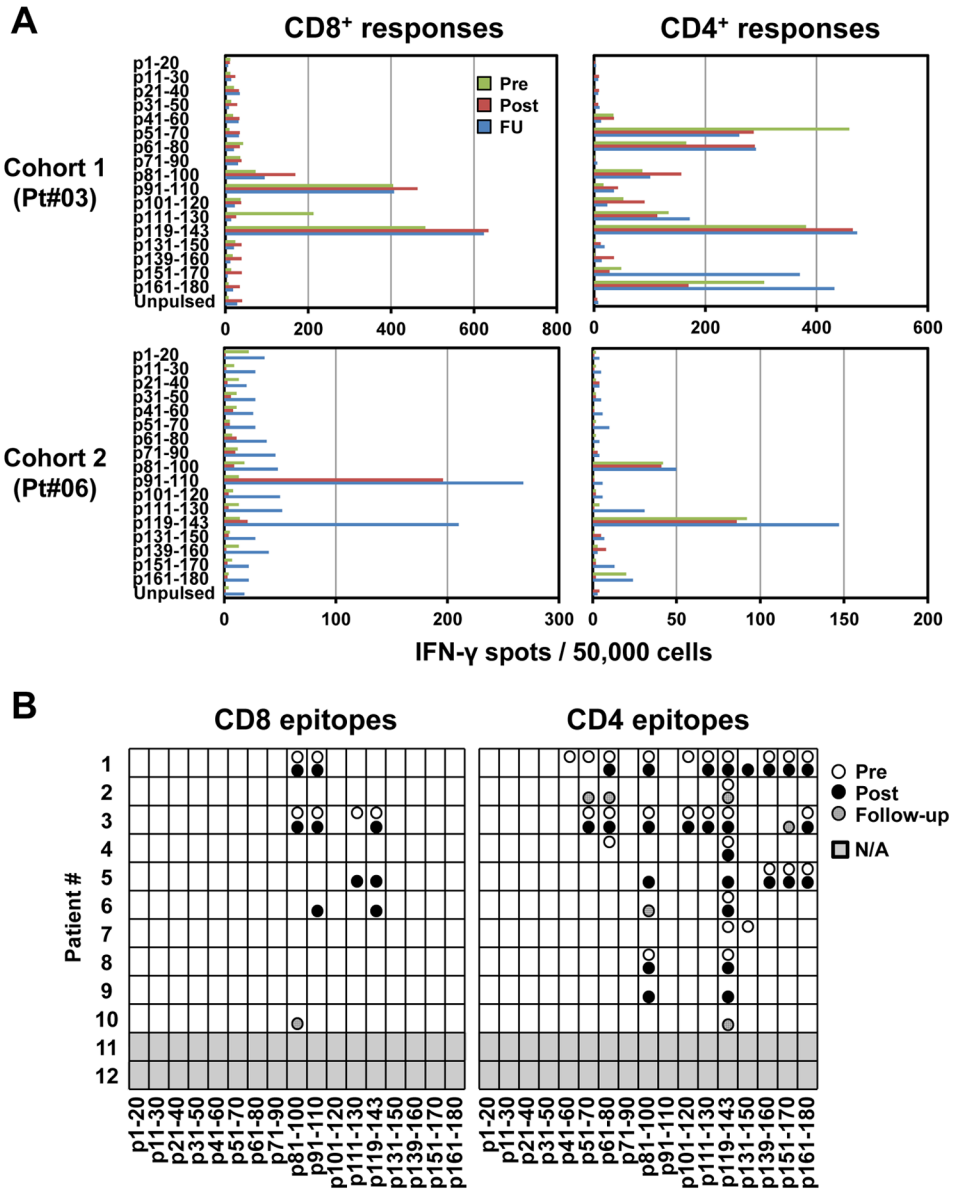

Figure 5. Epitopes of CD4$^+$ and CD8$^+$ T cells pre- and post-therapy

NY-ESO-1-specific CD8$^+$ and CD4$^+$ T cell responses against 20-mer peptides were tested by ELISPOT assays following *in vitro* stimulation. **A,** Responses of CD8$^+$ and CD4$^+$ T cells pre-therapy, post-therapy, and at the time of follow-up (FU; 6 months after the last treatment), from patients #03 and #06. For patient #03, the post-treatment time point shown for both CD8+ and CD4+ is day 113. For patient #06, the post-treatment time points shown are d57 and d85 for CD8+ and CD4+, respectively. **B,** Summary of NY-ESO-1 epitopes recognized by CD8$^+$ or CD4$^+$ T cells. A positive response was defined as a spot count > 50 out of 50,000 cells, and at least > 3× spot count of unpulsed targets.

Table 1

Summary of NY-ESO-1 specific immune responses

Patient Number	Pre-treatment NY-ESO-1 expression		Decitabine Dose	# of therapy cycles	Antibody			$CD8^+$ T cell			$CD4^+$ T cell		
	RT-PCR	IHC			Pre	Post	Follow-up	Pre	Post	Follow-up	Pre	Post	Follow-up
01	+	+	45 mg/m^2 × d1	2	++	+++	N/A	+	+	N/A	+	+	N/A
02	N/A	N/A	“”	4	++	++	+	−	−	−	+	−	+
03	+	+	“”	4	+++	+++	+	+	+	+	+	+	+
04	N/A	+	90 mg/m^2 × d1	2	−	N/A	N/A	−	−	N/A	+	+	N/A
05	−	−	“”	3	−	+	++	−	+	−	+	+	−
06	+	+	“”	3	+	+	+	−	+	+	+	+	+
07	−	−	“”	4	−	++	++	−	−	−	+	−	−
08	−	−	“”	2	−	+	N/A	−	−	N/A	−	−	N/A
09	−	−	“”	3	−	−	−	−	−	N/A	−	+	N/A
10	N/A	−	10 mg/m^2 × d5	4	−	+	+	−	−	+	−	+	+
11	−	−	“”	4	−	+	N/A	N/A	−	N/A	N/A	−	N/A
12	−	−	“”	1	−	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Antibody reciprocal titer key:

+: 100–10,000

++: 10,000–1,000,000

+++: > 1,000,000

N/A: sample not available.

Table 2

Clinical Responses

Patient Number	Decitabine Dose	Clincial Response[1]
01	45 mg/m^2 × d1	N/E[3]
02	“ ”	SD
03	“ ”	PR
04	90 mg/m^2 × d1	PD
05	“ ”	PD
06	“ ”	PD
07	“ ”	PD
08	“ ”	SD
09	“ “	SD
10	10mg/m^2 × d5	SD
11	“ “	SD
12	“ “	N/E[4]

N/E = Not Evaluable, safety assessment only

SD = Stable Disease; PR = Partial Response/Remission; PD = Progressive Disease

[1]The median duration of SD responses was 6.3 months (range = 3.9 to 7.8), and the PR duration was 5.8 months.

[3]Left study early; no post-treatment CT scan

[4]Left study early; contra-indicated corticosteroid use