Safety and Immunogenicity Study of NY-ESO-1b Peptide and Montanide ISA-51 Vaccination of Patients with Epithelial Ovarian Cancer in High-Risk First Remission

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Abstract

Purpose: The cancer-testis antigen NY-ESO-1 is expressed by >40% of advanced epithelial ovarian cancers and is a promising immunotherapeutic target. In this study, we describe the effects of vaccination with the HLA-A*0201 – restricted NY-ESO-1b peptide on patients with epithelial ovarian cancer in high-risk first remission.

Experimental Design: After primary surgery and chemotherapy, high-risk epithelial ovarian cancer patients in first clinical remission received NY-ESO-1b peptide and Montanide every 3 weeks for five vaccinations. Tumor expression was evaluated by immunohistochemistry. Toxicity was monitored using National Cancer Institute Common Toxicity Criteria Scale Version 2. NY-ESO-1 specific humoral immunity (ELISA), T-cell immunity (tetramer and ELISPOT), and delayed-type hypersensitivity were assessed on weeks 0, 1, 4, 7, 10, 13, and 16.

Results: Treatment-related adverse events included grade 1 fatigue, anemia, pruritus, myalgias, and hyperthyroidism and grade 2 hypothyroidism. There were no grade 3/grade 4 adverse events. Three of four patients (75%) with NY-ESO-1 – positive tumor showed T-cell immunity by tetramer (0.6-9.5%) and ELISPOT (range, 35-260 spots). Four of five patients (80%) with NY-ESO-1 – negative tumor showed T-cell immunity by tetramer (1.0-12.1%) and/or ELISPOT (range, 35-400 spots). With a median follow-up of 11.3 months, six of nine patients (67%) have recurred, with a median progression-free survival of 13 months (95% confidence interval, 11.2 months – not reached). Three of nine patients remain in complete clinical remission at 25, 38, and 52 months.

Conclusion: Vaccination of high-risk HLA-A*0201 – positive epithelial ovarian cancer patients with NY-ESO-1b and Montanide has minimal toxicity and induces specific T-cell immunity in patients with both NY-ESO-1 – positive and NY-ESO-1 – negative tumors. Additional study is warranted.

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Epithelial ovarian cancer is the leading cause of death from gynecologic malignancies in the United States. More than 25,000 cases are diagnosed annually, and there are an estimated 14,000 deaths/y due to this disease (1). Greater than 70% of ovarian cancer patients have advanced-stage disease at the time of diagnosis (2). Although a complete clinical remission (cCR) to initial chemotherapy can be anticipated for most patients, strategies to decrease recurrence are needed for >80% of patients with advanced ovarian cancer that will recur. Despite improvements in surgery and chemotherapy, the long-term survival rate remains at ~20% to 30% for advanced ovarian cancer (2–4). Suboptimal initial tumor debulking surgery, failure to normalize CA-125 after three cycles of chemotherapy, or positive second-look surgery are considered high-risk indications of recurrence due to micrometastatic disease despite apparent cCR; these "high-risk" patients have a short remission duration of 10 to 12 months and a recurrence rate of >70% (5–7). Given the minimal disease burden in the setting of cCR, these high-risk patients are ideal candidates for evaluating immune-modulating strategies.

The presence of immune cells within ovarian epithelial tumors has been correlated with improved clinical outcome; the presence of tumor-infiltrating lymphocytes within primary tumor is associated with improvement in both progression-free survival (PFS) and overall survival in patients with advanced ovarian cancer (8, 9). However, the presence of an immuno-suppressive milieu caused by regulatory T cells in ovarian cancer may confer a worse prognosis. High expression of FoxP3, a surrogate marker for quantitative tissue regulatory T content, has been associated with poor prognosis in terms of both PFS and overall survival in ovarian cancer patients (10). An inverse correlation between regulatory T-cell content and patient survival has also been shown, suggesting that regulatory T cells in ovarian cancer, which express intracellular CTLA-4, GITR, and FOXP3, may inhibit tumor-associated antigen–specific immunity *in vitro* and *in vivo* and contribute to tumor growth (11). Immune strategies are required to induce immune activation in patients with epithelial ovarian cancer while decreasing immune-suppressed states caused by regulatory T cells.

In recent years, ovarian cancer has been targeted by a variety of novel immune-based approaches, such as antibody therapy [e.g., oregovomab, a monoclonal antibody (mAb) therapy targeting the CA-125 antigen (12); ACA-125, an antiidiotypic antibody vaccine (13, 14); trastuzumab, a monoclonal humanized anti-HER2 antibody (15); and bevacizumab, an mAb targeting vascular endothelial growth factor-A (16)], cytokine therapy [e.g., IFN-γ (17, 18) and interleukin 2 (19)], and active immunization with proteins, such as Lewis-Y (20), MUC1 (21), heptavalent antigen-keyhole limpet hemocyanin (22), and, recently, NY-ESO-1 (23, 24). Evidence of potential clinical benefit from immunotherapy in epithelial ovarian cancer is suggested in the studies of oregovomab (12, 25, 26) and anti-CTLA-4 (27).

An ideal candidate antigen for immunotherapy of any cancer type should show both inherent immunogenicity and differential expression, with high frequency of expression in the cancer tissues and restricted expression in normal tissues. The cancer-testis antigens are highly expressed in subsets of a variety of cancers and are restricted in normal tissue expression to testis. Cancer-testis antigens currently consist of 90 family members, including NY-ESO-1 (28, 29). The full-length NY-ESO-1 cDNA was cloned, encoding a protein of 180 amino acids (30); the gene encoding NY-ESO-1 has been mapped to chromosome Xq28. A gene, LAGE-1, encoding an antigen closely related to NY-ESO-1 has also been mapped to chromosome Xq28 (31).

Although initially identified on the basis of serologic recognition, the cancer-testis antigen NY-ESO-1 is also recognized by autologous CTLs. NY-ESO-1–derived epitopes recognized in the context of MHC class I and MHC class II alleles have been identified, and specific circulating antibodies to NY-ESO-1 have been detected in ∼40% of patients with tumors expressing NY-ESO-1 (32–34). Antibodies and cellular immune responses against NY-ESO-1 have now been observed in multiple cancer patients who express the antigen on their tumors (35). Studies have also revealed that ovarian tumor expression of NY-ESO-1 is increased in frequency with advancing clinical stage (36).

The NY-ESO-1b peptide has the sequence SLLMWITQC (position 157-165). NY-ESO-1 peptides and proteins have been given to patients on previous protocols. To date, more than 200 patients have received vaccination with NY-ESO-1 either as a peptide or as full-recombinant NY-ESO-1 protein with various adjuvants (37, 38). These clinical data show an extensive safety profile for vaccination with NY-ESO-1, with related toxicities primarily limited to minor discomfort, erythema, and swelling at the injection site, and grades 1 and 2 influenza-like symptoms and arthralgias. No patient has developed grade 3 or grade 4 toxicity either in the acute setting or with long-term follow-up. Vaccination with these agents has shown augmentation of immune response in multiple clinical settings.

In this paper, we summarize the results of a phase 1 study investigating the safety and immunogenicity of NY-ESO-1b peptide and Montanide ISA-51 vaccination of patients with epithelial ovarian cancer in high-risk first remission. We also report clinical follow-up descriptively. This study is novel in that it is the first evaluation of vaccination with NY-ESO-1b peptide and Montanide ISA-51 in high-risk epithelial ovarian cancer patients in the adjuvant setting. We report safety data for the entire cohort, as well as extensive immunologic evaluations of the vaccine.

Materials and Methods

Study design. This was a phase 1 clinical study that was approved and monitored by the Memorial Sloan-Kettering Cancer Center Institutional Review Board. The study was designed to enroll nine patients; nine patients were deemed sufficient to assess the primary and secondary end points of toxicity and immunogenicity. The HLA-A*0201–specific NY-ESO-1b peptide SLLMWITQC (position 157-165; 100 μg) mixed with 0.5 mL of Montanide ISA-51 was given by s.c. injection once every 3 wk for a total of five doses (weeks 1, 4, 7, 10, and 13). After the final injection of peptide, there was a 3-wk follow-up period; patients returned for a final visit and immunologic monitoring at week 16. Thereafter, patients returned for regular follow-up with their treating physicians. Patients in clinical remission were contacted every 6 mo to ascertain disease status.

Patient selection. Patients with high-risk epithelial ovarian cancer (defined by suboptimal initial debulking surgery, failure to normalize CA-125 after three cycles of chemotherapy, or positive second-look surgery) in cCR, as documented by CT scan and CA-125 after completion of primary surgery and chemotherapy, were eligible for HLA testing. Eligible patients for HLA testing were required to have a Karnofsky performance status of ≥60 and an expected survival of ≥6 mo and to be willing and able to give informed consent. Laboratory requirements included hemoglobin at ≥9 g/dL, WBC count at ≥3,000/mm³, absolute neutrophil count at ≥1,500/mm³, platelets at ≥100,000/mm³, serum bilirubin at ≤1.5 times the institutional upper limit of normal, aspartate aminotransferase and alanine aminotransferase at ≤2.5 times the upper limit of normal, and serum creatinine at ≤1.5 times the upper limit of normal (or a creatinine clearance at >60 mL/min). Patients with autoimmune disease, prior or ongoing treatment with immunosuppressive therapy, other known malignancy within the past 3 y, New York Heart Association class III or class IV heart disease, or ongoing infection requiring prolonged antibiotic or hospitalization were ineligible. Standard HLA testing was done on patients who fulfilled the above criteria. Because this was an HLA-restricted peptide vaccine, eligibility was limited to patients who were homozygous or heterozygous to this HLA-A*0201 haplotype.

The study had a two-step consent process. Patients initially signed consent for HLA testing and testing of their primary tumor for NY-ESO-1 expression. A second consent was then used for HLA-A*0201 eligible patients for treatment with the vaccine. Initially, the vaccine was also restricted to patients who were positive for primary tumor

expression of NY-ESO-1; however, the study was amended after 3 mo to include both patients negative and positive for primary tumor expression of NY-ESO-1 in light of the data that tumor cell expression of NY-ESO-1 increases with advanced clinical stage. Dynamic changes in the antigenic content of tumors, such as the loss of expression of NY-ESO-1 after treatment, have also been observed.

Immunohistochemistry to determine ovarian tumor NY-ESO-1 tissue expression. Five-micrometer paraffin sections were applied to slides for immunohistochemistry (Superfrost Plus, Menzel). Slides were heated at 60°C for 2 h, deparaffinized, and rehydrated in xylene and graded alcohols. Antigen retrieval was done by heating slides in a vegetable steamer (Black and Decker) for 30 min in DAKO hipH retrieval solution (DAKOCytomation). Unspecific antibody binding was blocked with 5% bovine serum albumin/PBS solution for 30 min at room temperature, and primary anti-NY-ESO-1 antibody E978 was applied overnight at 4°C. As a secondary reagent, the Powervision kit (Immunovision Technologies) was used. Endogenous peroxidase was blocked with a 1% H_2O_2 solution for 20 min. Diaminobenzidine tetrahydrocholoride (Biogenex) served as a chromogen, and counter-stains were done with Gill's hematoxylin (39).

Clinical monitoring. All patients enrolled in the study were confirmed to be in cCR at the initiation of therapy, as documented by CA-125 at <35 units/mL, physical examination, and CT scan without evidence of disease. Patients were assessed for disease progression by CA-125 during the study at weeks 7 and 16. CT scan was done after completion of the study (week 16). Complete blood count and full chemistry panel were done at enrollment before initiation of therapy (week 0) and at weeks 1, 4, 7, 10, 13, and 16. After completion of the study, patients returned to the care of their primary treating oncologist, and monitoring was at the discretion of the treating physician.

Toxicity assessment. Safety and tolerability end points were determined throughout the duration of the study (weeks 1-16) by using the National Cancer Institute Common Toxicity Criteria Scale Version 2. Patients were observed for toxicity for 0.5 h immediately after each vaccination and were assessed for toxicity before each scheduled vaccination after week 1 (weeks 4, 7, 10, and 13). Patients returned for a final toxicity check 3 wk after their final vaccination (week 16).

Clinical outcome. Although clinical outcome was not an end point of our phase 1 study, patients were evaluated for disease progression at the time of study completion (week 16) and subsequently every 6 to 12 wk until disease progression as a matter of routine follow-up to the treating physician. Time to progression was calculated from the date of completion of all initial chemotherapy to the date of documented relapse and was estimated using the Kaplan-Meier method.

Immune monitoring (ELISA). Following the methods outlined by Stockert et al. (34), patient plasma samples were analyzed by ELISA for seroreactivity to recombinant proteins NY-ESO-1, LAGE-1, MAGE-1, MAGE-3, and p53. Plasma was serially diluted from 1:100 to 1:100,000 and added to low-volume 96-well plates (Corning) coated with 1 µg/mL antigen and blocked with PBS containing 5% nonfat milk. After incubation, plates were washed with PBS containing 0.2% Tween and rinsed with PBS. Plasma IgG (total or subclasses) bound to antigens was detected with alkaline-phosphatase conjugated–specific mAbs (Southern Biotech). After addition of ATTOPHOS substrate (Fisher Scientific), absorbance was measured using a fluorescence reader Cytofluor Series 4000 (PerSeptive Biosystems). A reciprocal titer was calculated for each plasma sample as the maximal dilution still significantly reacting to a specific antigen. Specificity was determined by comparing seroreactivity among various antigens tested. In each assay, sera of patients with known presence or absence of specific reactivity were used as controls. A positive result was defined as extrapolated reciprocal titers of >100.

Peptides and viral vectors. Synthetic NY-ESO-1 peptides 157 to 165 (SLLMWITQC), modified 157 to 165A (SLLMWITQA) and 145 to 174 (LQLSISSCLQQLSLLMWITQCFLPVFLAQP) or control peptide influenza matrix 58 to 66, were obtained from Multiple Peptide Systems. Adenovirus recombinant for full-length NY-ESO-1 (adeno-NY-ESO-1) were previously described (40).

In vitro sensitization with peptides or adenoviral constructs (23, 40). CD8+ T lymphocytes were separated from peripheral blood lymphocytes of cancer patients by antibody-coated magnetic beads (Dynabeads; Dynal) and seeded into round-bottomed 96-well plates (Corning) at a concentration of 5×10^5 cells per well in RPMI 1640 supplemented with 10% human antibody serum (NABI), L-glutamine (2 mmol/L), penicillin (100 units/mL), streptomycin (100 µg/mL), and 1% nonessential amino acids. As antigen-presenting cells, peripheral blood lymphocytes depleted of CD8+ and CD4+ T cells were either pulsed with 10 µmol/L peptide or infected with adenovirus recombinant for full-length NY-ESO-1 at 1,000 IU/cell, overnight at 37°C in 250 µL serum-free medium (X-VIVO-15, BioWhittaker). Pulsed or infected antigen-presenting cells were then washed, irradiated, and added to the plates containing CD8+ T cells at a concentration of 1×10^6 antigen-presenting cells per well. After 8 h, interleukin 2 (10 units/mL; Roche Molecular Biochemicals) and interleukin 7 (20 ng/mL; R&D Systems) were added to culture wells, and this step was repeated every 3 to 4 d until the cells were harvested for testing.

Tetramer and phenotype assay (23). HLA-A2 tetrameric complexes were assembled with NY-ESO-1b–derived peptides NY-ESO-1b (157-165), modified NY-ESO-1b-A (157-165A), or control peptide influenza matrix 58 to 66. Tetramer assays were conducted systematically in all patients after a single presensitization at culture days 7, 10, and 14. Presensitized CD8+ T cells in 50 µL PBS containing 3% FCS (Summit Biotechnology) were stained with PE-labeled tetramer for 15 min at 37°C before addition of PerCP-labeled anti-CD8 mAb (BD Biosciences), FITC-labeled anti-CCR7 mAb (Caltag), and allo-phycocyanin–labeled anti-CD45RA (Caltag) for 15 min on ice. After washing, results were analyzed by flow cytometry (FACSCalibur, Becton Dickinson). A positive result was defined as a percentage of NY-ESO-1b tetramer–positive CD8+ cells reproducibly at >0.1% and appearing clustered in dot plots.

ELISPOT assay (23). ELISPOT assays were conducted systematically in all patients after a single presensitization at culture days 10 and 14 and later. For ELISPOT assays, flat-bottomed, 96-well nitrocellulose plates (MultiScreen-HA, Millipore) were coated with IFN-γ mAb (4 µg/mL; 1-D1K, Mabtech) and incubated overnight at 4°C. After washing with RPMI, plates were blocked with 10% human antibody–type serum for 2 h at 37°C. Presensitized CD8+ T cells (5×10^4 and 1×10^4) and 5×10^4 target T2 cells, pulsed overnight with 10 µmol/L peptides, were added to each well and incubated for 20 h in RPMI 1640 without serum. Plates were then washed thoroughly with water containing 0.05% Tween 20 to remove cells, and IFN-γ mAb (0.2 µg/mL; 7-B6-1-biotin, Mabtech) was added to each well. After incubation for 2 h at 37°C, plates were washed and developed with streptavidin-alkaline phosphatase (1 µg/mL; Roche) for 1 h at room temperature. After washing, substrate (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium, Sigma) was added and incubated for 10 min. After final washes, plate membranes displayed dark violet spots representing IFN-γ secreting CD8+ T cells that were counted with the CTL immunospot analyzer and software (Cellular Technologies). Positive results were defined as the number of NY-ESO-1b–specific spots of >30, and more than thrice the number of spots for the irrelevant control.

Delayed-type hypersensitivity. Delayed-type hypersensitivity (DTH) testing was done at baseline (week 0) and after vaccination at weeks 7 and 16. Ten micrograms of peptide at a concentration of 0.1 mg/mL in 8% DMSO were injected intradermally at a separate site from the vaccination to give a visible and palpable skin depot. The extent and intensity of DTH reaction was documented by measuring visible redness, palpable induration, and other signs of local skin irritation or necrosis. Assessment of DTH reaction was done at 48 h postinjection, and the diameter of the reaction was documented.

Dose-limiting toxicity criteria. For an adverse event to be defined as a dose-limiting toxicity, it was required to be definitely, probably, or possibly related to the administration of the investigational agent. For this study, dose-limiting toxicities were defined as ≥grade 3

autoimmune phenomena, ≥grade 3 hematologic and nonhematologic toxicities, bronchospasm, or generalized urticaria.

Statistical considerations. This was a pilot study to assess the safety of repeated dose of NY-ESO-1b peptide mixed with Montanide ISA-51 in patients with ovarian, primary peritoneal, or fallopian tube cancer expressing NY-ESO-1 or LAGE-1. Planned accrual was nine subjects. Patients who experienced a dose-limiting toxicity would be removed from the study (41).

The population immune response curves, as measured by ELISA, tetramer analysis, and IFN-γ producing T-cell responses were estimated at baseline and at weeks 4, 7, 10, 13, and 16. For each assay, the population response curve was estimated by computing the average of the nine T-cell responses at each time period.

Results

Patient characteristics. The characteristics of the study population are presented in Table 1. The median age of the study population was 54 years (range, 37-66 years). Seven patients (78%) were clinical stage IIIC at initial diagnosis, and two (22%) were stage IV. Eight patients (89%) had papillary serous histology, and one patient (11%) had histology characterized as adenocarcinoma with papillary features, consistent with a Mullerian primary. All nine treated patients were high grade. All patients had at least one of three high-risk features: suboptimal initial debulking surgery, failure to normalize CA-125 after three cycles of chemotherapy, or positive second-look surgery. One patient had two high-risk features, and one patient had three. Five of nine patients (55%) had suboptimal initial cytoreductive surgery, two (22%) had failure to normalize CA-125 after three cycles of chemotherapy, and five (55%) had positive second-look surgery. All patients were in complete clinical first remission as documented by CT scan and CA-125 at the time of protocol enrollment. All patients had tumor tested for NY-ESO-1, but expression of NY-ESO-1 was not a criterion for study inclusion after the first 3 months of the study. Four of nine patients (44%) had tumor positive for NY-ESO-1 expression (Fig. 1).

NY-ESO-1 immunohistochemistry and patient HLA results. Overall, 46 patients signed informed consent and had HLA testing done. Seventeen of 46 patients (37%) were HLA-A*0201 positive. Three of these patients were screened before the amendment of the protocol to include both patients negative and positive for primary tumor expression of NY-ESO-1
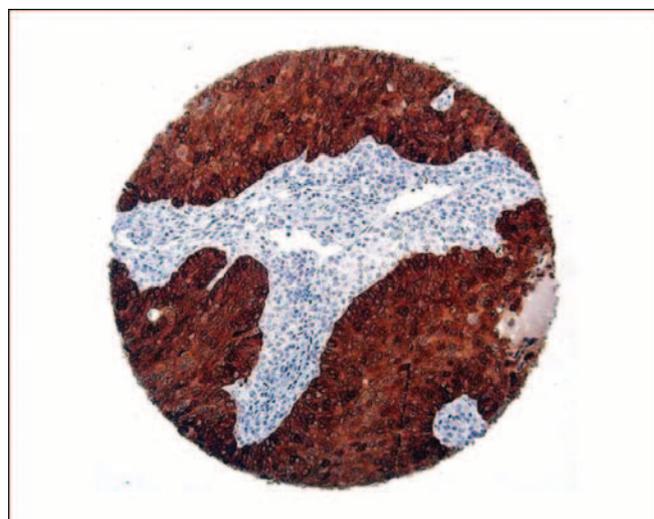

Fig. 1. NY-ESO-1 immunohistochemistry. An ovarian tumor cell staining positive for NY-ESO-1 by immunohistochemistry.

and were excluded on the basis of negative primary tumor expression of NY-ESO-1. The remaining four withdrew consent for participation after HLA testing and never consented for treatment with the vaccine. After informed consent was obtained, 10 of 17 (59%) were enrolled on the protocol. Of these 10, one patient was removed from the study before initiation of therapy for progression of disease shown in her screening CT scan. Nine patients (the target goal) completed the therapy regimen. One patient did not return for immunologic testing on week 16 after completion of vaccination therapy.

Toxicity. The vaccination therapy was well tolerated (Table 2). Treatment-related adverse events considered possibly or likely related to vaccine administration included grade 1 anemia, injection site pruritis, and rash in one of nine patients (11%) each, grade 1 fatigue and myalgias in two of nine patients (22%) each, and grade 2 hypothyroidism in one patient (11%). There were no grade 3/grade 4 adverse events. The time course of toxicity onset ranged between the first and fourth vaccination, with most symptoms occurring before the third vaccination. Fatigue was noted after the first vaccination in two patients, with a duration of ∼2 months; anemia after the second vaccination in one patient, which persisted until the completion of the study; injection site pruritis after the second vaccination in one patient, with a duration of 3 weeks; and injection site rash after the fourth vaccination in one patient, which resolved after 3 days. Myalgias developed in two patients—one after the first

Table 1. Epithelial ovarian cancer patient demographics

Age, y	
Median	54
Range	37-66
Stage, *n* (%)	
IIIC	7 (78%)
IV	2 (22%)
Grade, *n* (%)	
3	9 (100%)
Histology, *n* (%)	
Papillary serous	8 (89%)
Adenocarcinoma NOS	1 (11%)
Tumor expression NY-ESO-1, *n* (%)	
Positive	4 (44%)
Negative	5 (56%)

Table 2. Patient toxicities

Type of toxicity	Grade			
	1	2	3	4
Fatigue	2	0	0	0
Anemia	1	0	0	0
Hypothyroidism	0	1	0	0
Myalgia	2	0	0	0
Pruritis	1	0	0	0
Rash	1	0	0	0

and the other after the second vaccination; both myalgias were self limited and resolved before the next vaccination. One patient developed grade 2 hypothyroidism after five doses of vaccine, likely drug related, and required levothyroxine replacement therapy. Her hypothyroidism has persisted, and she continues to be stable on levothyroxine. No patients experienced treatment interruption or delay secondary to toxicity.

One patient who completed the study without any unusual toxicity was noted in routine follow-up at Memorial Sloan-Kettering Cancer Center to develop subclinical hyperthyroidism by screening TSH at 3 months after completion of vaccination. She was followed-up expectantly, and the hyperthyroidism was resolved without therapeutic intervention.

ELISA results. The ELISA data showing presence of serum antibodies specific for NY-ESO-1 are depicted in Fig. 2 (*left*). Two of nine patients (22%) had preexisting antibodies to NY-ESO-1, as shown by positive ELISA on week 0. The remaining six of nine patients (67%) had no preexisting NY-ESO-1 antibody at baseline and did not show an ELISA response during or after completion of vaccination.

Cellular responses. For cellular assays, CD8$^+$ T cells were analyzed *ex vivo* whenever possible for specificity to NY-ESO-1b by tetramer staining, but no responses could be detected in any patient (not shown). Therefore, CD8$^+$ T cells from all patients were presensitized with peptide NY-ESO-1b for all available time points of vaccination, as well as with adenovirus recombinant for NY-ESO-1, long NY-ESO-1 peptide 145 to 174, or analogue peptide 157 to 165A for multiple selected time points. Results are expressed as the average of at least three independent repeat assays in duplicates from the best presensitization method.

Tetramer results. The tetramer data showing the percentage of CD8$^+$ T cells with specificity for NY-ESO-1b are depicted in Fig. 2 (*center column*). All samples were additionally tested with tetramers containing analogue peptide NY-ESO-1 157-165A, which showed very similar results to the tetramers with wild-type peptide (not shown). Three of four treated patients (75%) with NY-ESO-1–positive tumor showed CD8$^+$ T-cell immunity by positive tetramer (range, 0.6-9.1%; Fig. 2A). Three of five treated patients with NY-ESO-1b–negative tumor showed CD8$^+$ T-cell immunity by positive tetramer (range, 1.0-12.1%; Fig. 2B). The median onset of NY-ESO1b–specific CD8$^+$ T-cell response was 11.5 weeks (range, 7-16 weeks). For all patients with demonstrated tetramer response, this response remained present at the week 16 evaluation (3 weeks after final vaccination). One of the two patients with preexisting NY-ESO-1 seropositivity (as shown by positive ELISA at week 0) had a weak but detectable CD8$^+$ T-cell reactivity to NY-ESO-1b at the initiation of the study, whereas the other one was negative by tetramer before vaccination (at week 0). The latter patient ultimately developed a CD8$^+$ T-cell response to NY-ESO-1b detected by tetramers starting at week 10. Results represent the average of at least three repeat assays in duplicate.

ELISPOT results. The ELISPOT data showing IFN-γ secretion of CD8$^+$ T cells with specificity for NY-ESO-1b is depicted in Fig. 2 (*right column*). Three of four treated patients (75%) with NY-ESO-1–positive tumor showed CD8$^+$ T-cell immunity by positive ELISPOT (range, 35-260 spots; Fig. 2A). Three of five treated patients (60%) with NY-ESO-1–negative tumor showed CD8$^+$ T-cell immunity by ELISPOT (range, 35-400 spots; Fig. 2B). The median duration of onset of ELISPOT

response was 11 weeks (range, 7-16 weeks). All ELISPOT responses generated remained sustained at week 16. For the two of nine patients (22%) with preexisting NY-ESO-1 seropositivity as shown by positive ELISA at week 0, CD8$^+$ T-cell reactivity to NY-ESO-1b at the initiation of the study was unexpectedly negative by ELISPOT before vaccination (at week 0). Both of these patients ultimately developed a CD8$^+$ T-cell response detectable by ELISPOT, one at week 13 and one at week 16. Results represent the average of at least three repeat assays in duplicate.

Phenotype of tetramer-positive cells. Tetramer-stained CD8$^+$ T cells specific for NY-ESO-1b were further analyzed for surface phenotypic markers and were found to be consistently CCR7-negative and CD45RA-negative in all responding patients (Fig. 3). This is characteristic of effector memory populations, although culture conditions in the presence of cytokines may have influenced this phenotype. The *in vitro* stimulation method, either using peptide or adenovirus recombinant for NY-ESO-1, did not seem to result in different phenotypes of effectors. There was no difference in the phenotype and effector function of cultured CD8$^+$ T cells from patients with NY-ESO-1–positive (Fig. 3A) or NY-ESO-1–negative (Fig. 3B) tumors by reverse transcription–PCR, although this phenotype may not necessarily reflect that of the original *in vivo* repertoire due to the extended culture period.

Recognition of naturally processed NY-ESO-1 by vaccine-induced CD8$^+$ T cells. To test the capacity of vaccine-induced T cells to recognize naturally processed NY-ESO-1 antigen, presensitized CD8$^+$ T cells from most patients were tested by ELISPOT against melanoma tumor cell lines known to express NY-ESO-1 and HLA-A2 (SK-MEL-37, NW-MEL-38). We were not able to show specific recognition of these tumor cell lines throughout vaccination (not shown). It is important to note, however, that in all patients responding to the vaccine, as measured by tetramer or ELISPOT, NY-ESO-1b–specific CD8$^+$ T-cell responses were efficiently elicited *in vitro* from full-length naturally processed NY-ESO-1 encoded by recombinant adenovirus, as efficiently as with peptide presensitization (Fig. 3A and B and data not shown). The capacity of cells to expand after adenovirus recombinant for NY-ESO-1 stimulation was independent of serologic status. This suggests that the vaccine with NY-ESO-1b peptide was still able to generate high-avidity T-cell responses recognizing naturally processed NY-ESO-1 antigen, although the exact frequency of such cells remains to be determined.

DTH results. No patients had preexisting DTH response at baseline. At evaluation at 7 weeks and after completion of therapy at 16 weeks, none of the nine patients developed DTH response.

Clinical outcome. Although clinical outcome was not an end point of our phase 1 study, patients were evaluated for disease progression throughout the study by CA-125 and physical examination and at the time of study completion with CT scan. They subsequently returned to routine clinical follow-up and were monitored per clinical standard until disease progression as a matter of routine follow-up by their treating physician. With a median long-term follow-up of 11.3 months, six of nine patients have recurred, with a median PFS of 13 months (95% confidence interval, 11.2 months–not reached), and three of nine patients, all with NY-ESO-1–negative tumors, remain in complete remission at intervals of 25, 38, and 52 months to date. One of three (33%) had an extremely robust immune response with both positive tetramer (12%) and ELISPOT
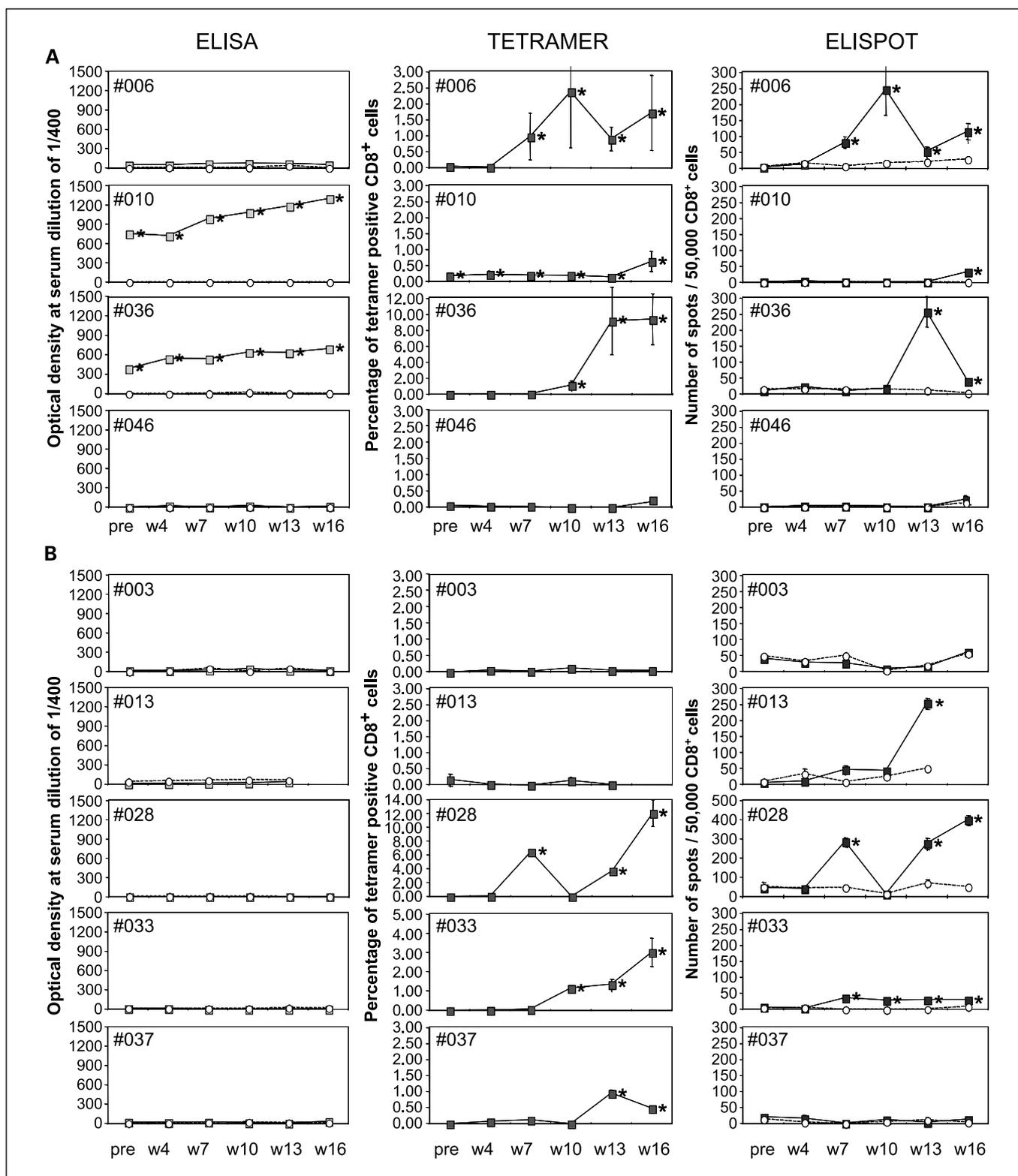
Fig. 2. Each box represents a vaccinated patient for all available time points, with patients with NY-ESO-1 – positive tumors on the top (*A*) and NY-ESO-1 – negative tumors on the bottom (*B*). ELISA (*left column*) antibody responses specific for recombinant NY-ESO-1 protein (*filled squares*) or irrelevant MAGE-3 protein (*open circle*) are shown as absorbance values at serum dilution 1:400 titers. Asterisks represent significant results, defined as extrapolated reciprocal titers of >100. Tetramer (*center column*) CD8^{+} T-cell responses specific for peptide NY-ESO-1b (*filled squares*) are shown as a percentage of CD8^{+} cells after presensitization with either NY-ESO-1 peptide or recombinant adenovirus. Results represent the average of at least three repeat assays; error bars, SD. Asterisks represent significant results, defined as a percentage of NY-ESO-1b-tetramer – positive CD8^{+} cells reproducibly of >0.1% and appearing clustered in dot plots. ELISPOT (*right column*) CD8^{+} T-cell responses specific for peptide NY-ESO-1b (*filled squares*) or irrelevant peptide (*open circle*) are shown as a number of spots representing IFN-γ secretion out of 50,000 CD8^{+} cells after presensitization with either NY-ESO-1 peptide or recombinant adenovirus. Results represent the average of at least three repeat assays in duplicates; error bars, SD. Asterisks represent significant results, defined as a number of NY-ESO-1b-specific spots of >30 and more than thrice the number of spots for the irrelevant control.

A

B
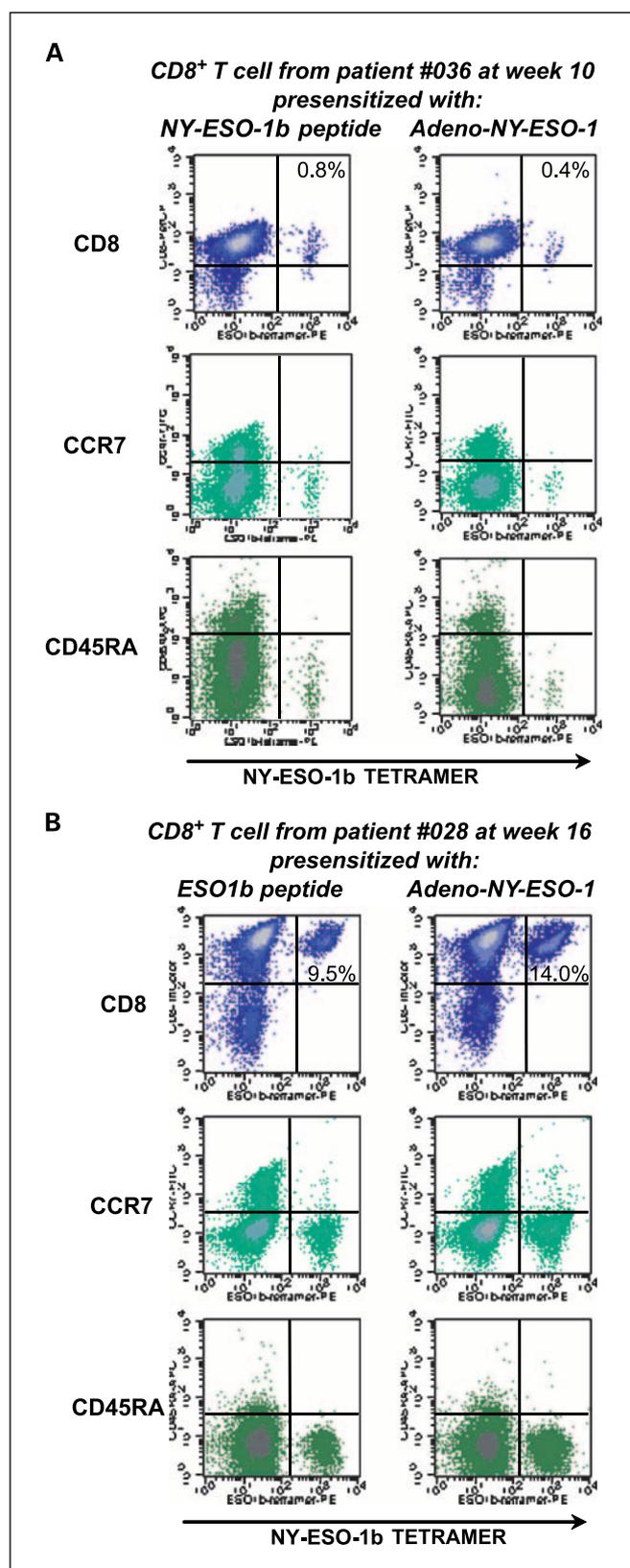
Fig. 3. Phenotype. Representative tetramer staining of CD8+ T cells from NY-ESO-1 tumor – positive patient 036 at week 10 (*A*) or NY-ESO-1b tumor – negative patient 028 at week 16 (*B*) after presensitization with NY-ESO-1b peptide (*left*) or adenovirus recombinant for full-length NY-ESO-1b (*adeno-NY-ESO-1b*; *right*). Cells were costained for CD8 (*top*), CCR7 (*middle*), and CD45RA (*bottom*). Values indicate percentage of tetramer-positive cells.

(400 spots)—the highest seen in our study. The remaining two of three (66%) had negative tetramer (0.10%) for both, but one of two had a strongly positive ELISPOT (250 spots).

Discussion

The cancer-testis antigen NY-ESO-1 shows selective expression; is present at a high frequency in epithelial ovarian cancer (40%); and is immunogenic, generating recognized antibody and cellular responses in subsets of patients (32–36). As such, it represents an attractive target for directed immunotherapy in epithelial ovarian cancer. The purpose of this study was to investigate the safety and immunogenicity of vaccination using the NY-ESO-1b peptide with Montanide immune adjuvant in a subset of high-risk epithelial ovarian cancer patients in first clinical remission. Because this was a single-peptide vaccine, study eligibility was restricted to those patients who were HLA-A*0201 positive.

The vaccine was generally well tolerated, resulting in only grade 1 and grade 2 toxicities, such as fatigue, myalgia, and skin rash. In general, the onset of symptoms was early in the course of vaccination, and the symptoms were self limited and resolved without the need for topical steroid therapy or other medical interventions, except in one patient. One patient developed hypothyroidism after five doses of vaccination but before her final week 16 visit. This patient, who was tumor negative for NY-ESO-1 expression, was also noted to show the highest levels of immunogenicity seen in our study, tetramer (12.1%) and ELISPOT (400 spots), with immunogenicity confirmed positive beginning at week 7 and persisting throughout weeks 13 to 16. This patient remains in cCR at >38 months after completion of chemotherapy. Her hypothyroidism was easily controlled with levothyroxine. Given this apparently related autoimmunity, it was notable that a second patient developed thyroid symptoms (hyperthyroidism) within 4 months of completing vaccination; however, this patient did not show immunogenicity during vaccination. Her hyperthyroidism remained grade 1, detected on screening TSH, and resolved without therapeutic intervention, and her PFS of 11 months is within the standard duration for PFS in high-risk first remission. Thyroid toxicity has been described in association with Montanide given as an adjuvant for a dendritic cell vaccine in conjunction with granulocyte-macrophage colony-stimulating factor and interleukin-2 (42), but is not a well-described toxicity of Montanide. Although there is clear evidence in the literature that the induction of autoimmunity in immunotherapy may be a marker for successful immunologic activation (43) and/or clinical efficacy, there is no clear correlation between the possible induction of autoimmunity with immunogenicity. The finding in one patient who had high immunologic activation showed autoimmunity, and a prolonged clinical remission is certainly intriguing. However, given that NY-ESO-1, a cancer-testis antigen, is not expressed on normal thyroid tissue, it seems likely that the possible autoimmune effect seen was a consequence of the Montanide or of some combination of Montanide and the vaccine rather than the vaccine alone.

In contrast to previous findings (44), the two patients with preexisting antibodies to NY-ESO-1 did not have detectable spontaneous CD8+ T-cell response to peptide NY-ESO-1b by ELISPOT, but both ultimately developed a positive response after vaccination. The remainder of the patients who were

seronegative for NY-ESO-1 did not seroconvert during vaccination. This result was expected with a 9-mer peptide vaccine, which was designed to elicit T-cell responses rather than antibody responses. Stimulation of CD8^{+} response was seen in six of nine patients as shown by positive tetramer and/or ELISPOT. Onset of CD8^{+} T-cell responses to NY-ESO-1b was noted primarily between weeks 7 and 10 (two or three vaccinations), although one patient had preexisting immunity and another showed immune stimulation beginning at week 4 (one vaccination). This suggests that multiple vaccinations were necessary to induce immune responsiveness. Immune response, once stimulated, persisted throughout the duration of the surveillance period. Indeed, specific CD8^{+} T-cell responses at week 16 were generally heightened compared with responses in the previous weeks for both tetramer and ELISPOT, suggesting that immunogenicity increased with repeated exposure to NY-ESO-1b peptide and Montanide and that vaccination on an every 3-week schedule was sufficient to maintain immunogenicity after it was generated (i.e., sufficient for boost). The question remains as to what the optimal schedule for maintenance or boost is; one would expect that with cessation of vaccination, unless CD4^{+} T-cell activation had been induced, immunogenicity would wane; however, an optimal boost schedule after priming may not require vaccinations as frequently as every 3 weeks. The optimal schedule for maintenance of immunogenicity after immunologic priming remains an intriguing question in need of further investigation. Maintenance of immunity may also be augmented by concurrent stimulation of CD4^{+} T cells; future studies should involve more class II epitopes as well (45).

It is important to note that CD8^{+} T-cell responses were also detected in these NY-ESO-1 peptide–vaccinated patients after presensitization using adenovirus encoding full-length NY-ESO-1 (Fig. 3) and that this was observed in all patients responding to the vaccine, regardless of their serologic status for NY-ESO-1. This indicates that the NY-ESO-1 antigen was naturally processed by antigen-presenting cells and allowed the stimulation of specific precursors primed *in vivo* by the vaccine. These results with recombinant adenovirus sensitization argue in favor of a vaccine-induced repertoire capable of recognizing naturally processed NY-ESO-1 antigen. Nevertheless, there is a paradox: whereas adenovirus-NY-ESO-1 may stimulate specific CD8^{+} T cells to grow, the effectors presensitized in this fashion still fail to recognize tumor cells expressing NY-ESO-1 and HLA-A2 on a bulk cell line level. Recently, it has been shown that the presensitization method may influence the capacity of CD8^{+} T-cell effectors against NY-ESO-1b from peptide-vaccinated patients to recognize tumor cells *in vitro* (46). This work shows that a subset of peptide vaccine–induced effectors have the potential to recognize and eliminate tumor cells.

Time to progression was not considered a primary end point of this phase 1 study of nine patients. Yet, we can observe that, with a median follow-up of 11.3 months, at least three patients have displayed long remissions (range, 25-52 months) well beyond the expected range of 10 to 12 months. A fourth patient showed recurrence after a long disease-free interval, with a PFS of 28 months. Of the three patients who remain in cCR, all three had primary tumors that were negative for expression of NY-ESO-1 and were antibody negative. This is too small of a sample from which to derive conclusions, but it raises interesting questions regarding NY-ESO-1 tumor expression, tolerance, and induction of immunogenicity with vaccination. The frequency of ovarian tumor expression of NY-ESO-1 is increased in advanced-stage patients, but we do not know if this expression is dynamic and increases with advancing clinical stage; if so, it is possible that these high-risk patients with primary tumor negative for NY-ESO-1 expression had NY-ESO-1–positive tumor cells present as micrometastases. These cells, in small quantity, may be inadequate to induce tolerance to NY-ESO-1, and vaccination may stimulate immune recognition of NY-ESO-1–positive micrometastatic tumor cells. Conversely, patients with NY-ESO-1–positive tumors with extensive exposure to NY-ESO-1 antigen on their tumor may have become tolerant to the NY-ESO-1 antigen (47). If this was the case, vaccination with a single peptide and Montanide could be insufficient for breaking tolerance in these patients. No correlation between homozygosity and heterozygosity to HLA-A*0201 and immune responsiveness was shown.

This study showed that vaccination with NY-ESO-1b peptide and Montanide was safe, well tolerated, and able to induce immunogenicity in both NY-ESO-1–positive and NY-ESO-1–negative patients. Some autoimmune events were observed. Long remission durations were seen in three NY-ESO-1–negative patients. A limitation of this study included the necessity of HLA restriction to HLA-A*0201–positive patients; thus, restricting it to a small fraction of high-risk epithelial ovarian cancer patients. A second limitation of peptide vaccination is the restriction of immunogenicity to a limited epitope, which may or may not be recognized on the tumor itself and which may or may not be capable of generating a true *in vivo* immune response without other immunologic adjuvants to boost immune stimulation. Future studies that take these questions into account are warranted.

Disclosure of Potential Conflicts of Interest

Acknowledgments

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