Efficacy of vaccination with recombinant vaccinia and fowlpox vectors expressing NY-ESO-1 antigen in ovarian cancer and melanoma patients

Kunle Odunsi[a,b,1], Junko Matsuzaki[a,b,2], Julia Karbach[c,2], Antje Neumann[c], Paulette Mhawech-Fauceglia[d], Austin Miller[e], Amy Beck[a,b], Carl D. Morrison[d], Gerd Ritter[f], Heidi Godoy[a], Shashikant Lele[a], Nefertiti duPont[a], Robert Edwards[g], Protul Shrikant[b], Lloyd J. Old[f], Sacha Gnjatic[f], and Elke Jäger[c,1]

Departments of [a]Gynecologic Oncology, [d]Pathology, and [e]Clinical Biostatistics and [b]Center for Immunotherapy, Roswell Park Cancer Institute, Buffalo, NY 14263; [c]Medizinische Klinik, Hämatologie–Onkologie, Krankenhaus Nordwest, Frankfurt 60488, Germany; [g]Department of Obstetrics, Gynecology, and Reproductive Sciences, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213; and [f]Ludwig Institute for Cancer Research, New York Branch at Memorial Sloan-Kettering Cancer Center, New York, NY 10065

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Recombinant poxviruses (vaccinia and fowlpox) expressing tumor-associated antigens are currently being evaluated in clinical trials as cancer vaccines to induce tumor-specific immune responses that will improve clinical outcome. To test whether a diversified prime and boost regimen targeting NY-ESO-1 will result in clinical benefit, we conducted two parallel phase II clinical trials of recombinant vaccinia-NY-ESO-1 (rV-NY-ESO-1), followed by booster vaccinations with recombinant fowlpox-NY-ESO-1 (rF-NY-ESO-1) in 25 melanoma and 22 epithelial ovarian cancer (EOC) patients with advanced disease who were at high risk for recurrence/progression. Integrated NY-ESO-1-specific antibody and $CD4^+$ and $CD8^+$ T cells were induced in a high proportion of melanoma and EOC patients. In melanoma patients, objective response rate [complete and partial response (CR+PR)] was 14%, mixed response was 5%, and disease stabilization was 52%, amounting to a clinical benefit rate (CBR) of 72% in melanoma patients. The median PFS in the melanoma patients was 9 mo (range, 0–84 mo) and the median OS was 48 mo (range, 3–106 mo). In EOC patients, the median PFS was 21 mo (95% CI, 16–29 mo), and median OS was 48 mo (CI, not estimable). $CD8^+$ T cells derived from vaccinated patients were shown to lyse NY-ESO-1-expressing tumor targets. These data provide preliminary evidence of clinically meaningful benefit for diversified prime and boost recombinant pox-viral-based vaccines in melanoma and ovarian cancer and support further evaluation of this approach in these patient populations.

effector function | T cell epitopes

The ability to induce robust clonal expansion and effector and memory differentiation of antigen-specific T cells is an important goal of cancer vaccines. To achieve this goal, several approaches have been used in clinical cancer vaccine studies. Although promising results on prolongation of overall survival (OS) have been reported in recent cancer vaccine studies (1), the optimal strategy for generating integrated Ab, CD4, and CD8 responses with potential to control tumor growth has yet to be determined. In this regard, the use of recombinant orthopox vectors such as vaccinia or avipox (fowlpox and/or canarypox) has been shown to induce more robust T-cell responses to tumor antigens (TAs) in animal models and humans, compared with the use of the TA protein in adjuvant (2).

Our group has focused on the "cancer-testis" antigen NY-ESO-1, as a prototype tumor antigen for human cancer vaccine studies because of its unique characteristics of tissue restricted expression and inherent immunogenicity (3). We have assessed the immunogenicity of NY-ESO-1-based candidate vaccines in early-phase clinical trials under the sponsorship of the Cancer Vaccine Collaborative (4–6). In a previous pilot study, we tested priming immunization with recombinant vaccinia-NY-ESO-1 (rV-NY-ESO-1), followed by booster vaccinations with recombinant fowlpox-NY-ESO-1 (rF-NY-ESO-1) in patients with various advanced solid tumors (4). We demonstrated that rV-NY-ESO-1 and rF-NY-ESO-1 constructs are safe and induced NY-ESO-1-specific Ab responses and/or specific $CD8^+$ and $CD4^+$ T-cell responses directed against a broad range of NY-ESO-1 epitopes (4). To test the clinical efficacy of rV-NY-ESO-1 and rF-NY-ESO-1 prime boost approach, we conducted two parallel phase II clinical trials in epithelial ovarian cancer (EOC) and melanoma patients at high risk for disease recurrence. Although patients with advanced EOC often achieve complete clinical remission after primary surgery and chemotherapy, the majority ultimately die of recurrent/progressive disease (7). Similarly, patients with stage IV malignant melanoma have a poor prognosis, and those with resected stage III/IV melanoma have a high risk of recurrent/progressive disease (8). Therefore, approaches to minimize the risk of progressive disease and enhance the OS of EOC and melanoma patients are desirable. We report here clinical and immunological results on advanced EOC and melanoma patients who received diversified prime and boost vaccination with rV-NY-ESO-1 and rF-NY-ESO-1 following completion of their primary therapy.

Results

Patients. ***Melanoma patients.*** Twenty-five melanoma patients with NY-ESO-1-expressing tumors were entered into a clinical trial at Krankenhaus Nordwest, Frankfurt (Trial LUD00-014). The melanoma patients presented with stages III (28%) and IV (72%) disease. The median duration of follow-up for melanoma patients was 32 mo (range, 9–106 mo), respectively. Additional characteristics of melanoma and EOC patients are presented in Table S1. ***Ovarian cancer patients.*** Twenty-two EOC patients with NY-ESO-1-expressing ovarian cancer, who had completed surgery and adjuvant platinum based chemotherapy for primary disease, were entered into a clinical trial (Protocol I13303/LUD02-012) at Roswell Park Cancer Institute. The majority of EOC patients presented with grade 3 tumors (96%), at stages III/IV (91%),

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[1]To whom correspondence may be addressed. E-mail: kunle.odunsi@roswellpark.org or elke.jaeger@licr.org.

[2]J.M. and J.K. contributed equally to this work.

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with serous histology (82%) and were optimally debulked at surgery (73%). The median duration of follow-up for the EOC patients was 48 mo (range, 23–66 mo). The trial was conducted in parallel with the melanoma trial in Frankfurt.

Toxicity. No major (>grade II) treatment-related toxicity was observed in any patient in the two studies. Transient injection site pain was seen in all patients. No systemic hypersensitivity reactions were observed.

Impact of rV-NY-ESO-1 and rF-NY-ESO-1 Vaccination on Progression-Free and Overall Survival in Melanoma Patients. The primary endpoints for the melanoma patients were response rates and PFS. Four of the 25 melanoma patients had completely resected disease at study entry. In the remaining 21 patients with measurable disease, objective response rates were as follows: 2/21 (9.5%) complete response (CR), 1/21 (4.8%) partial response (PR), and 1/21 (4.8%) mixed response (MR). Considering that 11/21 patients (52.4%) demonstrated stabilization of disease, the combined percentages of objective responses and stable disease (SD) amounted to a clinical benefit rate (CBR) of 71.5% in melanoma patients (Table S2). The median time to progression in the 11 patients with disease stabilization was 6.5 mo (range, 1–21 mo). Overall, the median PFS in all 25 melanoma patients was 9 mo (range, 0–84 mo). The median OS was 48 mo (range, 3–106 mo) and 13/25 (52%) melanoma patients were still alive after 31 mo of follow-up. Imaging studies from one of the complete responders (patient no. 5) are shown in Fig. S1.

Impact of rV-NY-ESO-1 and rF-NY-ESO-1 Vaccination on Progression-Free and Overall Survival in Ovarian Cancer Patients. Because the ovarian cancer patients had no measurable disease at study entry, the primary efficacy endpoint was progression-free survival (PFS). For the 22 EOC patients, the median time to disease progression/recurrence was 21 mo (95% CI, 16–29 mo), and median OS was 48 mo (CI, not estimable) (Table S3).

Vaccination with rV-NY-ESO-1 and rF-NY-ESO-1 Generates Anti-NY-ESO-1 Abs. ***Melanoma patients.*** Ten of 25 melanoma patients (40%) were baseline seropositive. Ab responses were noted in 5/15 (33%) baseline seronegatives, and 2 of these (patient nos. 7 and 8) seroconverted rapidly after the first vaccination. Interestingly, a decrease in Ab titers was noted in four patients during the course of continued vaccination with the most significant decrease in patient no. 5 from baseline of 1:100,000 to 1:400 within 22 mo (Table 1 and Table S2). Because this patient also demonstrated complete response (Fig. S1), the decrease in Ab titers may be related to the reduced tumor burden.

Ovarian cancer patients. Three out of 22 EOC patients (nos. 6, 19, and 20) (14%) were baseline seropositive for anti-NY-ESO-1 Ab, and all three remained seropositive during the course of immunization.

Table 1. Summary of Ab, CD4, and CD8 responses in ovarian cancer and melanoma patients

	Ovarian (n = 22)	Melanoma (n = 25)
Antibody responses		
Baseline seropositives	3	10
Baseline seronegatives	19	15
Seroconversions	8/19 (42%)	5/15 (33%)
Total posttreatment seropositives	11/22 (50%)	15/25 (60%)
$CD4^+$ T-cell responses		
Preexisting	15/22 (68%)	12/23 (52%)
Postvaccination	20/22 (91%)	18/23 (78%)
$CD8^+$ T-cell responses		
Preexisting	3/22 (14%)	10/25 (40%)
Postvaccination	10/22 (45%)	22/25 (88%)

Interestingly, Ab titers increased with vaccination in all three patients, with the highest increase in patient no. 20 (baseline titer of 1:2,600 rising to 1:80,000 at the completion of all vaccinations and remained at this level 6 mo later). Induction of Ab responses was noted in 8/19 (42%) baseline seronegative EOC patients and occurred rapidly after the priming injection with rV-NY-ESO-1 in >50% of seroconverted patients (Table 1 and Table S3).

$CD4^+$ and $CD8^+$ T-Cell Responses to NY-ESO-1. ***Melanoma patients.*** Spontaneous $CD8^+$ T-cell responses were found in 10/25 (40%) of melanoma patients (Table 1), and after vaccination, 22/25 (88%) patients had detectable $CD8^+$ T-cell responses to the vaccine (Table S2). Detectable $CD4^+$ T-cell responses were observed in 52% of melanoma patients at baseline (Table 1). After vaccination, $CD4^+$ T-cell responses were detected in 18/23 (78%) of melanoma patients (Table S2).

Ovarian cancer patients. Preexisting $CD8^+$ T cells were noted in 3/22 (14%) EOC patients. Ten of 22 (46%) patients had a detectable $CD8^+$ T-cell responses following vaccination. Spontaneous $CD4^+$ T-cell responses were detected in 68% of patients (Table 1). Further increases in the magnitude of $CD4^+$ T-cell responses in patients with preexisting $CD4^+$ T-cell responses and de novo induction of $CD4^+$ T cells in those without preexisting responses were found in 20/22 (91%) of EOC patients (Table S3).

A summary of NY-ESO-1-specific $CD4^+$ and $CD8^+$ T-cell responses observed in both patient populations is presented in Table 1, and a summary of responses observed in individual patients are presented in Tables S2 and S3. Together, the results of the two parallel studies indicate that heterologous prime/boost vaccination with rV-NY-ESO-1 and rF-NY-ESO-1 leads to induction of Ab and/or $CD8^+$ and/or $CD4^+$ T-cell responses in the majority of EOC and melanoma patients.

Analysis of Frequency, Specificity, and Effector Function of Vaccine-Induced $CD8^+$ and $CD4^+$ T-Cell Responses. In a subset of EOC and melanoma patients, the frequencies of $CD8^+$ T cells were further analyzed using HLA multimers for HLA-A2, HLA-B35, and HLA-Cw3. In the example shown in Fig. 1*A*, EOC patient no. 4 (baseline seronegative, HLA-$A2^+$, and HLA-$B35^+$) received all vaccinations and was analyzed by multimer. There was rapid induction of HLA-B35 restricted $CD8^+$ T cells that persisted for up to 12 mo following completion of vaccination. Fig. 1*B* illustrates tetramer analysis in a baseline seronegative melanoma patient (no. 21) that developed a high magnitude of vaccine induced HLA-Cw3/p92-100 specific $CD8^+$ T-cell response. Next, we determined effector function of vaccine induced $CD8^+$ T cells by IFN-γ enzyme-linked immunospot (ELISPOT) and intracellular cytokine staining (ICS). As illustrated in Fig. 1*C*, melanoma patients nos. 20 and 21 developed vaccine-induced $CD8^+$ T cells specific for NY-ESO-1 epitope region p81-110. In patients with pre-existing $CD8^+$ T-cell responses to NY-ESO-1, there was further expansion of $CD8^+$ T-cell frequencies in ovarian cancer (Fig. 2*A*) and melanoma (Fig. 2*B*) patients.

There was further expansion of NY-ESO-1-specific $CD4^+$ T cells in patients with preexisting (Fig. S2*A*) immunity to NY-ESO-1; or de novo induction of NY-ESO-1-specific $CD4^+$ T cells in patients without preexisting $CD4^+$ T-cell immunity (Fig. S2*B*). Together, these results indicate induction of Ab, $CD4^+$ and $CD8^+$ T cells in EOC and melanoma patients treated with rV-NY-ESO-1 and rF-NY-ESO-1. These NY-ESO-1-specific $CD8^+$ and $CD4^+$ T cells were detectable in a subset of patients up to 6 and 12 mo following the last vaccination indicating longevity of the vaccine-induced T-cells (Fig. 1*A*). Next, we established NY-ESO-1-specific $CD4^+$ T-cell lines in a subset of ovarian cancer patients pre- and post-vaccination, as previously described (9). We determined Th1, Th2, and Th17 differentiation based on IFN-γ, IL-4, IL-13, or IL-17 production upon stimulation with NY-ESO-1 pooled peptides. Although the IFN-γ/IL-13 ratio did not change during the course of

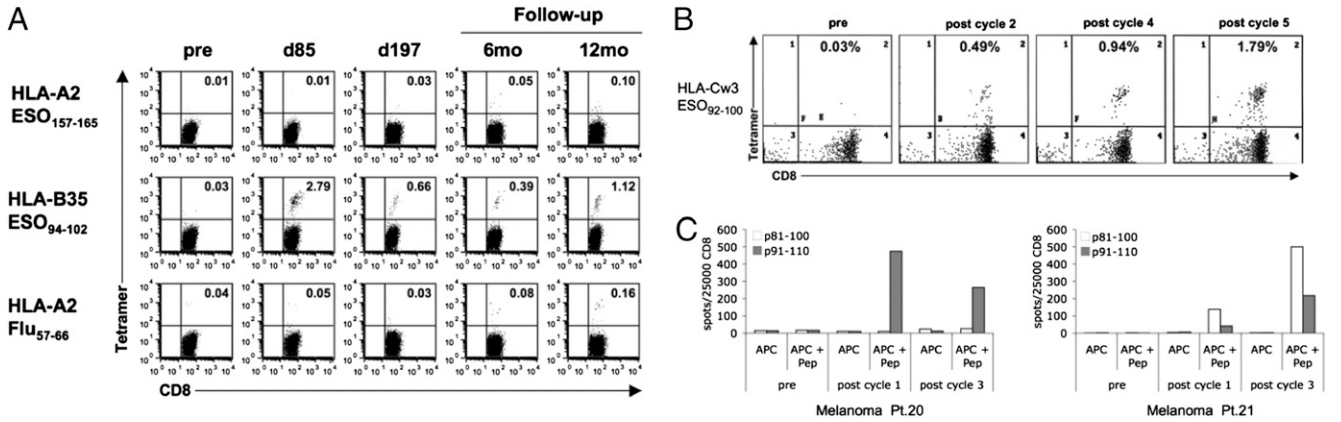

Fig. 1. NY-ESO-1-specific CD8 responses are induced by vaccination from baseline seronegative patients. (*A*) The frequency of NY-ESO-1-tetramer$^+$ (HLA-A2 and B-35) cells at the indicated time points following vaccination in EOC patient no. 4. The number in right upper quadrant indicates percentage of tetramer$^+$CD8$^+$ cells following in vitro stimulation with cognate NY-ESO-1 peptides. (*B*) NY-ESO-1-specific (HLA-Cw3) CD8$^+$ T-cell analysis in melanoma patient no. 21. (*C*) Effector function was determined by ELISPOT assays on day 12 after in vitro presensitization with NY-ESO-1 peptides. Numbers of NY-ESO-1-specific IFN-γ spots were shown against peptide-pulsed and -unpulsed autologous dendritic cells.

vaccination, the IFN-γ/IL-4 and IFN-γ/IL-17 ratios were increased after vaccination (Fig. S3*A*). Moreover, the ability to recognize full-length NY-ESO-1 protein (Fig. S3*B*) and T-cell receptor (TCR) avidity were enhanced by vaccination (Fig. S3*C*). Similarly, post-vaccine NY-ESO-1-specific CD4$^+$ T-cell clones generated form melanoma patients demonstrated both IFNγ and lytic effector function (Fig. S4). The changes in CD4$^+$ T-cell differentiation were confined to NY-ESO-1-specific T cells because direct *ex-vivo* phenotypic analysis of peripheral blood mononuclear cells (PBMCs) derived total CD4$^+$ T cells indicated no significant changes in the frequencies of Treg, Th1/Th2, and Th17 cells after vaccination in both EOC and melanoma patients (Fig. S5). In addition, although EOC patients demonstrated significantly higher expression of LAG-3 and lower expression of PD-1 than melanoma patients in the CD8$^+$ T-cell compartment, there were no differences between pre- and postvaccination expression of inhibitory [LAG-3, PD-1, cytotoxic effector T cell (CTL)A-4] or stimulatory (ICOS) receptors in the ovarian cancer and melanoma patients (Fig. S6). Together, the results indicate that the vaccine regimen enhanced Th1 differentiation, recognition of naturally processed antigen, and TCR avidity of NY-ESO-1-specific CD4$^+$ T cells.

T-Cell Epitope Clusters Induced by rV-NY-ESO-1 and rF-NY-ESO-1 Vaccination. ***Ovarian cancer patients.*** Vaccine-induced CD8$^+$ T-cell epitopes were clustered in the p81-110 region of NY-ESO-1 in EOC patients. Less frequent epitopes were located in the p119-160 regions.

Melanoma patients. In melanoma patients, spontaneous and vaccine-induced CD8$^+$ T-cell responses were predominantly directed against NY-ESO-1 epitopes within regions p81-110 and p151-170 and, less frequently, against epitopes within region p119-143.

In both patient populations, CD4$^+$ T-cell responses were directed against a broader region of the NY-ESO-1 protein. Overall, vaccine-induced CD4$^+$ and CD8$^+$ T-cells were directed against the same epitopes as found in patients with spontaneous NY-ESO-1 reactivity (Fig. S7).

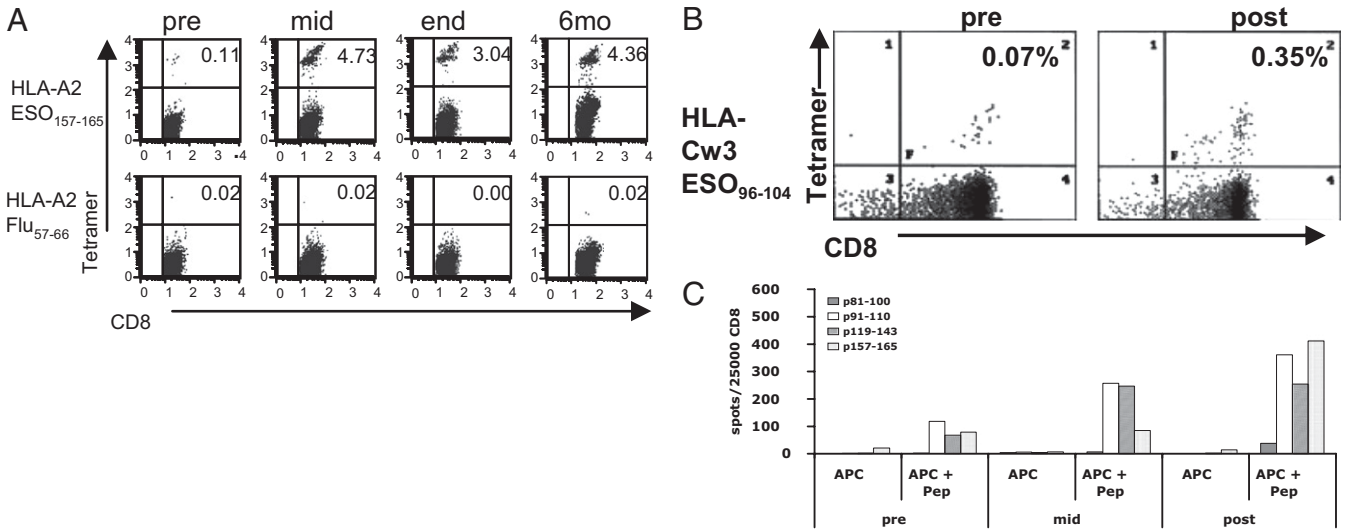

Fig. 2. Vaccination enhances NY-ESO-1-specific CD8$^+$ T-cell response in baseline seropositive patients. (*A*) Expansion of HLA-A2/ESOp157-165 tetramer$^+$ cells in EOC patient no. 20 by vaccination. (*B*) Direct ex vivo HLA-Cw3/ESOp96-104 tetramer staining in melanoma patient no. 19. (*C*) NY-ESO-1-specific CD8$^+$ T-cell responses against peptides p91-110, p119-143, and p157-165 were enhanced by vaccination as shown by ELISPOT assay after in vitro presensitization with NY-ESO-1 peptides.

Tumor Reactivity of Vaccine-Induced NY-ESO-1-Specific CD8$^+$ T Cells. To further characterize the effector function of vaccine elicited CD8$^+$ T cells, polyclonal populations of tetramer reactive cells derived from vaccinated EOC patients were tested for IFN-γ and CD107, following stimulation with NY-ESO-1$^+$ and NY-ESO-1$^-$ tumor lines. As shown in Fig. 3*A*, whereas vaccine-induced polyclonal HLA-A2 restricted NY-ESO-1-specific CD8$^+$ T cells recognized MZ-Mel-19 (A2$^+$ESO$^+$) tumor line, SK-Mel-23 (A2$^+$ESO$^-$) line was not recognized. In the case of melanoma patients, CD8$^+$ T-cell populations were tested for tumor recognition in ^{51}Cr release assays on HLA-matched NY-ESO-1$^+$ and NY-ESO-1$^-$ tumor lines. As shown in Fig. 3*B*, CD8$^+$ T-cells specific for NY-ESO-1 p157-165 and p92-110 efficiently recognized HLA-matched NY-ESO-1-expressing tumor cell lines. These results indicate that rV/rF-NY-ESO-1 vaccines can generate tumor-reactive T cells in both patient populations.

Impact of Vaccine-Induced Immune Responses on Clinical Outcome. Given the remarkable ability of rV/rF-NY-ESO-1 to induce tumor-reactive immune responses, we next asked whether these immune responses are predictive of clinical outcome. In melanoma patients, the median PFS and OS of patients with and without NY-ESO-1-specific CD8$^+$ T-cell responses were 9 vs. 7 mo ($P = 0.16$) and 82 vs. 15 mo ($P = 0.007$), respectively (Fig. 4 *A* and *B*). Because there were detectable CD8$^+$ T-cell responses in the majority (88%) of melanoma patients following vaccination, we sought to determine the impact of integrated Ab, CD4$^+$, and CD8$^+$ T-cell responses on clinical outcome as previously described (4) (Tables S2 and S3). We found that patients who were seropositive and showed CD8$^+$ T-cell reactivity at baseline that remained stable or was broadened during the course of vaccination demonstrated a significantly improved PFS (median, 19.5 mo) compared with (*i*) seronegative patients who remained seronegative but developed CD4 and/or CD8 T-cell responses (median, 9 mo) and (*ii*) seronegative patients who seroconverted and developed CD4 and/or CD8 T-cell responses (median, 3.75 mo) (log rank $P = 0.05$; Fig. 4*C*).

Similar analysis in ovarian cancer patients revealed that (*i*) seronegative patients who remained seronegative but developed CD4 and/or CD8 T-cell responses and (*ii*) seronegative patients who seroconverted and developed CD4 and/or CD8 T-cell responses demonstrated improved OS (median, 52.4 and 48.4 mo, respectively) compared with other categories of patients (median, 14.5months) ($P < 0.0001$) (Fig. S8). The results were not impacted by clinicopathologic variables such as age, grade, stage, histology and performance status in both melanoma and ovarian cancer patients. A summary of tumor-infiltrating CD8$^+$ T cells and CD8$^+$/Treg ratio in a small subset of EOC patients is presented in Table S4. Although the CD8$^+$/Treg ratio consistently decreased at the time of tumor recurrence, the small number of cases precluded analysis of the relationship between CD8$^+$/Treg ratio and clinical outcome.

Discussion

The NY-ESO-1 tumor antigen is frequently expressed in ovarian cancer and melanoma, eliciting both cellular and humoral immune responses in a proportion of patients with advanced NY-ESO-1-expressing tumors (3, 10). In a previous phase I study, we demonstrated that a diversified prime/boost regimen using recombinant vaccinia as prime and recombinant fowlpox boost efficiently delivered NY-ESO-1 tumor antigen to the immune system leading to the generation of integrated Ab, CD4 and CD8 T-cell responses (4). In the present report, we tested the clinical efficacy of this approach in two parallel phase II clinical trials focusing on ovarian cancer and melanoma, two cancer types where patients treated for advanced disease are at extremely high risk for recurrence/progression (70% and 80%, respectively) (7, 8). Therefore, vaccine strategies to extend remission and disease control rates in these patients are highly desirable.

Consistent with the notion that malignant melanoma is a highly immunogenic tumor, the baseline frequency of humoral and CD8$^+$ T-cell immunity to NY-ESO-1 in melanoma patients (40%) was significantly higher than for ovarian cancer patients (14%). In contrast, preexisting CD4$^+$ T-cell response to NY-ESO-1 was more frequent in ovarian cancer than in melanoma patients (68% Vs 52% respectively). Despite these baseline differences, the proportion of patients that seroconverted, or mounted CD8$^+$ and/or CD4$^+$ T-cell responses to NY-ESO-1 was similar in ovarian cancer and melanoma, indicating that the rV-NY-ESO-1/rF-NY-ESO-1 approach is capable of inducing Ab and CD4$^+$ and CD8$^+$ T-cell responses regardless of disease type. However, postvaccination, the total number of melanoma patients with NY-ESO-1-specific CD8$^+$ T-cell responses was greater than for ovarian cancer patients (88% vs. 46%, respectively). Because we also demonstrated robust effector function (including tumor recognition) of vaccine induced CD8$^+$ T cells, this could explain the strong relationship between induction of CD8$^+$ T cells and survival in melanoma patients.

The phase II clinical trials reported here were designed to measure PFS as the primary endpoint. Because of the limited sample size and nonrandomized study design, OS was measured as an exploratory endpoint. We noted that median PFS and OS in our ovarian cancer study population were 21 and 48 mo respectively. The ovarian cancer patients that benefitted the most were (*i*) seronegative patients who remained seronegative but developed CD4 and/or CD8 T-cell responses and (*ii*) seronegative patients who seroconverted and developed CD4 and/or CD8 T-cell responses. While there are significant limitations in comparing these results with data from large phase III studies, median PFS and OS were 16.0 and 44.1 mo respectively in the largest ever phase III study recently conducted by the Gynecologic

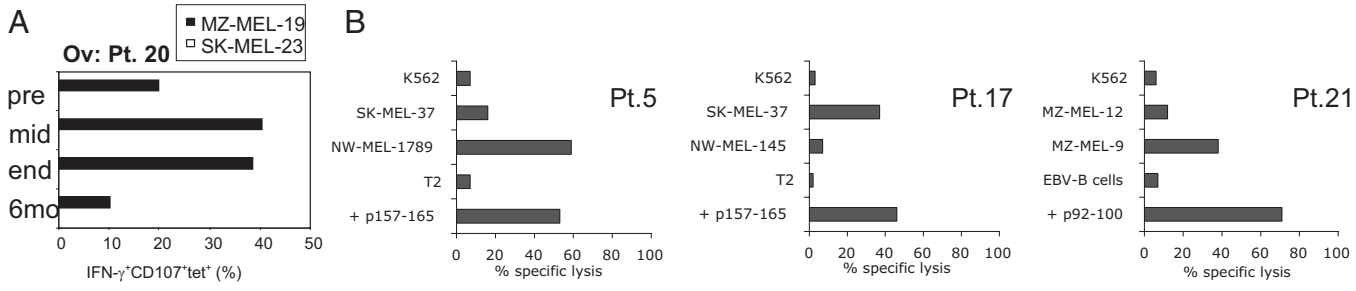

Fig. 3. Vaccine-induced CD8$^+$ T cells are capable of recognizing NY-ESO-1+ tumor targets. (*A*) Tumor reactivity of CD8$^+$ T cells in EOC patient no. 20 was assessed by ICS against HLA-A2$^+$ melanoma lines; MZ-MEL-19 (ESO$^+$) or SK-MEL-23 (ESO$^-$). IFN-γ and CD107 expression were determined by gating on HLA-A2/ESOp157-165 tetramer$^+$CD8$^+$ cells. (*B*) Cytotoxicity of vaccine-induced CD8$^+$ T-cells against HLA-matched NY-ESO-1$^+$ tumor cell lines MZ-MEL-9 (Cw3$^+$), NW-MEL-1789 (A2$^+$), and SK-MEL-37 (A2$^+$). NW-MEL-145 (A2$^+$), and NW-MEL-12 (Cw3$^+$) were NY-ESO-1-negative and used as control cell lines. CD8 T cells of melanoma patient nos. 17 and 5 were generated by using autologous tumor cell line for prestimulation; CD8 T cells of patient no. 21 were generated by prestimulation with NY-ESO-1 p91-110.

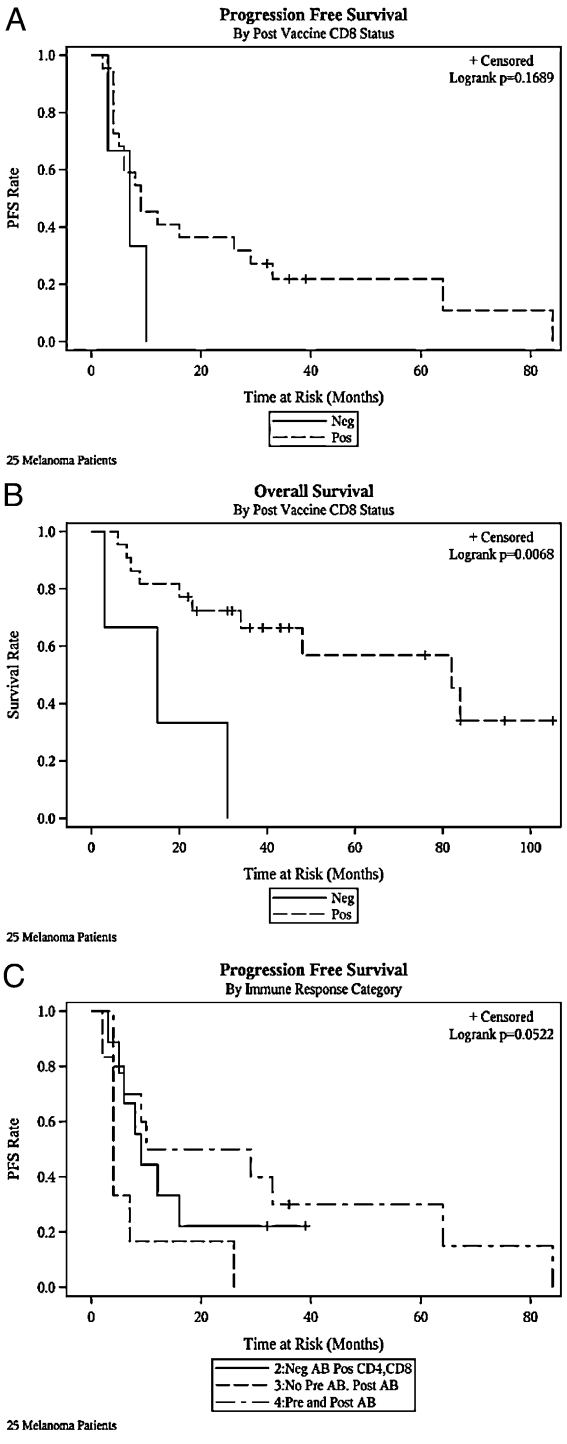

Fig. 4. The relationship between NY-ESO-1-specific immune responses and clinical outcome in melanoma patients. (*A*) $CD8^+$ T-cell response vs. PFS of melanoma patients. (*B*) $CD8^+$ T-cell response vs. OS of melanoma patients. (*C*) Baseline seropositive patients with $CD8^+$ T-cell reactivity demonstrated a significantly improved TTP (median, 19.5 mo) compared with other groups of patients.

Oncology Group (GOG protocol 0182) (11). Because EOC patients with small residual disease appear to have a survival advantage when treated with i.p. platinum-based chemotherapy compared with the standard i.v. regimen (7), it is likely that the clinical outcome from rV/rF-NY-ESO-1 will be further improved in EOC patients that receive i.p. chemotherapy.

For melanoma patients, PFS and OS were significantly extended in patients with detectable NY-ESO-1-specific immune responses compared with historical data from cohorts who receive standard chemotherapy with dacarbazine (12), or immunotherapy of the vaccine type, or with anti-CTLA-4 Ab (13) or with patients who did not develop any NY-ESO-1-specific immune response. Moreover, 71% of melanoma patients with measurable disease demonstrated tumor regression or disease stabilization. The melanoma patients that derived the most benefit were those with preexisting NY-ESO-1-specific humoral and T-cell immunity. Compared with ovarian cancer, this striking and remarkable clinical outcome in melanoma patients may reflect the higher frequency of detectable preexisting NY-ESO-specific $CD8^+$ T cells that are then further expanded by vaccination, and receive $CD4^+$ Th1 help, leading to a strong capacity to control the outgrowth of NY-ESO-1-expressing melanoma cells in vivo. Taken together with the previous observation that patients who had a clinical benefit (stable disease or partial response) from anti-CTLA-4 Ab administration in a phase II clinical study showed detectable NY-ESO-1-specific immune responses during treatment (14, 15), it is reasonable to conclude that further amplification of preexisting, or de novo induction of NY-ESO-1 immunity is beneficial in melanoma patients. Because the three ovarian cancer patients with preexisting NY-ESO-1 humoral immunity did not demonstrate improved PFS or OS compared with other EOC categories, we propose that this category of ovarian cancer patients may represent a population with high disease burden, given that they progressed rapidly and did not complete the planned courses of vaccination.

In conclusion, two parallel phase II studies, albeit of limited size, using heterologous prime-boost vaccination with rV-NY-ESO-1 and rF-NY-ESO-1 showed clinical benefit among patients with melanoma and ovarian cancer at high risk for relapse. Possible reasons for the clinical efficacy of the viral vaccines may be the intensity and breadth of epitope specificity of immune responses generated, that were similar to those observed after vaccination with NY-ESO-1 protein (6), or in patients with spontaneous NY-ESO-1 immunity. Epitope-specific T-cell responses elicited by the vaccine were shown to be tumor-reactive, a finding that not only supports the therapeutic potential of the effector cells but also the susceptibility of melanoma and ovarian cancer cells to lysis by antigen-specific T cells in vitro and in vivo. Our findings suggest that further evaluation of this approach, in combination with strategies to counteract negative regulation of immune responses, is warranted in these patient populations.

Patients and Methods

Study Protocols and Patient Population. ***EOC patients.*** The rV-NY-ESO-1 and rF-NY-ESO-1 clinical study (Protocol I13303/LUD02-012) was approved by the Institutional Review Board at Roswell Park Cancer Institute for stage II-IV EOC patients who have completed surgery and first-line chemotherapy. Expression of NY-ESO-1 and/or LAGE-1 was detected in tumors by RT-PCR and/or immunohistochemistry, as described previously (3). Patients received one intradermal injection of rV-NY-ESO-1, 3.1×10^7 plaque-forming units (PFU), followed by monthly s.c. injections of rF-NY-ESO-1, 7.41×10^7 PFU for 6 mo. ***Melanoma patients.*** The rV-NY-ESO-1 and rF-NY-ESO-1 protocol (LUD00-014) was approved by the ethics committee of the Landesärztekammer Hessen in Frankfurt, for stages III and IV melanoma patients. Patients with NY-ESO-1/LAGE-expressing melanoma were treated in six cohorts. Patients in cohort 1–4 (patient nos.1–5) received rV-NY-ESO-1 and rF-NY-ESO-1 at four different doses of $3.1 \times 10^7/3.1 \times 10^8$ and $7.41 \times 10^7/7.41 \times 10^8$ PFU, respectively. Patients in cohort 5 (patient nos. 6–12) received two vaccinations with rV-NY-ESO-1 at a dosage of 3.1×10^7 PFU, followed by two vaccinations with rF-NY-ESO-1 at a dose of 7.41×10^7 PFU at 4-wk intervals. Patients in cohort 6 (patient nos. 13–25) received one i.m. injection of rV-NY-ESO-1, 3.1×10^7 PFU, followed by monthly s.c. injections of rF-NY-ESO-1, 7.41×10^7 PFU for 3 mo. In the absence of toxicity and disease progression that required other therapeutic interventions, melanoma patients could continue to receive monthly s.c. injections of rF-NY-ESO-1 at a dosage of 7.41×10^7 PFU.

Antibody Responses and Analysis of NY-ESO-1-Specific T Cells. Analysis of serum samples for NY-ESO-1 by ELISA was performed by using a method described previously (4, 5). Ex vivo or in vitro analysis of NY-ESO-1-specific T cells were performed as described previously (4, 5). *SI Patients and Methods* provides further details and information regarding these analyses.

Statistical Analysis. The primary objective of the phase II studies was to demonstrate improved efficacy for the proposed treatment, where improved efficacy means extending the PFS. Additional measures of clinical benefit are CR, PR, SD, PFS, and OS. Standard non- and semiparametric statistical procedures were completed in the statistical environment R. Specific procedures used included Kendall tau, Wilcoxon signed-rank test, McNemar test of symmetry, Kaplan–Meier survival estimator, and Cox regression model.

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