Vaccination with an NY-ESO-1 peptide of HLA class I/II specificities induces integrated humoral and T cell responses in ovarian cancer

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NY-ESO-1 is a "cancer-testis" antigen expressed in epithelial ovarian cancer (EOC) and is among the most immunogenic tumor antigens defined to date. The NY-ESO-1 peptide epitope, $ESO_{157–170}$, is recognized by HLA-DP4-restricted $CD4^+$ T cells and HLA-A2- and A24-restricted $CD8^+$ T cells. To test whether providing cognate helper $CD4^+$ T cells would enhance the antitumor immune response, we conducted a phase I clinical trial of immunization with $ESO_{157–170}$ mixed with incomplete Freund's adjuvant (Montanide ISA51) in 18 $HLA\text{-}DP4^+$ EOC patients with minimal disease burden. NY-ESO-1-specific Ab responses and/or specific HLA-A2-restricted $CD8^+$ and HLA-DP4-restricted $CD4^+$ T cell responses were induced by a course of at least five vaccinations at three weekly intervals in a high proportion of patients. There were no serious vaccine-related adverse events. Vaccine-induced $CD8^+$ and $CD4^+$ T cell clones were shown to recognize NY-ESO-1-expressing tumor targets. T cell receptor analysis indicated that tumor-recognizing $CD4^+$ T cell clones were structurally distinct from non-tumor-recognizing clones. Long-lived and functional vaccine-elicited $CD8^+$ and $CD4^+$ T cells were detectable in some patients up to 12 months after immunization. These results confirm the paradigm that the provision of cognate $CD4^+$ T cell help is important for cancer vaccine design and provides the rationale for a phase II study design using $ESO_{157–170}$ epitope or the full-length NY-ESO-1 protein for immunotherapy in patients with EOC.

HLA-DP4 | peptide epitope | tumor recognition | vaccine

There is increasing evidence that the immune system has the ability to recognize tumor-associated antigens expressed in human malignancies and to induce antigen-specific humoral and cellular immune responses to these targets. In epithelial ovarian cancer (EOC), support for the role of immune surveillance of tumors comes from our recent observation indicating that the presence of intraepithelial $CD8^+$-infiltrating T lymphocytes in tumors is associated with improved survival of patients with the disease (1). Although the majority of women with advanced-stage ovarian cancer respond to first-line chemotherapy, most of these responses are not durable, and >70% of patients die of recurrent disease within 5 years of diagnosis. Therefore, the development of strategies to enhance the potential of tumor antigen-specific $CD8^+$ T and $CD4^+$ T cells is urgently needed for extending remission rates in this disease. In this regard, cancer-testis antigens, a unique class of antigens that demonstrate high levels of expression in adult male germ cells but generally not in other normal adult tissues and aberrant expression in a variable proportion of a wide range of different cancer types, are promising candidates for immunotherapy. Among cancer-testis antigens, NY-ESO-1 (2) is one of the most spontaneously immunogenic tumor antigens described so far. Previously, we reported that NY-ESO-1 is a promising target for specific immunotherapy of EOC (3).

Although the majority of cancer vaccine trials have focused on eliciting antigen-specific $CD8^+$ T cells, a growing body of evidence indicates that $CD4^+$ T cells play a pivotal role in orchestrating these responses. The multiple roles of antigen-specific $CD4^+$ T cells include the provision of help to $CD8^+$ T cells during the primary and secondary immune responses, direct cytolysis, and activation of B cells for production of tumor antigen-specific Abs. Therefore, we have focused on the NY-ESO-1 epitope, $ESO_{157–170}$, a naturally processed helper epitope that is recognized by $CD4^+$ T cells in the context of HLA-DPB1*0401 and *0402 (4), prevalent MHC class II alleles present in ≈43–70% of Caucasians. Moreover, the NY-ESO-1 HLA-DP4 epitope has HLA-A2 ($ESO_{157–165}$) (5) and HLA-A24 ($ESO_{158–166}$) (6) motifs embedded in its natural sequence. In this study, we evaluated whether active immunization with $ESO_{157–170}$ would elicit NY-ESO-1-specific $CD4^+$ and $CD8^+$ T cell responses in ovarian cancer patients with minimal disease burden. In addition, we characterized NY-ESO-1-specific $CD8^+$ and $CD4^+$ T cell receptor (TCR) repertoires in conjunction with functional analysis of vaccine-elicited T cell clones.

Results

Patients. Eighteen EOC patients (HLADPB1*0401 or *0402) with NY-ESO-1-expressing tumors who had completed adjuvant chemotherapy for primary or recurrent disease were entered into the trial (protocol no. LUD02-011), which was sponsored by the Cancer Vaccine Collaborative program of the Cancer Research Institute/Ludwig Institute for Cancer Research (www.cancerresearch.org). All patients gave written informed consent and were able to be evaluated for toxicity and immunological and tumor response. Nine patients were either HLA-A2 or HLA-A24 (HLA-A2, five; HLA-A24, five; HLA-A24 and -A2, one). The majority of patients presented with grade 3 tumors (89%) at stage IIIC (89%) with serous histology (94%), and 45% had received two to eight previous lines of chemotherapy. Additional patient characteristics are presented in supporting information (SI) Table 2.

Toxicity. No major (more than grade II) treatment-related toxicity was observed in any patient. Transient injection site pain was seen in all patients, and systemic hypersensitivity reactions were not observed.

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Abbreviations: CTL, cytotoxic incomplete T lymphocyte; ELISPOT, enzyme-linked immunospot; EOC, epithelial ovarian cancer; IFA, incomplete Freund's adjuvant; ICS, intracellular cytokine staining; T-APC, target antigen-presenting cell; TCR, T cell receptor; Th1, T helper 1.

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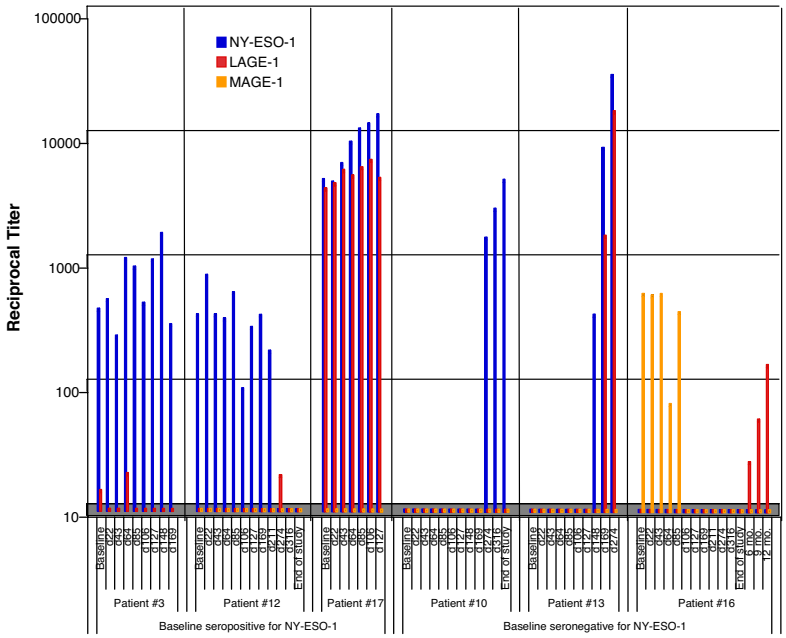

Fig. 1. NY-ESO-1 serum Ab was assessed by ELISA before and after vaccination. Two baseline seronegative patients (10 and 13) converted to seropositive during the course of immunization.

Vaccination with NY-ESO-$1_{157–170}$ in Combination with Incomplete Freund's Adjuvant (IFA) Generates Anti-NY-ESO-1 Abs. Three patients (nos. 3, 12, and 17) were baseline seropositive for anti-NY-ESO-1 Ab, and all three remained seropositive during the course of immunization (Fig. 1). Ab titers for patient 3 increased from 1:360 at baseline to a peak of 1:2,000; Ab titers for patient 17 increased from 1:4,500 to 1:15,000 with immunization; and patient 12 became seronegative 1 year after completing immunization. Two baseline seronegative patients converted to seropositive during the course of immunization. Patient 10 became seropositive on day 274 (after 11 vaccinations) with a titer of 1:1,500 and peaking at 1:4,500 after 14 immunizations. Patient 13 became seropositive on day 169 (after 7 vaccinations) with a titer of 1:380, increasing to a titer of 1:31,108 after 11 immunizations. The patient also became seropositive for LAGE-1 on day 211 (1:1,228), increasing to 1:15,873 on day 274 (Fig. 1). Serological responses to unrelated recombinant proteins MAGE-1, -3, -4, and -10 were used as internal controls. Although patient 16 was seropositive for MAGE-1 at baseline, there was no increase in Ab titer during the course of NY-ESO-1 immunization. However, the patient developed weakly detectable Ab to LAGE-1 long after completion of vaccine therapy (Fig. 1).

Vaccination with NY-ESO-$1_{157–170}$ in Combination with IFA Evokes a Strong $CD4^+$ T Cell Response. Induction of specific $CD4^+$ T cells was analyzed by IFN-γ enzyme-linked immunospot (ELISPOT) and intracellular staining after a single *in vitro* stimulation with $ESO_{157–170}$. Patients 1 and 3 had preexisting peptide-reactive $CD4^+$ T cells, and patient 3 was also baseline seropositive for NY-ESO-1 (SI Table 3). In both patients, the frequency of peptide reactive $CD4^+$ T cells increased during the course of immunization. Of the remaining 16 patients, 13 demonstrated detectable $ESO_{157–170}$-reactive $CD4^+$ T cells during the course of immunization (SI Table 3). The data indicate that $ESO_{157–170}$-reactive $CD4^+$ T cells could be detectable by IFN-γ ELISPOT as early as the third (day 43) vaccination in the majority of patients. As shown in Fig. 2*A*, there was a significant relationship between the number of vaccines and the maximal number of detectable $ESO_{157–170}$-reactive $CD4^+$ T cells (Kendall's τ; $P = 0.003$). In the example shown, patient 18 completed all 15 vaccinations, and $ESO_{157–170}$-reactive $CD4^+$ T cells were detected by IFN-γ intracellular staining (ICS) (Fig. 2*B*) and confirmed by IFN-γ ELISPOT (SI Fig. 7). Together, the data show that the proportion of patients with $CD4^+$ T cell response at the end of the study (83%) was significantly greater than the proportion at the start of therapy (11%) (McNemar's test; $P = 0.0009$).

$CD4^+$ T Cells Induced by Vaccination with NY-ESO-$1_{157–170}$ in Combination with IFA Recognize Tumor Targets. We previously showed that peptide vaccine-induced, NY-ESO-$1_{157–170}$-specific $CD4^+$ T helper 1 (Th1) cells had limited capacity in recognizing naturally processed NY-ESO-1 protein in two patients using IFN-γ ELISPOT (7). We extended this analysis to functional tumor recognition by vaccine-induced NY-ESO-$1_{157–170}$-specific $CD4^+$ T cells in additional patients. The results showed that NY- ESO-$1_{157–170}$-specific $CD4^+$ T cells induced by immunization were able to recognize tumor targets more by production of IFN-γ than TNF-α. In the example shown in Fig. 3 for patient 18, vaccine-induced polyclonal NY-ESO-$1_{157–170}$-specific $CD4^+$ T cells were able to recognize $DP4^+ESO^+$

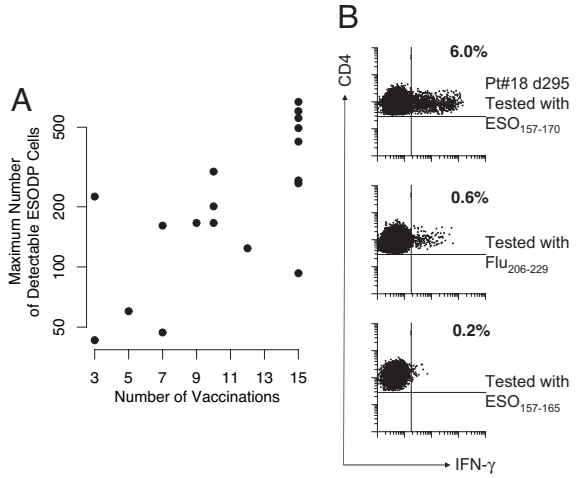

Fig. 2. $CD4^+$ T cell response to $ESO_{157–170}$ vaccine. (*A*) There is a significant relationship between the number of vaccines and the maximal number of detectable $ESO_{157–170}$-reactive $CD4^+$ T cells ($P = 0.002$) when data from all patients are examined cumulatively. (*B*) $CD4^+$ T cells were stimulated with $ESO_{157–170}$ peptide and tested against target cells (T-APC loaded with $ESO_{157–170}$ peptide or irrelevant peptide Flu $NP_{206–229}$, Flu NP) by IFN-γ ICS.

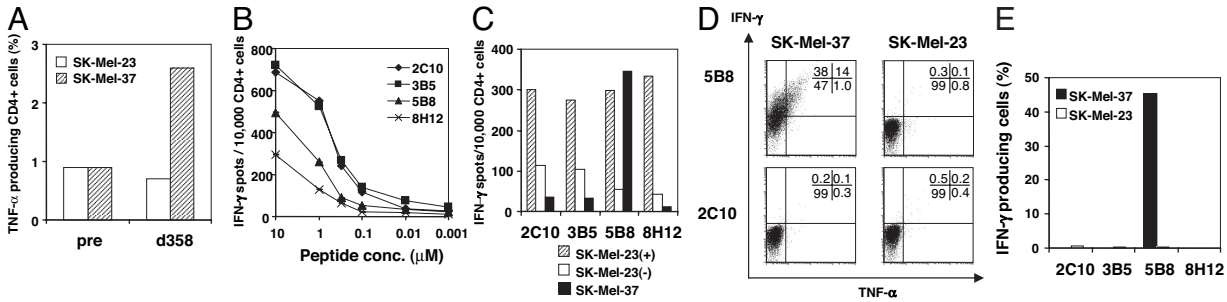

Fig. 3. Tumor recognition by vaccine-induced CD4$^+$ T cells. (*A*) $ESO_{157–170}$-specific CD4$^+$ T cells were assessed for recognition of ESO$^+$DP4$^+$ SK-Mel-37 or ESO^{-ve}DP4$^+$ SK-Mel-23 by intracellular TNF-α staining. (*B*) Reactivity of CD4$^+$ T cell clones to $ESO_{157–170}$ peptide with concentration ranging from 10 μM to 1 nM. (*C*) Only clone 5-B-8 recognized SK-Mel-37, and SK-Mel-23 was only recognized by all clones when pulsed with $ESO_{157–170}$. (*D* and *E*) Assessment of tumor recognition by IFN-γ ICS.

SK-Mel-37 tumor line, but not DP4$^+$ESO$^-$ SK-Mel-23 tumor line (Fig. 3*A*). To confirm this observation, $ESO_{157–170}$-reactive CD4$^+$ T cells were sorted by using IFN-γ capture and cloned by limiting dilution. We identified four clones that showed $ESO_{157–170}$-specific response by using a peptide-pulsed, target antigen-presenting cell (T-APC). All of the clones (3-B-5, 2-C-10, 5-B-8, and 8-H-12) could recognize as little as 0.1 μM $ESO_{157–170}$ peptide (Fig. 3*B*). Although all clones recognized SK-Mel-23 when pulsed with $ESO_{157–170}$ peptide, only clone 5-B-8 strongly recognized SK-MEL-37 by IFN-γ ELISPOT (Fig. 3*C*), intracellular IFN-γ, and TNF-α (Fig. 3 *D* and *E*). Clones 2-C-10 and 3-B-5 (with higher avidity than clone 5-B-8) and clone 8-H-12 (with lower avidity than 5-B-8) did not recognize SK-Mel-37 or SK-Mel-23 (Fig. 3 *D* and *E*). Up to 24% and 5% of IFN-γ-secreting 5-B-8 cells also were positive for TNF-α and IL-2, respectively, whereas only 0.6%, 0.3%, and 0.3% were positive for IL-4, -5, and -10, respectively (SI Fig. 8). These results indicate a predominant Th1 cytokine differentiation of $ESO_{157–170}$ peptide vaccine-induced CD4$^+$ T cells. In another example from patient 8, polyclonal $ESO_{157–170}$-reactive CD4$^+$ T cells (SI Fig. 9) recognized SK-Mel-37 by IFN-γ ELISPOT and ICS.

Vaccination with NY-ESO-$1_{157–170}$ in Combination with IFA Induces HLA-A2-Restricted NY-ESO-1-Specific CD8$^+$ T Cells. We sought to determine whether $ESO_{157–170}$ peptide vaccine in combination with IFA also induced HLA-A2-restricted ($ESO_{157–165}$) and/or HLA-A24-restricted ($ESO_{158–166}$) CD8$^+$ T cell responses in immunized patients. There was no demonstrable HLA-A24-restricted CD8$^+$ T cell reactivity by A24/$ESO_{158–166}$ multimer and IFN-γ ELISPOT. Although patient 10 had weakly detectable CD8$^+$ T cells on day 85 by using A2/$ESO_{157–165}$ multimer (0.19%), there was no evidence of IFN-γ production by ELISPOT or ICS (data not shown). In contrast, patients 2, 14, and 18 clearly demonstrated CD8$^+$ T cell reactivity by A2/$ESO_{157–165}$ multimer, ELISPOT, and ICS (example from patient 18 shown in Fig. 4). Overall, the results indicate induction of HLA-A2-restricted CD8$^+$ T cells by $ESO_{157–170}$ in three of the five (60%) HLA-A2$^+$ patients and in none of the five HLA-A24$^+$ patients.

Vaccine-Induced NY-ESO-1-Specific CD8$^+$ T Cells Also Recognize Tumor Targets. To characterize the effector function of vaccine-elicited CD8$^+$ T cells from patient 18, tetramer-reactive cells were tested for IFN-γ and CD107 after coculture with autologous $ESO_{157–165}$-loaded T-APC. As shown in Fig. 5*A*, we observed IFN-γ and CD107 only when CD8$^+$ T cells encountered $ESO_{157–165}$ but not irrelevant flu peptide. In addition, although not all $ESO_{157–160}$ tetramer-reactive cells demonstrated effector function, a significant proportion ($\leq$65%) was positive for IFN-γ and/or CD107. This polyclonal population of CD8$^+$ $ESO_{157–160}$ tetramer-reactive cells also recognized MZ-MEL-19 (HLA-A2$^+$ and NY-ESO-1$^+$), but not SK-MEL-23 (HLA-A2$^+$ and NY-ESO-1$^-$) (Fig. 5*B*). To determine tumor recognition at the clonal level, CD8$^+$ $ESO_{157–165}$ tetramer-reactive cells were cloned by limited dilution. Four clones (2-5-C, 3-4-D, 5-5-B, and 5-6-B) showed 95–97% multimer reactivity (SI Fig. 10). All of the cytotoxic incomplete T lymphocyte (CTL) clones seemed to have comparable avidity for peptide (Fig. 5*C*) and recognized allogeneic NY-ESO-1-expressing melanoma cells, indicating that their avidity was high enough to recognize the level of antigen expressed by tumor cells. All of the clones efficiently recognized the HLA-A2$^+$ESO$^+$ cell line, MZ-MEL-19 (Fig. 5 *D* and *E*). In contrast, none of the four clones recognized the HLA-A2$^+$ESO$^-$ cell line, SK-MEL-23. A second example in SI Fig. 11 shows tumor recognition by two vaccine-elicited CD8$^+$ T cell clones (6-C-4 and 6-G-8) from patient 2.

TCR Usage of Vaccine-Induced NY-ESO-1-Specific CD8$^+$ and CD4$^+$ T Cell Clones. To determine the molecular basis of tumor recognition, we analyzed TCR diversity of clonal populations of vaccine-elicited,

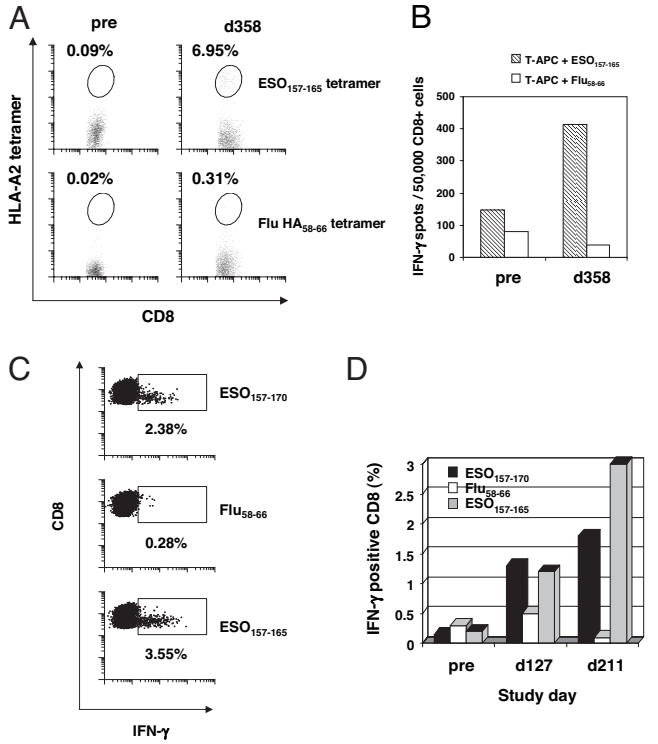

Fig. 4. CD8$^+$ T cell response to $ESO_{157–170}$ vaccine. (*A*) CD8$^+$ T cells were stimulated with $ESO_{157–170}$ peptide and stained with HLA-A2/$ESO_{157–165}$ multimer or HLA-A2/Flu $HA_{58–66}$ multimer. (*B*) IFN-γ production by $ESO_{157–170}$ specific CD8$^+$ T cells was analyzed by ELISPOT. (*C* and *D*) Confirmation by IFN-γ ICS.

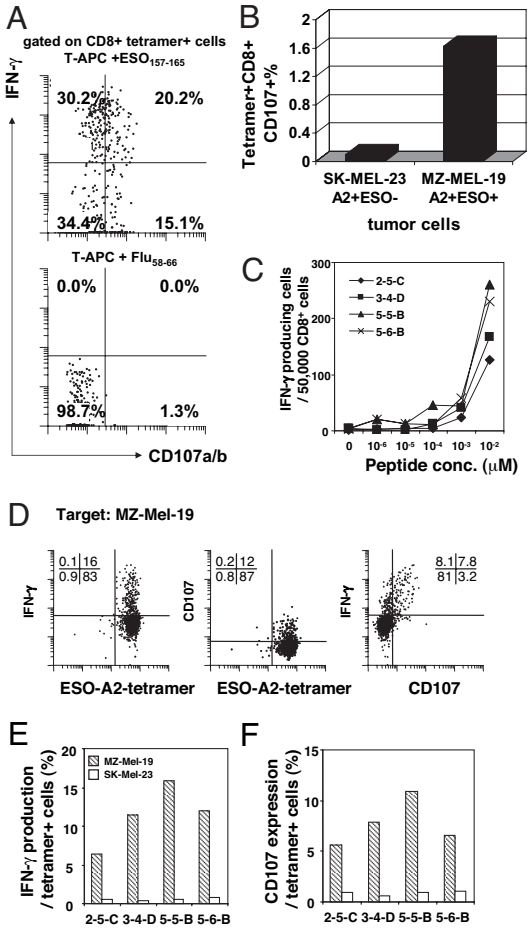

Fig. 5. Tumor recognition by vaccine-induced CD8$^+$ T cells. (*A*) After vaccine, $ESO_{157–170}$-specific CD8$^+$ T cells from patient 18 were tested against ESO-1b or irrelevant peptide Flu-HA in a CD107a/b surface and IFN-γ ICS assay. (*B*) HLA-A2/$ESO_{157–165}$ multimer-reactive CD8$^+$ T cells recognized HLA2$^+$ESO$^+$ cell line MZ-MEL-19 but not HLA-A2$^+$ESO$^-$ line SK-MEL-23. (*C*) Reactivity of CD8$^+$ T cell clones to $ESO_{157–165}$ peptide (range 10 nM to 1 pM). (*D–F*) All four CD8$^+$ T cell clones recognized HLA2$^+$ESO$^+$ MZ-Mel-19 but not HLA2$^+$ESO$^-$ SK-Mel-23.

$ESO_{157–165}$-specific CD8$^+$ CTLs and $ESO_{157–170}$-specific CD4$^+$ T cells obtained from patient 18. For CD8$^+$ T cell clones, we found identical usage of Vβ and Jβ, consistent with similarities in peptide avidity and tumor recognition (Table 1). All clones also had identical CDR3 lengths and sequences. In contrast, there was functional clustering of TCR usage by CD4$^+$ T cells based on tumor-recognition properties. Although TCR usage by non-tumor-reactive clones (including the two clones with higher peptide avidity than 5-B-8; i.e., 2-C-10 and 3-B-5) was identical (Vβ6.1 and Jβ2.5), clone 5-B-8 (able to recognize NY-ESO-1-expressing tumor cells) used Vβ6.7 and Jβ6.7 with different CDR3 lengths and sequences (Table 1). These results show that although peptide avidity is an important determinant of tumor recognition by vaccine-elicited effector cells, TCR structural diversity also is critical for effector function.

Characterization of Long-Lived NY-ESO-1-Specific CD8$^+$ and CD4$^+$ T Cell Responses. To determine the longevity of vaccine-induced, NY-ESO-1-specific CD8$^+$ and CD4$^+$ T cell responses, peripheral mononuclear cells were obtained from patients without evidence of disease for at least 6 months after completion of vaccination. We observed detectable NY-ESO-1-specific CD4$^+$ T cells in all patients tested at 6 and 12 months. To address the possibility that the observed response might not be related to $ESO_{157–170}$ peptide vaccination, the results were confirmed by presensitizing CD4$^+$ T cells with a pool of 17 peptides, 20-mer in length, overlapping with 10 aa, and covering the full length of the NY-ESO-1 protein (data not shown). As illustrated in Fig. 6*A*, $ESO_{157–170}$-specific CD4$^+$ T cells were detectable in patient 2 at 6 months after the last vaccination with $ESO_{157–170}$ peptide plus IFA. The CD4$^+$ T cells recognized autologous T-APC cells pulsed with $ESO_{157–170}$ peptide, ESO$^+$DP4$^+$ melanoma cell line SK-Mel-37 but not ESO$^-$DP4$^+$ melanoma cell line SK-Mel-23. These results indicate that vaccination with $ESO_{157–170}$ peptide plus IFA induced long-lived tumor reactive NY-ESO-1-specific CD4$^+$ T cells.

Table 1. Assessment of TCR BV (B variable) usage of vaccine-elicited CD8$^+$ A2/NY-ESO-$1_{157–165}$ multimer T cells and CD4$^+$ DP4/NY-ESO-$1_{157–170}$ T cells for patient 18

Clone	Vβ	CDR3β	Jβ
A2 (CD8)			
2-5-C	1	CAS SPGQGPEQ YFG	2.7
3-4-D	1	CAS SPGQGPEQ YFG	2.7
5-5-B	1	CAS SPGQGPEQ YFG	2.7
5-6-B	1	CAS SPGQGPEQ YFG	2.7
DP4 (CD4)			
2-C-10	(6)	CAS SLVAAEGTQ YFG	2.5
3-B-5	(6)	CAS SLVAAEGTQ YFG	2.5
5-B-8	(6)	CAS SLVPDSAYEQ YFG	2.7
8-H-12	(6)	CAS SLVAAEGTQ YFG	2.5

Next we characterized long-lived CD8$^+$ T cell responses to $ESO_{157–170}$ peptide plus IFA vaccination. CD8$^+$ T cells were presensitized with $ESO_{157–170}$ (DP4), $ESO_{157–165}$ (ESO-1b), pooled ESO peptides, or Flu HA peptide. HLA-A2-restricted $ESO_{157–165}$-specific CD8$^+$ T cells were detected at 6 months in patient 2 from CD8$^+$ T cells presensitized with either ESO-DP4 peptide or pooled ESO peptides by A2/$ESO_{157–165}$ multimer (SI Fig. 12). Moreover, the A2/$ESO_{157–165}$ multimer-reactive cells also recognized ESO$^+$A2$^+$ melanoma cells SK-Mel-37 and MZ-Mel-19 by IFN-γ ICS and CD107 (Fig. 6*B*). For patient 18, although A2/$ESO_{157–165}$ multimer-reactive CD8$^+$ cells were detectable 6 months after the last immunization, at 13 months, the cells were no longer detectable (SI Fig. 13), suggesting that vaccine-elicited CD8$^+$ T cells persisted for at least 6 months but <1 year.

Clinical Results. The median time to disease progression/recurrence from start of vaccination was 19.0 months (Kaplan–Meier 95% confidence interval: 9.0 months→∞). Although a Cox proportional hazards model with a time-varying covariate indicated the number of vaccines to be significantly related to disease-free survival (log rank, one-sided test; $P = 0.035$), this relationship could also be due to the reduced number of vaccines in patients with progressing disease. In one patient (patient 2) with measurable disease at enrollment, there was complete regression of metastatic disease after 10 immunizations (SI Fig. 14 *A* and *B*). This response correlated with induction of CD4$^+$ and CD8$^+$ T cell responses (SI Table 3 and SI Fig. 11). However, the patient developed recurrence of disease 8 months after discontinuation of vaccine. The patient did not receive cytotoxic chemotherapy during these intervening 8 months. She underwent secondary tumor-reductive surgery, followed by additional chemotherapy. Although the patient had detectable functional NY-ESO-1-specific CD8$^+$ and CD4$^+$ T cells 6 months after vaccination (Fig. 6 and SI Fig. 12) (i.e., 2 months before detectable recurrence) and the recurrent tumor was more highly infiltrated by CD8$^+$ and CD4$^+$ T cells compared with the primary tumor (SI Fig. 14 *C* and *D*), expression of NY-ESO-1 was no longer detectable by RT-PCR or immunohistochemistry (SI Fig. 14 *E* and *F*), suggesting antigenic loss as a possible mechanism of immune evasion in the patient.

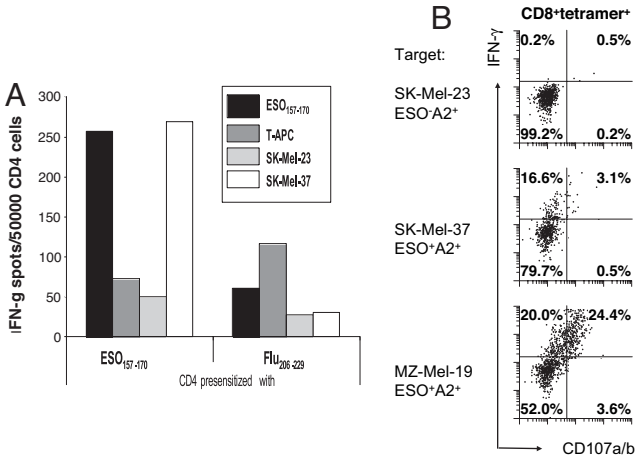

Fig. 6. Long-lived T cell immunity. (*A*) $ESO_{157–170}$-specific $CD4^+$ T cell response at 6 months for patient 2. (*B*) A2/$ESO_{157–165}$ multimer-reactive $CD8^+$ T cells recognized SK-Mel-37 and MZ-Mel-19, but not SK-Mel-23, when tested for CD107a/b and IFN-γ by ICS.

Discussion

During the past decade, remarkable progress has been made in understanding the interactions between the immune system and cancer. Importantly, evidence from correlative studies indicates that the presence of tumor-infiltrating lymphocytes may be associated with improved clinical outcome in several human cancers, including EOC (1, 8). In the first human study of NY-ESO-1 vaccination, $ESO_{157–165}$ peptide in conjunction with granulocyte/macrophage colony-stimulating factor was shown to induce HLA-A2-restricted $CD8^+$ T cell responses in patients without preexisting NY-ESO-1 immunity (9), although these peptide-induced $CD8^+$ T cell responses were generally of low affinity and did not recognize naturally processed NY-ESO-1 (10). Subsequently, recombinant NY-ESO-1 protein in a saponin-based adjuvant (ISCOMATRIX) was used to immunize stages III and IV melanoma patients after tumor resection (11). More recently, patients with a range of tumor types were immunized with recombinant vaccinia NY-ESO-1 and recombinant fowlpox NY-ESO-1 (12). These vaccine strategies induced high-titered NY-ESO-1 Ab, $CD4^+$, and $CD8^+$ T cell responses in a high proportion of patients. Nonetheless, because of their ease of production, peptide vaccines remain attractive candidates for clinical use. In the current study, we have chosen an NY-ESO-1-derived peptide with dual HLA class I and II specificities, in combination with IFA to immunize a homogenous population of patients with ovarian cancer.

Our results indicate that vaccination with NY-ESO-$1_{157–170}$ induced integrated Ab, $CD4^+$, and $CD8^+$ T cell responses in ovarian cancer patients (SI Table 4). The frequency of seroconversion that we observed is lower than that achieved with immunization with NY-ESO-1 protein/ISCOMATRIX (11) and recombinant vaccinia/fowlpox NY-ESO-1 (12) vaccines. One possible explanation for the lower frequency of B cell responses in our study is the relatively short length (14-mer) of the immunogen used compared with the full-length NY-ESO-1 used in the previous studies. Moreover, recent studies attempting to map B cell epitopes from spontaneous or full-length NY-ESO-1 vaccine-induced responses have demonstrated more frequent Ab responses to the N-terminal half compared with the C-terminal half of NY-ESO-1 (13), the location of the immunogen in the present study. Although induction of Ab response may be desirable for promoting T cell responses by *in vivo* cross-priming (14), there are reports indicating that T cell responses may be skewed to Th1 type in the absence of B cell responses in some systems (15). In this regard, our major objective of inducing tumor-reactive $CD4^+$ and $CD8^+$ T responses with this 14-mer peptide was achieved.

Based on our previous observations that direct *ex vivo* detection of NY-ESO-1-specific $CD4^+$ and $CD8^+$ T cells in peripheral blood is rare (even in patients with preexisting immunity to NY-ESO-1), we developed and optimized methods for amplifying NY-ESO-1-specific $CD4^+$ and $CD8^+$ T cell responses to include an *in vitro* stimulation step (16, 17). The absence of detectable NY-ESO-1-specific T cells in healthy donors and the short *in vitro* stimulation step strongly suggest that NY-ESO-1-specific T cells in vaccinated patients have been primed *in vivo*. Our analyses indicate that the majority of patients developed detectable $ESO_{157–170}$-reactive $CD4^+$ T cells. In contrast, only 4 of 14 (29%) patients developed class II-restricted reactivity to a related HLA-DP4-restricted epitope, $ESO_{161–180}$, in a study by Khong *et al.* (18). Although this result may be related to the differential ability of the peptides to induce class II-restricted immune responses, only two vaccinations were administered to the majority of patients in the study by Khong *et al.* (18). Our finding indicating a significant relationship between higher number of vaccinations and the maximal number of detectable $ESO_{157–170}$-reactive $CD4^+$ T cells supports the notion that peptide vaccination may require prolonged administration to demonstrate efficacy.

A relevant finding in this study is the demonstration of tumor recognition by vaccine-elicited $CD8^+$ and $CD4^+$ T cells. For $CD8^+$ T cells, previous clinical studies suggest that peptide vaccination with $ESO_{157–165}$ and $ESO_{157–167}$ generated NY-ESO-1-specific T cells that recognized peptides but not tumors (18, 19). Moreover, vaccination with $ESO_{157–167}$ elicited $CD8^+$ T cell responses against a cryptic HLA-A2 epitope (amino acids 159–167) that were not tumor-reactive (19, 20). Although we used a 14-mer peptide that included the cryptic NY-ESO-1 epitope, we have previously shown that the processing of longer peptides requires internalization and the action of the proteasome (21). Thus, the requirement for trimming 3 aa from the C terminus of $ESO_{157–170}$ could explain why we did not observe generation of T cells against the cryptic epitope in the current study. Because we consistently observed induction of $CD4^+$ T cells in the majority of patients, we propose that simultaneous induction of NY-ESO-1-specific $CD4^+$ T cells might have enhanced the effector function of vaccine-elicited $CD8^+$ T cells. In support of this hypothesis, vaccine-elicited $CD4^+$ T cells also demonstrated a predominant Th1 cytokine profile that could enhance the quality of $CD8^+$ T cells. In this regard, cognate $CD4^+$ Th1 cells could provide IL-2 to $CD8^+$ T cells (22) and promote induction of $CD8^+$ T cell responses through dendritic cell activation by CD40–CD40L interactions (23). Because our *in vitro* stimulation method for detecting NY-ESO-1-specific $CD4^+$ and $CD8^+$ T cells does not directly detect circulating precursor cells but rather cells proliferating in response to a cognate NY-ESO-1 epitope *in vitro*, the demonstration of tumor recognition is suggestive of the potential for recall responses and *in vivo* antitumor efficacy.

To gain insight into the structural basis of the functional differences between CTLs elicited from previous $ESO_{157–165}$ (ESO1b) peptide vaccine trials and the current study, we analyzed the TCR repertoire from one patient. Although TCR BV4.1 was dominant in previous $ESO_{157–165}$ (ESO1b) peptide vaccine trials (10), we found BV1 to be dominant in the current study and a striking homology of the CDR-3 regions of CTL clones that highly correlated to similar functional characteristics, such as tumor recognition and cytokine production. Although it is possible that differences in TCR usage could be shaped by host factors, such as allelic polymorphism in TCR gene segments (24) or recognition of autologous peptides by thymocytes in a different HLA context during negative selection in the thymus (25), our findings suggest that simultaneous induction of $CD4^+$ T cells by $ESO_{157–170}$ vaccination may be associated with recruitment of a functionally distinct repertoire of $CD8^+$ T cells with enhanced tumor-recognizing properties.

The generation of functional memory T cells is one of the major goals of cancer vaccines. In our study, we detected long-lived functional $ESO_{157–170}$-specific $CD4^+$ T cells and $ESO_{157–165}$ $CD8^+$ T cells 6 months after completion of immunizations in all patients and at 12 months in some patients. Although longevity of immune responses was not addressed in previous NY-ESO-1 peptide vaccine studies (9, 18, 19), sustenance of vaccine-induced responses might also be related to the dual MHC class I and II specificities of the peptide vaccine in the current study. Importantly, it has been reported that $CD4^+$ Th cells are required in determining the magnitude and persistence of CTL responses (26).

Although our study was designed as a phase 1 clinical trial, we noted encouraging clinical results. Considering that almost half of the study population consisted of patients who received between two and eight previous lines of chemotherapy for ovarian cancer, the finding of median progression-free survival of 19.0 months was striking. In general, after completion of front-line treatment for recurrent disease, EOC patients have progressively shorter and predictable progression-free intervals. Thus, progression-free survival after first-line i.v. platinum-based chemotherapy is ≈18 months (27) and is reduced significantly to 16–18 weeks in patients receiving second-line chemotherapy (28). Nevertheless, because a significant proportion of patients in our study still developed progression/recurrence of disease, we question the potential mechanisms of immune escape in the ovarian cancer population. We previously showed that the beneficial prognostic effect of $CD8^+$ tumor-infiltrating lymphocytes in ovarian cancer patients is adversely affected by $CD4^+CD25^+FOXP3^+$ regulatory T cells (Tregs) with immunosuppressive properties (1). Although we did not observe any significant expansion of Tregs by vaccination in the current study (data not shown), we previously showed that vaccination with an $ESO_{157–170}$ peptide failed to modulate the suppressive effect of Tregs on high-avidity NY-ESO-1-specific T cell precursors (7). In the current report, we found lack of NY-ESO-1 expression in recurrent tumors in a subset of patients, suggesting antigen loss as a potential mechanism of immune escape. Together the findings from our previous (1, 7) and current studies argue for future development of a multimodal immunization strategy in EOC to (*i*) actively counteract the effects of Tregs, (*ii*) minimize tumor antigen loss through epigenetic modulation, and (*iii*) incorporate multiple antigenic targets containing $CD8^+$ and $CD4^+$ T cell epitopes in the vaccine constructs.

Materials and Methods

Study Protocol and Patient Population. The NY-ESO-1 peptide-based phase I clinical study (protocol no. LUD02-011) was approved by the Institutional Review Board at Roswell Park Cancer Institute. Patients must have had histologically documented NY-ESO- or LAGE-1-expressing EOC or primary peritoneal carcinoma, stages II–IV at diagnosis. Expression of NY-ESO-1 and/or LAGE-1 was detected in tumors by RT-PCR and/or immunohistochemistry as described (3). The NY-ESO-1 peptide sequence for immunization was $ESO_{157–170}$ (SLLMWITQCFLPVF). The vaccine was composed of 100 μg of $ESO_{157–170}$ and 500 μl of IFA and was injected s.c. once every 3 weeks. In the absence of toxicity and disease progression that required other therapeutic interventions, patients received up 15 injections.

NY-ESO-1 Serum Ab. NY-ESO-1-specific Abs were measured in the serum by ELISA on the day of each vaccination and at 6 and 12 months after the last vaccination as described (3).

Analysis of NY-ESO-1-Specific T Cells. For the analysis of $CD8^+$ T cells, $ESO_{157–170}$ and a pool of synthetic overlapping 18- to 20-mer NY-ESO-1 peptides covering the entire NY-ESO-1-protein sequence were used for *in vitro* stimulation. $ESO_{157–165}$ and $ESO_{158–168}$ peptides also were used for *in vitro* stimulation in HLA-A2 and HLA-A24 patients, respectively. In all patients, purified $CD4^+$ T cells were stimulated with DP4 peptide ($ESO_{157–170}$). Presensitized $CD8^+$ T cells were stained with phycoerythrin-labeled HLA-A2 or HLA-A24 multimers as previously described (16). Presensitized $CD4^+$ T cells were tested for intracellular IFN-γ secretion; $CD8^+$ T cells were tested for IFN-γ and CD107a/b expression against target cells as previously described. All mAbs were obtained from BD PharMingen (San Diego, CA).

ELISPOT Assay. Presensitized $CD8^+$ or $CD4^+$ T cells were assessed by ELISPOT as described (17). A response was considered positive when spot numbers in triplicate assays in the presence of target cells significantly exceeded the cutoff value, corresponding to the number of nonspecific spots in the presence of flu-NP (for $CD4^+$ cells) or flu-HA (for CD8+ cells) peptide loaded on target cells (cutoff was mean ± 3 SD). A detailed description of cell lines, assay for tumor recognition, and molecular TCR repertoire analysis is available in *SI Materials and Methods*.

Statistical Analysis. Standard nonparametric and semiparametric statistical procedures were completed in the statistical environment. Specific procedures used include Kendall's τ, Wilcoxon's signed rank test, McNemar's test of symmetry, Kaplan–Meier survival estimator, and the Cox regression model.

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