

Phase I Trial of Overlapping Long Peptides from a Tumor Self-Antigen and Poly-ICLC Shows Rapid Induction of Integrated Immune Response in Ovarian Cancer Patients

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Abstract

Purpose: Long peptides are efficiently presented to both CD4⁺ and CD8⁺ T cells after intracellular processing by antigen-presenting cells. To investigate the safety and *in vivo* immunogenicity of synthetic overlapping long peptides (OLP) from a human tumor self-antigen, we conducted a phase I clinical trial with OLP from cancer-testis antigen NY-ESO-1 in various adjuvant combinations.

Experimental Design: Twenty-eight patients with advanced ovarian cancer in second or third remission were enrolled sequentially in three cohorts and received at least one vaccination. Patients in Cohort 1 ($n = 4$) received 1.0 mg OLP, Cohort 2 ($n = 13$) received OLP in Montanide-ISA-51, and Cohort 3 ($n = 11$) received OLP + 1.4 mg Poly-ICLC in Montanide-ISA-51 on weeks 1, 4, 7, 10, and 13. Humoral and cellular responses were evaluated by standardized immunomonitoring techniques (ELISA, ELISPOT assay, intracellular cytokine staining, and tetramer staining).

Results: The vaccine was generally well tolerated with injection site reactions and fatigue that resolved. NY-ESO-1-specific antibody and CD8⁺ T cells were undetectable after vaccination with OLP alone, but were found in 6 of 13 (46%) and 8 of 13 (62%) patients, respectively, after vaccination with OLP+Montanide, and in 10 of 11 (91%) and 10 of 11 (91%) patients, respectively, after vaccination with OLP+Montanide+Poly-ICLC. NY-ESO-1-specific CD4⁺ T cells were detected in all patients with greater frequency and polyclonality when Montanide-ISA-51 was used for vaccination. Inclusion of Poly-ICLC as an adjuvant further accelerated the induction of NY-ESO-1-specific immune responses.

Conclusions: The current study shows that NY-ESO-1 OLP vaccine is safe and rapidly induces consistent integrated immune responses (antibody, CD8⁺ and CD4⁺) in nearly all vaccinated patients when given with appropriate adjuvants. *Clin Cancer Res*; 18(23); 6497–508. ©2012 AACR.

Introduction

Cancer vaccines using short-minimal peptides from tumor antigens present limitations because they can only be used in patients with known HLA alleles that present

these epitopes in the absence of natural processing; alternatively, full-length protein vaccines often suffer from lack of consistent CD8⁺ T-cell induction, likely due to inefficient cross-presentation of the exogenous antigen by antigen-presenting cells (APC; ref. 1). In contrast, synthetic long peptides are efficiently presented to both CD4⁺ and CD8⁺ T cells by professional APCs as well as nonprofessional APCs (2). The cross-presentation of long peptides to CD8⁺ T cells requires proteasomal degradation, indicating that internalization and natural processing by APCs is required before loading on HLA molecules. The superior induction of CD8⁺ T cells *in vivo* by long peptides versus short peptides was shown in mice (3) and in humans by vaccinating with long peptides against human papillomavirus (4). However, the safety and immunogenicity of long-peptide vaccines targeting human self-antigens has not been sufficiently evaluated. In contrast to prophylactic vaccinations against pathogenic antigens, the efficacy of therapeutic cancer vaccination targeting self-antigens is potentially limited because of self-tolerance and tumor-induced immune suppression

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Translational Relevance

Human cancer vaccines have shown limited clinical efficacy to date, and choosing the right combination of antigen and adjuvant requires guidance from in-depth immunomonitoring. Therapeutic vaccines with a tumor self-antigen are achievable in mice, especially in combination with toll-like receptor (TLR) ligands, but questions remain in humans. How to achieve induction of antigen-specific immune responses in all patients? Does prior exposure of the immune system to tumor self-antigens limit the ability to vaccinate? The present study in patients with ovarian cancer shows that overlapping long peptides (OLP) from a tumor-derived self-antigen induce strong immune responses in nearly all patients when combined with Poly-ICLC and Montanide-ISA-51. Inclusion of Poly-ICLC significantly enhanced vaccine immunogenicity compared with OLP alone or OLP in Montanide-ISA-51. Tumor antigen presence or systemic immunosuppression did not negatively affect the vaccine's immunogenicity. These results provide a scientific rationale for using long peptides and TLR ligands in future human immunotherapeutic developments.

mechanisms. The specific effects of prior exposure of the immune system to antigen-expressing tumors on the quantity and quality of vaccine-induced immune responses have not been clearly investigated in human clinical trials.

NY-ESO-1 is an intracellular cancer/testis antigen found in a wide range of tumor types, but its expression in adult normal tissues is limited to the germ cells of the testis (5). In a subset of patients with NY-ESO-1-expressing tumor, naturally occurring immune responses to NY-ESO-1 are detectable in an integrated fashion, that is, they consist of spontaneous anti-NY-ESO-1 serum antibody responses together with NY-ESO-1-specific CD8⁺ and CD4⁺ T-cell responses (6, 7). Recently, we reported a significant correlation between spontaneous integrated immune responses against NY-ESO-1 and clinical benefit in patients with advanced melanoma receiving cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) blockade (8).

To induce NY-ESO-1 responses *in vivo*, a variety of NY-ESO-1-based vaccines such as human leukocyte antigen (HLA) class I and/or class II-binding peptides, recombinant viruses, DNA, and recombinant protein with or without adjuvants or delivery systems have been studied (5, 9–15). Although NY-ESO-1-specific serologic and cellular responses have been individually reported in patients vaccinated with several of these formulations, no vaccine has yet been shown to reliably induce antibody, CD4 and CD8 T-cell responses together, simultaneously, in all vaccinated patients. Strategies for a tumor vaccine that consistently induces an integrated immune response are therefore warranted.

Toll-like receptor (TLR) ligands are recognized as having adjuvant properties for augmenting T-cell responses to vaccines, but there have been very few controlled studies of

TLR agonists in addition to standard adjuvants. In the present open-label phase I study, we investigated a novel preparation of overlapping long peptides (OLP) from NY-ESO-1 while also testing 2 different adjuvant preparations (Montanide and Poly-ICLC) and a negative control group. Because neither NY-ESO-1 OLP nor their adjuvant combinations had been administered before, patients with advanced ovarian cancer enrolled sequentially and received: (i) NY-ESO-1 OLP alone (Cohort 1, $n = 4$), (ii) OLP in Montanide (Cohort 2, $n = 13$), or (iii) OLP and Poly-ICLC (polyinosinic-polycytidylic acid-stabilized by lysine and carboxymethylcellulose) in Montanide (Cohort 3, $n = 11$). The primary endpoint was safety and the secondary endpoint was immunogenicity (NY-ESO-1-specific antibody, CD4⁺ and CD8⁺ T cells). In addition, we retrospectively asked whether expression of NY-ESO-1 in the patients' tumors affected these endpoints. Induction of humoral and cellular immune responses was evaluated by our previously validated immunomonitoring techniques, to address vaccine-induced antibody, CD8⁺ and CD4⁺ T cells specific of NY-ESO-1.

Materials and Methods

Eligibility criteria

Eligible patients in this open-label, single institution phase I study had stage II to IV histologically documented epithelial carcinoma arising in the ovary, fallopian tube, or peritoneum (trial identifiers: NCT00616941, MSK07-152, LUD2006-001). Patients initially received cytoreductive surgery and chemotherapy with at least one platinum-based chemotherapy regimen. Following relapse, they returned to a second or third complete clinical remission with additional chemotherapy treatment. Remission was defined as CA125 less than 35 U/mL, unremarkable physical examination, and no definite evidence of disease by computed tomography. Nonspecific lymph nodes or soft tissue abnormalities 1 cm or less were permitted. Cytotoxic chemotherapy was to have concluded at least 4 weeks before the start of the study. Other requirements included Karnofsky Performance Status (KPS) 70% or more, neutrophil count 1.5×10^9 /L or more, hemoglobin 10 g/dL or more, platelets 80×10^9 /L or more, and aminotransferases 2.5 \times upper limit of the normal range or less. Patients with prior autoimmune diseases, severe allergic reactions, and serious intercurrent illness were excluded. All patients provided written informed consent for this Institutional Review-approved protocol. An Investigational New Drug application was filed by the Ludwig Institute for Cancer Research (LICR, NY).

Treatment plan

A total of 28 patients were sequentially enrolled in 3 cohorts. Patients received at least 1 subcutaneous immunization in rotating sites on the upper arms regardless of the expression of NY-ESO-1 in tumor tissues. Patients in Cohort 1 received 1.0 mg NY-ESO-1 OLP in 0.5 mL diluent; Cohort 2 received 1.0 mg NY-ESO-1 OLP in 0.5 mL diluent + 0.5 mL Montanide-ISA-51 VG (total of 1.0 mL); and Cohort 3 received 1.0 mg NY-ESO-1 OLP in 0.3 mL diluent + 0.7 mL (1.4 mg) Poly-ICLC + 1.0 mL Montanide-ISA-51 VG (total

of 2.0 mL administered in 2 syringes containing 1.0 mL each). Vaccines were administered on weeks 1, 4, 7, 10, and 13 with final study safety assessment on week 16. Delayed Type Hypersensitivity testing was conducted with 1 mg lyophilized NY-ESO-1 OLP at pretreatment and at week 16.

Evaluation during study

Pretreatment evaluations included history, physical and radiologic examination, vital signs, KPS assessment, and laboratory tests including complete blood cell counts, electrolytes and renal function, hepatic profile, thyroid stimulating hormone, prothrombin time, serum CA125 level, and urinalysis. Subsequent visits included vital signs and physical examination, toxicity assessment using CTCAE version 3.0, and serial laboratory studies to include complete blood cell counts, electrolytes and renal function, and hepatic profile. CT imaging was obtained every 2 months while on study, and standard RECIST criteria were used for disease progression. Blood samples for research studies (HLA typing, humoral and cellular immunity) were obtained pretreatment and at weeks 4, 7, 10, 13, and 16.

Statistical considerations

The primary objective of the study was to assess the safety of repeated vaccination with NY-ESO-1 OLP with or without the immunoadjuvants Montanide and Poly-ICLC administered every 3 weeks for 5 vaccinations. The secondary endpoint was to characterize the immune response with NY-ESO-1 OLP vaccination with or without the immunoadjuvants. The plan was for Cohort 1 (3 patients), Cohort 2 (9 patients), and Cohort 3 (9 patients) to accrue sequentially, based on safety requirements and availability of Poly-ICLC. Dose adjustment was not permitted. For the evaluation of safety, dose-limiting toxicity (DLT) was defined as \geq grade II allergic reaction, \geq grade III autoimmune reaction, \geq grade III hematologic or nonhematologic toxicity including fever, or \geq grade III injection site reaction. A total of 21 to 24 patients (if DLT was experienced) were planned. If 1 of 3 patients had DLT, an additional 3 patients would be added. If 1 of 6 patients had DLT, the arm would be considered safe and an additional 3 patients would be added. If an additional patient had DLT, the arm would be discontinued. The immunologic data was to be reported descriptively. Population and individual immune response curves generated 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 all patients. If at least 5 of 9 patients in Cohort 2 or 3 developed immune response (true response range 23%–28%), treatment would be considered worthy of further study. Time to disease progression was not an endpoint of this phase I study but would be recorded. Reciprocal antibody titers by ELISA were considered significant if ≥ 100 . Antigen-specific T-cell responses by enzyme-linked immunosorbent spot (ELISPOT) assays were considered significant if spot count was more than 50 out of 50,000 cells and at least 3 times more than the number of spots obtained with control nonpulsed target cells. Remaining data were analyzed using Prism 5 software (GraphPad

Software, Inc.) by unpaired 2-tailed *t* test or Kaplan–Meier method and log-rank test.

Immunohistochemical evaluation of NY-ESO-1 expression

Expression of NY-ESO-1 was assessed retrospectively by immunohistochemistry using E978 on archival paraffin blocks with representative areas of the surgical tumor resection specimens (16). Focal expression ($<5\%$ of tumor with E978 staining) was considered significant.

Synthetic peptides

The following 4 30/32mer peptides (vaccine OLP) were synthesized under cGMP conditions by Multiple Peptide Systems, and formulated as lyophilized powder in 25 mg palmitoyl oleoyl phosphatidyl choline (POPC) and acetic acid by Phares: NY-ESO-1₇₉₋₁₀₈; NY-ESO-1₁₀₀₋₁₂₉; NY-ESO-1₁₂₁₋₁₅₀; and NY-ESO-1₁₄₂₋₁₇₃. Because most of NY-ESO-1 epitopes recognized by CD4⁺ and CD8⁺ T cells are in the central to C-terminus hydrophilic region of the protein and because of high homology to LAGE-1 (5), the first 78 amino acids of NY-ESO-1 and the last 7 amino acids were excluded from the vaccine design. To enable efficient cross-presentation, the selected length of vaccine OLP was 30 amino acids with an overlap of 9 amino acids to include most of the possible CD4⁺ and CD8⁺ T-cell epitopes. Peptides were designed to avoid any strong anchor HLA-binding motif at the C-terminus to prevent the generation of cryptic epitopes (17). All vaccine peptides were characterized by high-performance liquid chromatography and mass spectrometry. A different set of 20-mer OLP (assay OLP, purchased from Bio-Synthesis or GenScript), covering the entire sequence of NY-ESO-1 with an overlap of approximately 10 amino acids, were used for the analyses of antibody mapping and T-cell responses and to avoid detection of immune responses to potential impurities of the vaccine OLPs.

Measurement of serum antibody

Plasma was obtained before and after vaccination by centrifugation of whole blood and stored at -80°C . Recombinant tumor antigen NY-ESO-1 and control dihydrofolate reductase (DHFR) proteins were used to coat plates and measure specific serum antibody levels in ELISA as described previously (18). In some experiments, 20-mer synthetic OLP were used as the coating antigen. A reciprocal titer was estimated from optical density readings of serially diluted plasma samples as described (18). Negative control sera from healthy individual and positive control sera for each antigen from patients with cancer were always included in all assays. The anti-human immunoglobulin antibodies used as secondary reagents were: alkaline phosphatase (AP)-labeled goat-anti-human IgG (polyclonal antisera; Southern Biotech), biotinylated mouse-anti-human IgG1 (Clone JDC-1; BD Pharmingen), AP-labeled mouse anti-human IgG2 (clone HP6002; Southern Biotech), AP-labeled mouse anti-human IgG3 (clone HP6050; Southern Biotech), AP-labeled mouse anti-human IgG4 (clone HP6023; Southern Biotech), AP-labeled goat-anti-human

IgA (polyclonal antisera; Southern Biotech), AP-labeled mouse-anti-human IgD-AP (clone IADB6; Southern Biotech), AP-labeled goat-anti-human IgE (Clone HP6029; Southern Biotech), and AP-labeled goat-anti-human IgM (polyclonal antisera; Southern Biotech). To be considered significant, reciprocal titers had to be more than 100.

Monitoring of T-cell responses

Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation over Lymphocyte Separation Medium (Mediatech Inc) and aliquots were stored in liquid nitrogen. *In vitro* stimulation (presensitization) of T cells from patients with cancer was carried out as described (19). Briefly, CD8⁺ and CD4⁺ T cells were isolated from PBMC by magnetic beads (Invitrogen Dynal) and independently stimulated with T-cell-depleted cells that were pulsed overnight with NY-ESO-1 assay OLP (6 μmol/L/each peptide). In some experiments, cells were pulsed with reduced amount of OLP at 100 nmol/L for each peptide. Presensitization with adenovirus recombinant for NY-ESO-1 (1,000 units/cells) was also conducted in most patients but only for some time points because of the limitation in the number of PBMCs available. CD8⁺ and CD4⁺ T cells were cultured in the presence of 10 U/mL IL-2 (Roche Molecular Biochemicals) and 20 ng/mL IL-7 (R&D systems) in RPMI-1640 medium supplemented with 10% human AB serum (Gemini Bio-Products), 2 mmol/L L-Glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1% nonessential amino acids (RPMI + 10% pooled AB serum). The number of cytokine-producing NY-ESO-1-specific T cells was evaluated by ELISPOT assay and intracellular staining of cytokines at day 9 to 14 for CD8⁺ T cells and at day 19 to 23 for CD4⁺ T cells. Each assay was repeated twice. Tetramers were also assayed at day 9 to 14 for CD8⁺ T cells for patients with appropriate HLA-class I alleles. Autologous Epstein-Barr virus-transformed B-cell lines (EBV-B cells) were generated from a part of CD4⁺ CD8⁻ cells by supernatant from B95-8 cells in RPMI + 10% fetal calf serum (FCS) and used as target cells in assays. For 6 patients whose autologous EBV-B cells failed to grow (M06, M11, M15, M24, M26, M28), HLA-compatible allogeneic EBV-B cells were used as target cells in ELISPOT assays. In addition, a part of CD4⁺ T cells was polyclonally expanded with phytohemagglutinin (PHA, Remel) in RPMI + 10% pooled AB serum in the presence of low-dose IL-2 and IL-7 to be used as target cells (T-APC) of CD4⁺ T cells in repeat assays.

ELISPOT assay

IFN-γ ELISPOT assay was conducted as described previously (19). Briefly, nitrocellulose-coated microtiter plates (Millipore) were coated overnight with anti-IFN-γ monoclonal antibody (2 μg/mL 1-D1K, MABTECH) and blocked with 10% human serum (Sigma-Aldrich) in RPMI-1640 medium. EBV-B cells were pulsed with subpools of assay OLP (10 μmol/L) or infected with vaccinia virus encoding NY-ESO-1 or Influenza virus nucleoprotein (NP) gene overnight at 37°C. Indicated number of effector cells were cocultured with 5 × 10⁴ antigen-pulsed EBV-B cells for

24 hours in RPMI medium without serum. Plates were developed using 0.2 μg/mL biotinylated anti-IFN-γ mAb (7-B6-1-biotin, MABTECH), 1 U/mL streptavidin-alkaline phosphatase conjugate (Roche Diagnostics GmbH), and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma-Aldrich). The number of spots was evaluated using C.T.L. Immunospot analyzer and software (Cellular Technologies). Results were shown as the average number of spots from duplicate wells without subtracting the number of background spots against unpulsed target cells. To be considered significant, antigen-specific IFN-γ responses had to have a spot count more than 50 out of 50,000 cells and at least 3 times more than the number of spots obtained with control nonpulsed target cells.

Tetramer and intracellular cytokine staining assays

Tetramer assays were conducted using HLA-A2/NY-ESO-1₁₅₇₋₁₆₅, HLA-B35/NY-ESO-1₉₄₋₁₀₂, and HLA-Cw3/NY-ESO-1₉₂₋₁₀₀ complexes made by the Lausanne facility of the Ludwig Institute, in patients with relevant HLA alleles, as described (19). Responses were considered significant if tetramer-positive cells were 0.5% or more of CD8⁺ T cells and were appearing clustered. Intracellular Cytokine Staining assays were conducted using mAb for CD8, CD4, IFN-γ, IL-5, CD45RA, and CCR7, as described (19). Responses were considered significant if more than 0.5% and at least 3 times more than the numbers obtained with control nonpulsed target cells.

Results

Patient characteristics and disposition

A total of 28 patients with epithelial ovarian cancer, fallopian tube, or primary peritoneal cancer in second or third complete clinical remission were enrolled sequentially in 3 cohorts (Supplementary Table S1), between March 2008 and June 2011. The patient characteristics are listed in Table 1. Of the 28 patients, 26 (93%) had stage III disease at diagnosis, with predominantly serous histology. All

Table 1. Patient characteristics

Patient characteristics (N = 28)	
Median age (range)	57 (44–73 years)
Median # prior chemotherapy (range)	2.4 (2–4)
FIGO Stage at diagnosis	N (%)
II	1 (3.6)
III	26 (92.2)
IV	1 (3.6)
Histology	
Serous carcinoma	24 (85.8)
Endometrioid carcinoma	2 (7.1)
Carcinoma not otherwise specified	2 (7.1)
Second remission at start of study	18 (64.3)
Third remission at start of study	10 (35.7)

patients had received platinum/taxane-based therapy, and 64% of patients entered the study in second remission and 36% were in third remission. The median time from last chemotherapy to first vaccination was 2.8 months (ranging from 1.2–5.5 months). From 26 patients with available tumor specimens assessed retrospectively, 2 had strong and 7 had focal (<5% sample) but significant NY-ESO-1 expression by immunohistochemistry, consistent with the expected NY-ESO-1 expression frequency in advanced ovarian cancer (Supplementary Table S1). For Cohort 1, 1 out of 4 patients was replaced because of disease progression at the third vaccination. For Cohort 2, 4 of 13 patients were replaced (2 because of disease progression at second and fourth vaccinations, respectively, and 2 due to unrelated medical illness or complication—edema and pneumonia, respectively). For Cohort 3, 2 of 11 patients were replaced (1 because of disease progression at second vaccination and 1 because of significant noncompliance on the part of the patient). The overall number of patients completing study as planned was 16 of 28 (57%). The ability to complete all 5 vaccinations was 3 of 4 in Cohort 1, 8 of 13 in Cohort 2, and 5 of 11 in Cohort 3 (Supplementary Table S1). The main reasons for study discontinuation were progressive disease (4/28 patients) and early study closure (4/11 in Cohort 3).

Safety

The vaccine was generally well tolerated, with mostly grade I (10, 76.9%) and II (1, 7.7%) adverse events unlikely related to the vaccine, except for injection site reactions and fatigue that were definitely or possibly related, respectively, that resolved (Supplementary Table S2). In Cohort 3, 1 patient developed grade II panniculitis at the injection site (Supplementary Fig. S1) and 3 patients had simple grade II injection site reactions. While the injection site reactions were grade II (and technically not DLT), they were more robust than anticipated and persistent (Supplementary Fig. S1), and therefore further vaccinations were held in the last 4 patients from Cohort 3. It was felt prudent to consider a lower dose of Poly-ICLC and/or Montanide before further study. These patients were followed for safety and recurrence as per protocol (Supplementary Table S1). The only grade III (severe) adverse events occurred in 2 patients in Cohort 2: pneumonia (unrelated) and abnormal neutrophil count (possibly related; Supplementary Table S2).

Monitoring of humoral immune responses

Spontaneous and vaccine-induced humoral immune responses were investigated by measuring serum IgG against recombinant full-length NY-ESO-1 protein in ELISA. Results are summarized in Fig. 1A and titers for individual patients are shown in Supplementary Fig. S2. Two patients had significant (reciprocal titer ≥ 100) spontaneous anti-NY-ESO-1 antibody before vaccination (M01 and M28). Patients who received OLP alone (Cohort 1, $n = 4$) failed to develop detectable NY-ESO-1-specific antibody throughout the vaccination period, except for baseline seropositive patient M01 whose titers gradually increased during vaccination. In contrast, evidence of NY-ESO-1 serum antibody

was found in 6 of 13 (46%) patients from Cohort 2 and 10 of 11 (91%) patients in Cohort 3, showing that these peptides are immunogenic. Inclusion of Poly-ICLC in Cohort 3 led to accelerated seroconversions and significantly increased antibody titers compared with Cohort 2 (Fig. 1B). Patients were able to mount similar antibody responses to the vaccine regardless of NY-ESO-1 tumor expression, although the 2 patients with baseline seropositive also had the highest NY-ESO-1 tumor expression. Early discontinuation of vaccination in the last 4 patients (Supplementary Table S1) did not negatively impact antibody responses, as titers had already developed maximally after 2 or 3 injections and remained high during follow-up without additional vaccination.

Characterization of antibody responses

A mapping of epitopes recognized by vaccine-induced NY-ESO-1-specific antibody was determined using 20-mer overlapping peptides as antigen in ELISA. As shown in Fig. 1C and Supplementary Fig. S2, antibody responses seen to NY-ESO-1 protein were confirmed against these peptides and found clustered in 3 areas of NY-ESO-1 centered around amino acids approximately 80, 100 to 110, and approximately 135 to 149. These stretches coincided with hydrophilic areas of the NY-ESO-1 sequences used in the vaccine (Fig. 1C), similarly with what is expected from naturally occurring immune responses. Specific antibody titers were detected to all 4 30-mer overlapping peptides used in vaccination, with no clear evidence of immunodominance, although recognition of the most C-terminus peptide (NY-ESO-1₁₄₂₋₁₇₃) was comparatively less frequent. Antibody mapping of samples from Cohort 3 was more widespread and showed a greater coverage of NY-ESO-1 epitopes than those from Cohort 2, possibly reflecting differences in overall titers. No clear response was detected against NY-ESO-1 region 1 to 80 outside of the vaccine, except for patient M28 who had preexisting immunity. Thus, Poly-ICLC enhanced humoral immune responses by not only accelerating the responses and increasing the antibody titer but also by broadening the repertoire of antibodies.

We next investigated isotypes and class switching of NY-ESO-1 antibodies induced by OLP in different formulations. As shown in Fig. 1D, most OLP-induced antibodies were of IgG1 and IgG3 isotypes. NY-ESO-1-specific IgG2, IgG4, IgA, and IgM were also detected in some patients by the end of vaccination. There was no clear evidence of early IgM response when measured at week 4 or 7 (not shown).

Monitoring of CD8⁺ T-cell responses

All patients were analyzed for CD8⁺ T-cell responses against NY-ESO-1 at all time points available by IFN- γ ELISPOT assays and whenever possible by staining of intracellular cytokines and tetramers. In patients with appropriate HLA alleles, we also tested purified unstimulated CD8⁺ T cells for NY-ESO-1 tetramer staining and failed to detect clear evidence of positive cells from any of the samples tested, confirming the difficulty to detect

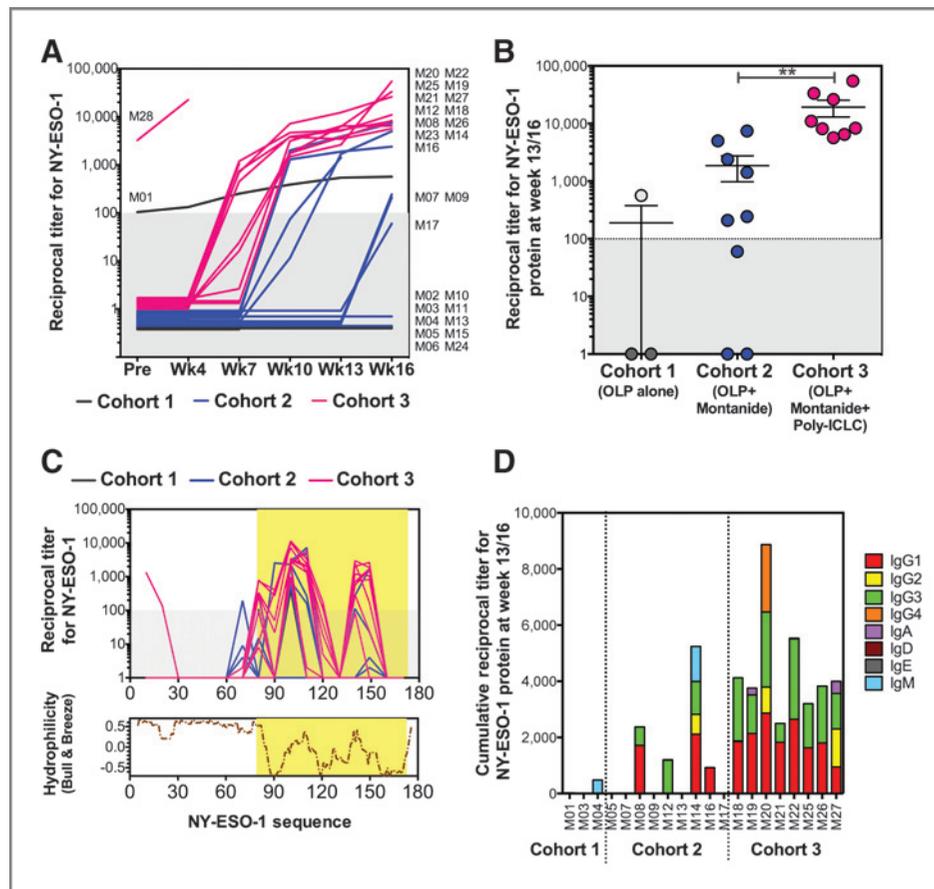


Figure 1. NY-ESO-1–specific humoral immune responses before and after vaccination with NY-ESO-1 OLP. **A**, reciprocal titers of plasma IgG antibodies were measured against recombinant NY-ESO-1 protein by ELISA throughout vaccination. Patients received OLP alone (Cohort 1, gray), OLP in Montanide (Cohort 2, blue), or OLP and Poly-ICLC in Montanide (Cohort 3, pink) at prestudy (pre), week (wk) 4, 7, 10, and 13, and plasma samples were collected before each injection and at week 16. Patient M01, who was baseline seropositive, is shown in light gray. **B**, comparison of maximal reciprocal titers achieved at week 13 or 16. Bars indicate average values \pm SEM. **, $P = 0.01$ by unpaired 2-tailed t test. **C**, mapping of epitopes recognized by vaccine-induced antibodies at week 16 was analyzed using 20-mer OLP in ELISA. Peaks represent titers against 20-mer individual peptides centered around indicated amino acids. Area covered by the vaccine indicated in yellow. Hydrophilicity profile of NY-ESO-1 plotted for reference. **D**, isotypes of NY-ESO-1–specific antibodies were determined using isotype-specific secondary anti-human immunoglobulin mAbs as indicated. Results were confirmed in multiple repeat assays and reciprocal titers were considered significant if 100 or more.

ex vivo responses to NY-ESO-1 from PBMC, even in patients with spontaneous immunity. Therefore, CD8⁺ T cells were stimulated once *in vitro* with a pool of 20-mer peptides from NY-ESO-1 using a protocol standardized to enable the amplification of NY-ESO-1-specific cells from circulating precursors without *de novo* induction. NY-ESO-1–specific CD8⁺ T cells were measured against peptide subpools covering C-terminal area 1 to 80 (outside of the vaccine), central area 71 to 130, and N-terminal area 119 to 180, to evaluate polyclonality.

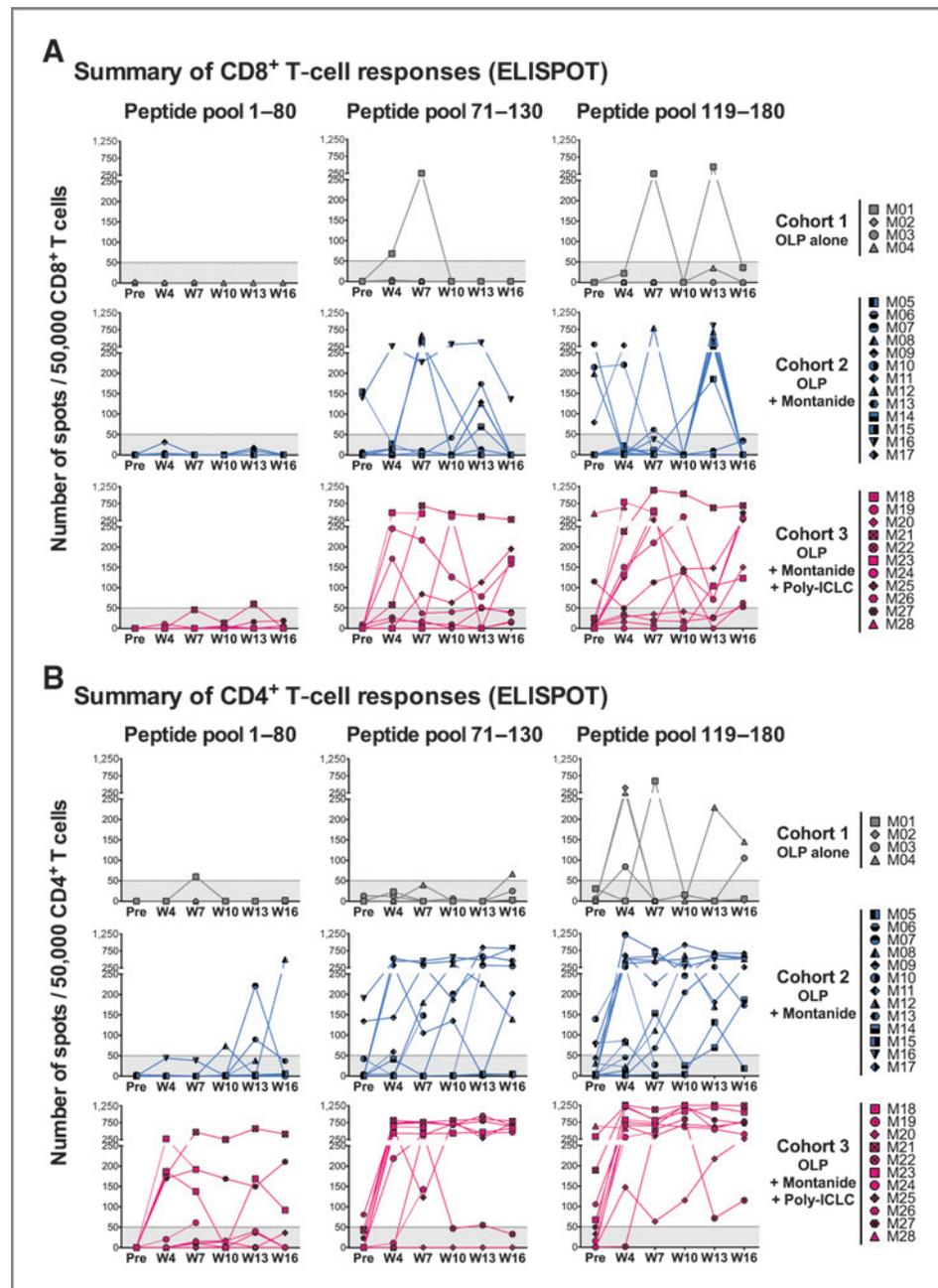
Figure 2A shows the magnitude of CD8⁺ T-cell responses detected by ELISPOT assays. From 4 patients in Cohort 1, CD8⁺ T-cell responses were detected only in baseline seropositive patient M01. From 8 of 13 (62%) patients in Cohort 2, weak and transient or sporadic CD8⁺ T-cell responses were detected from week to week, except for patient M16 who showed preexisting sustained responses. In contrast, 10 of 11 (91%) patients from Cohort 3 showed

evidence of CD8⁺ T-cell responses, and 7 of these were consistent and sustained throughout vaccination. Furthermore, Poly-ICLC helped in the rapid induction of NY-ESO-1–specific CD8⁺ T cells, apparent at week 4 after a single injection of OLP. Responses were polyclonal, as they could recognize at least 2 different areas of NY-ESO-1. Individual minimal epitopes recognized by these CD8⁺ T-cell responses were confirmed by tetramer assays in several patients with HLA alleles for which prevalidated tetramers were available (Fig. 3A). Corresponding ELISPOT responses against minimal peptide epitopes were also confirmed (not shown).

Quality of CD8⁺ T-cell responses

The ability of vaccine-induced CD8⁺ T cells to recognize naturally processed NY-ESO-1 was shown by sensitizing and expanding NY-ESO-1–specific CD8⁺ T cells using APCs transduced with adenovirus recombinant for full-length

Figure 2. Details of CD8⁺ and CD4⁺ T-cell responses for all patients throughout vaccination with NY-ESO-1 OLP. A, CD8⁺ T cells were stimulated once with T-cell-depleted PBMC pulsed with assay OLP and 10 to 13 days later, IFN- γ -producing NY-ESO-1-specific CD8⁺ T cells were enumerated by ELISPOT assays against EBV-transformed B cells pulsed with 3 subpools of 20-mer NY-ESO-1 peptides covering areas indicated. B, CD4⁺ T cells were stimulated once with T-cell-depleted PBMC pulsed with assay OLP and 20 to 23 days later, IFN- γ -producing NY-ESO-1-specific CD4⁺ T cells were enumerated by ELISPOT assays against EBV-transformed B cells pulsed with 3 subpools of 20-mer NY-ESO-1 peptides covering areas indicated. Results were confirmed in multiple repeat assays and a significant response was defined as a spot count more than 50 of 50,000 cells and at least more than 3 times the spot count of unpulsed targets (above gray area). Number of spots against unpulsed targets was not subtracted and was generally low (<50), but for samples with higher background spot numbers and where NY-ESO-1 responses failed to reach significance (i.e., <3 \times number of spots for unpulsed targets), results were artificially considered as 0 to avoid plotting false-positive results.



NY-ESO-1 (Fig. 3B). In addition, CD8⁺ T cells presensitized with NY-ESO-1 OLP were able to recognize target cells infected with vaccinia virus encoding full-length NY-ESO-1 (vvESO) in 4 of 16 patients tested after vaccination, including baseline NY-ESO-1-seropositive patient M28 (Fig. 3C). The absence of vvESO recognition in some patients could be ascribed to the preferential expansion of low-avidity T cells after *in vitro* presensitization with the regular 6 μ mol/L OLP, as using a lower dose (100 nmol/L) could recover reactivity to vvESO (Fig. 3D). Therefore, OLP vaccine induced CD8⁺ T cells of wide range of avidities, including some high-avidity responses comparable with

those observed in patients with spontaneous NY-ESO-1 immunity.

Monitoring of CD4⁺ T-cell responses

Monitoring of NY-ESO-1-specific CD4⁺ T cells is shown in Fig. 2B. All patients were analyzed for CD4⁺ T-cell responses against NY-ESO-1 at all time points available by IFN- γ ELISPOT assays and whenever possible by staining of intracellular cytokines. NY-ESO-1-specific CD4⁺ T cells were seen in all patients and were significantly enhanced by Montanide and further by Poly-ICLC. Polyclonal responses to at least 2 areas of NY-ESO-1 were seen as early

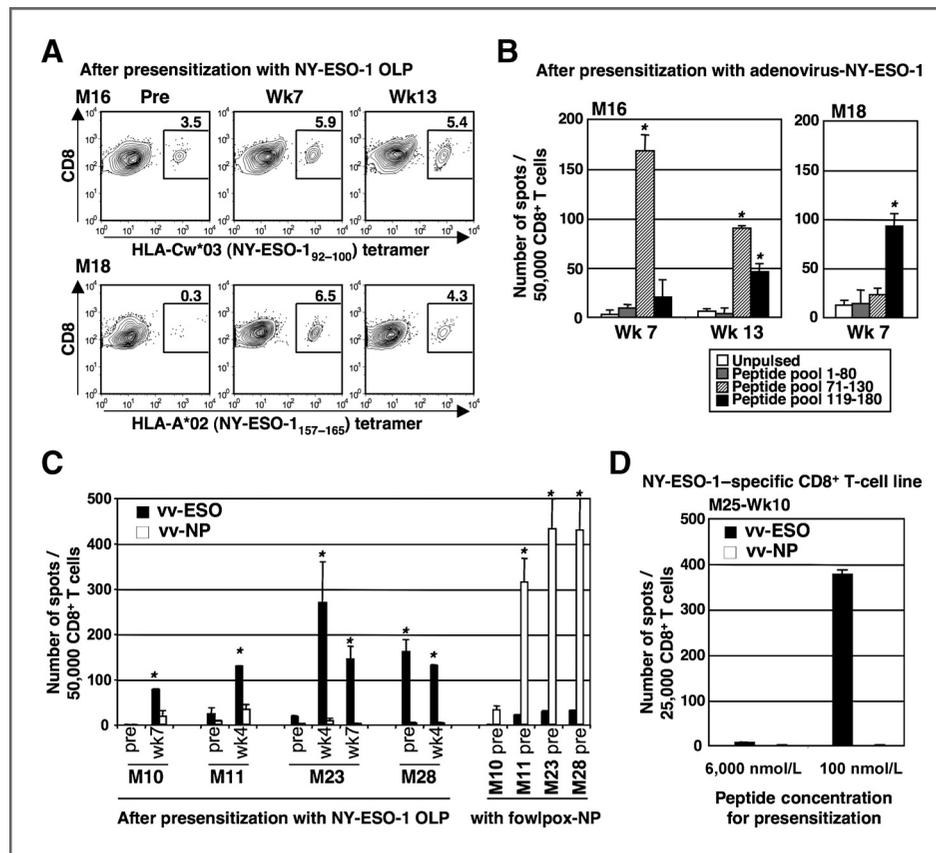


Figure 3. Characterization of OLP vaccine-induced CD8⁺ T cells. **A**, detection of vaccine-induced NY-ESO-1–specific CD8⁺ T cells by HLA tetramers. CD8⁺ T cells were stimulated with NY-ESO-1 OLP and after 10-day culture, NY-ESO-1–specific CD8⁺ T cells were enumerated by staining with indicated tetramers (pre, prestudy; wk, week). **B**, naturally processed adenovirus-induced NY-ESO-1 is able to expand OLP vaccine-induced CD8⁺ T cells. CD8⁺ T cells were stimulated with T-cell–depleted PBMC infected with adenovirus-encoding NY-ESO-1 and 10 days later, IFN- γ -producing NY-ESO-1–specific CD8⁺ T cells were enumerated by ELISPOT assays. Error bars indicate SD from duplicate wells. **C**, recognition of vaccinia virus–induced NY-ESO-1 by OLP vaccine-induced CD8⁺ T cells. CD8⁺ T cells were stimulated with NY-ESO-1 assay OLP and 9 to 14 days later, IFN- γ -producing CD8⁺ T cells were enumerated by ELISPOT assays. Target cells were autologous EBV-B cells infected with vaccinia virus–encoding NY-ESO-1 (vv-ESO) or influenza virus nucleoprotein (vv-NP) at multiplicity of infection (MOI) of 30. Control responses to vv-NP are shown following presensitization with fowlpox virus recombinant for NP. **D**, selective expansion of high-avidity CD8⁺ T cells by presensitization with peptides at low concentration. CD8⁺ T cells were stimulated with 6,000 or 100 nmol/L NY-ESO-1 OLP, and IFN- γ -producing CD8⁺ T cells were isolated after restimulation with peptide-pulsed T-APC, polyclonally expanded, and tested by ELISPOT assay against autologous EBV-B cells infected with vv-ESO or vv-NP. *, significant response as defined by spot count more than 50 of 50,000 cells and at least more than 3 times the spot count of unpulsed or control targets.

as after 1 vaccination in the majority of patients from Cohort 3. Most HLA-DP4⁺ patients also developed a CD4⁺ T-cell response to HLA-DP4–restricted epitope NY-ESO-1_{157–170} (not shown). These vaccine-induced CD4⁺ T-cell responses were confirmed by intracellular cytokine staining, with mostly IFN- γ and less IL-5 secretion (Supplementary Fig. S3). Interestingly, consistent CD4⁺ T-cell responses against the region excluded from the vaccine, NY-ESO-1_{1–78}, were observed in 3 patients from Cohort 3, and transiently in 6 other patients. Detectable presence of NY-ESO-1 in patient tumors did not seem to affect the number or nature of CD4⁺ T-cell responses generated.

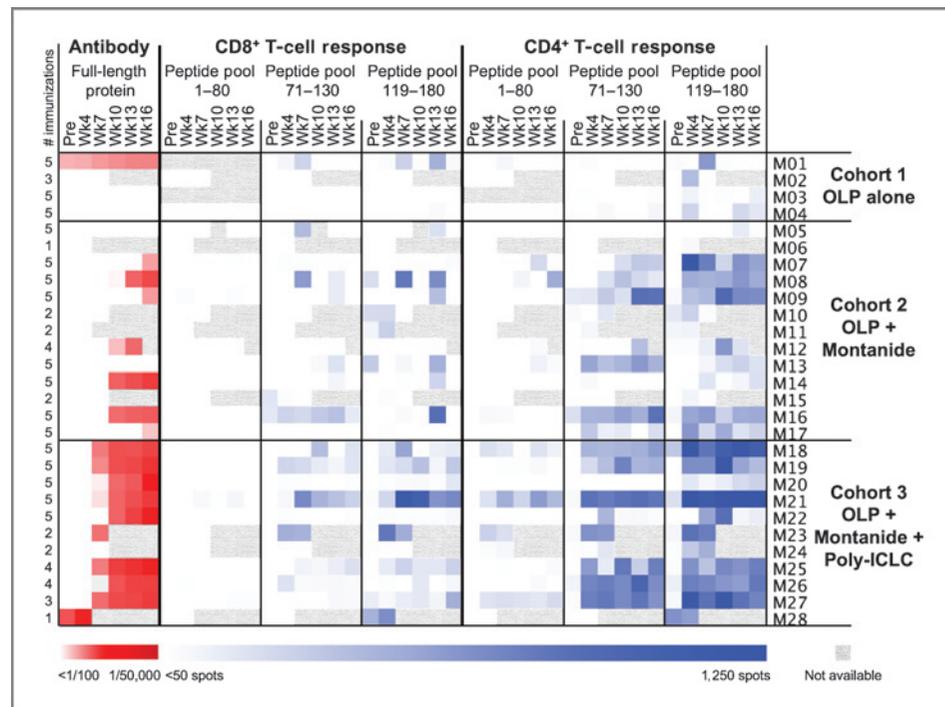
Results of NY-ESO-1–specific antibody, CD4⁺ and CD8⁺ T-cell responses are summarized in a heatmap format in Fig. 4. Collectively, occurrence of NY-ESO-1–specific CD4⁺ T cells together with antibody and CD8⁺ T-cell responses, that is, integrated immune responses, was seen in 1 of 4 patient

from Cohort 1, 4 of 13 patients from Cohort 2, and 10 of 11 patients from Cohort 3, indicating that Poly-ICLC significantly improved the immunization not only by accelerating and broadening immune responses, but also by making them more consistent (higher counts for CD4⁺ T-cell responses against each NY-ESO-1 peptide subpool at week 4 in Cohort 3 compared with Cohort 2, higher overall maximal CD4 T-cell response and more integrated Ab-CD8-CD4 responses in Cohort 3 than in Cohort 2, $P < 0.03$ by unpaired t test).

Impact of NY-ESO-1 tumor expression on antibody, CD4⁺ and CD8⁺ T-cell responses

The impact of NY-ESO-1 tumor expression on vaccine-induced immune responses was evaluated in Cohort 3 where 5 patients showed NY-ESO-1 tumor expression and 5 did not (Supplementary Table S1). Expression of

Figure 4. Summary of antibody, CD8⁺ and CD4⁺ T-cell responses throughout vaccination with NY-ESO-1 OLP. In shades of red, IgG antibody titers by ELISA against NY-ESO-1 protein from individual patient plasma samples represented as a heatmap from prestudy (pre) to week (wk) 16, with levels indicated in legend. In shades of blue, number of IFN- γ spots by ELISPOT of 50,000 CD8⁺ and CD4⁺ T cells following presensitization with a pool of 20-mer OLP covering all of NY-ESO-1, and tested against EBV-transformed B cells pulsed with 3 subpools of these peptides covering areas indicated; represented as heatmap with levels indicated in legend. In textured gray, samples and time points not available. Number of immunizations received for each patient shown on left.



NY-ESO-1 antigen by the patient tumors did not affect the induction of NY-ESO-1 antibody, as average maximal titers at the end of vaccination did not significantly differ for patients with and without NY-ESO-1 tumor expression (Fig. 5A, $P = 0.25$ at week 16, t test). Similarly, NY-ESO-1 CD8⁺ and CD4⁺ T-cell responses were very comparable in patients with and without NY-ESO-1 tumor expression (Fig. 5B, $P = 0.25$ at week 16, t test).

Impact of CD4⁺CD25⁺ regulatory T cells

Tumor vaccines have been reported to expand regulatory T cells (Tregs; refs. 4, 20). As reported previously (21, 22), NY-ESO-1-specific CD4⁺ T cells that were not detectable from whole CD4⁺ T-cell population before vaccination became detectable by removal of CD25⁺ T cells in several patients (Fig. 5C). In contrast to the prevaccination specimens, the effect of the removal of CD25⁺ T cells at week 4, 13, and 16 was not consistent, increasing some responses in patients while reducing others, or having no effect at all. From the observations that NY-ESO-1-specific IFN- γ -producing T cells were detectable in the presence of Tregs, and that removal of Tregs did not consistently enhance the expansion of effector CD4⁺ T cells after vaccination, it is unlikely that vaccination systematically induced NY-ESO-1-specific CD25⁺ Tregs able to actively suppress the expansion of NY-ESO-1-specific effector CD4⁺ T cells.

Long-term follow-up and clinical observations

At the last follow-up in February 2012, 3 of 4 patients had progressive disease (PD) in Cohort 1 (median 9 months, range 1–9), and 1 had no evidence of disease (NED) at 46 months. In Cohort 2, 10 of 13 had PD (median 4.5 months, range 1–15 months), and 3 were NED (median 41 months,

range 29–46 months). In Cohort 3, 9 of 11 patients had PD (median 6 months, range 2–22 months) and 2 were NED (median 21 months, range 17–25 months). Although the study was not randomized and the small number of patients in this study does not allow for definitive conclusions, time-to-progression was analyzed *posthoc* by the Kaplan–Meier method and no significant difference was found between cohorts overall. However, among 10 evaluable patients vaccinated with OLP+Montanide+Poly-ICLC (Cohort 3), those with baseline evidence of NY-ESO-1 tumor expression ($n = 5$) showed a significantly delayed time to recurrence compared with NY-ESO-1-negative patients (Fig. 5D). Such delayed time to recurrence was not observed for the other 2 cohorts when stratified by NY-ESO-1 expression.

Discussion

In this study, we showed that NY-ESO-1 OLP were immunogenic when used in combination with adjuvant. Both Montanide and Poly-ICLC significantly influenced the kinetics, magnitude, and quality of responses elicited by vaccination, resulting in integrated responses of antibody, CD8⁺ and CD4⁺ T cells for NY-ESO-1 in nearly all patients. Patients receiving vaccination with OLP in the presence of Montanide and Poly-ICLC developed significantly higher, earlier, and more frequent antibody, CD4⁺ and CD8⁺ T-cell responses to NY-ESO-1 than those vaccinated with OLP in Montanide alone or without adjuvant. Patients in this study had typical characteristics of second or third ovarian cancer remission, and experienced multiple prior chemotherapies. The rationale for inclusion of patients without NY-ESO-1 expression was to retrospectively evaluate the impact of expression on safety and immune responses. In addition, beside heterogeneity of expression that may lead to false-

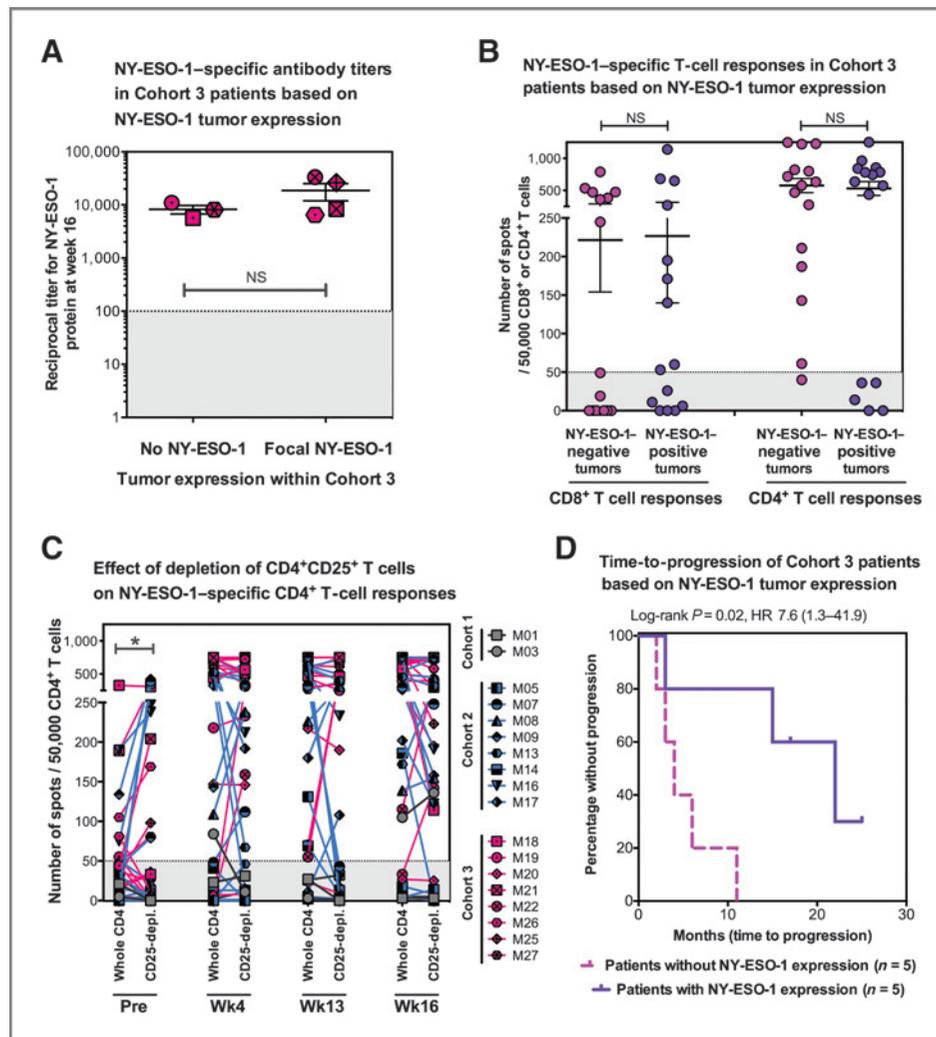


Figure 5. Effect of NY-ESO-1 tumor expression and of Treg-containing $CD4^+CD25^+$ T cells on vaccine-induced NY-ESO-1 immune responses. **A**, comparison of antibody titers achieved at week 16 for cohort 3 patients based on available NY-ESO-1 tumor expression data. NS, not significant difference in mean titers. **B**, comparison throughout vaccination of maximal $CD8^+$ and $CD4^+$ T-cell responses measured by IFN- γ ELISPOT against all subpools of NY-ESO-1 assay OLP for cohort 3 patients depending on original NY-ESO-1 tumor expression. NS, not significant difference in mean T-cell counts. **C**, effect of removal of $CD4^+CD25^+$ T cells on the *in vitro* expansion of NY-ESO-1-specific $CD4^+$ T cells. Whole $CD4^+$ T cells (Whole) or $CD4^+CD25^-$ T cells (CD25-depl) were stimulated once with T-cell-depleted PBMC pulsed with NY-ESO-1 assay OLP and after 20-day culture, NY-ESO-1-specific $CD4^+$ T cells were enumerated by ELISPOT assays. EBV-B cells pulsed with 20-mer OLP subpools 71 to 130 or 119 to 180 were used as target cells. CD25 (Treg)-depletion resulted in increased T-cell responses before vaccination at prestudy (pre), but not at later time points [week (wk)]. *, $P < 0.05$ by paired 2-tailed t test (also significant if considering Cohort 2 patients only, shown in blue). **D**, time-to-progression in patients from Cohort 3 (OLP+Montanide+Poly-ICLC) according to NY-ESO-1 expression status in their tumor as determined by immunohistochemistry (see Supplementary Table S1). When analyzed by Kaplan-Meier method, the curves were found significantly different in Log-rank (Mantel-Cox) test ($P = 0.02$), with a median survival of 22 months for patients with any NY-ESO-1 tumor expression (solid line) versus 4 mos for patients without the presence of NY-ESO-1 in their tumor (dotted line); HR = 7.6 [95% confidence interval (CI) of ratio: 1.3–41.9].

negative results, the frequency of NY-ESO-1 expression in cancer is known to increase with stage and grade, so that some patient may express NY-ESO-1 upon recurrence even if their original tumor was considered negative. NY-ESO-1 expression in the tumor was detected in 9 of 26 (35%) of patients, within expectations, and it did not seem to affect the safety or immunogenicity of the vaccine. Treatment with NY-ESO-1 OLP was well tolerated regardless of adjuvant, and was not associated with any clinical systemic toxicities attributable to autoimmunity. An increase in local injection

site reactions was seen in Cohort 3 patients receiving both Montanide and Poly-ICLC and led to the discontinuation of vaccinations in this cohort. The skin reactions may have been related to the volume of diluent required for the administration of both adjuvants and seemed to be a local irritant phenomenon, as observed in other studies (23).

Strong antibody responses against vaccine OLP, which excluded the most immunogenic N-terminal region for naturally induced antibodies, were induced in most patients after vaccination with OLP in Montanide, especially when

combined with Poly-ICLC. Titers were sustained even in patients who did not complete all planned immunizations. These results indicate that the antigen in a form of long peptide is a strong immunogen that induces antibody responses even against less immunogenic parts of the protein.

Vaccination with OLP coemulsified in Montanide and Poly-ICLC was also able to induce consistent antigen-specific CD8⁺ T cells in nearly all patients. This is in contrast to vaccination with whole NY-ESO-1 protein, against which less than 50% of patients develop CD8⁺ T-cell responses when coemulsified in Montanide with CpG7909, or even in delivery systems that facilitate cross-presentation of a formulated protein (12–14). While full-length protein vaccines usually result in only a limited repertoire of NY-ESO-1-specific CD8⁺ T cells in half of the treated patients (9, 12, 24), OLP vaccines emulsified in Montanide with Poly-ICLC generally induced frequent CD8⁺ T cells recognizing multiple epitopes and in some cases naturally processed NY-ESO-1. Although OLP vaccine induced frequent polyclonal-specific CD8⁺ T cells, these were found to have a wide range of avidities (Fig. 3), including high-avidity responses which were sometimes obscured by low-avidity cells following *in vitro* expansion, as observed in short-peptide vaccination (25). Combination vaccination with recombinant DNA or viruses (26), shown to induce high-avidity responses, or using a reduced dose of OLP vaccine should be explored to favor the expansion of high-avidity CD8⁺ T cells rapidly induced by OLP vaccine.

Vaccination with OLP in Montanide efficiently and consistently induced NY-ESO-1-specific CD4⁺ T cells recognizing multiple epitopes in vaccine OLP, regardless of NY-ESO-1 tumor expression. The responses were often elicited after a single vaccination, something not previously observed with short-peptide or protein vaccination (12, 27). Surprisingly, CD4⁺ T-cell responses against the NY-ESO-1_{1–80} region, which is weakly immunogenic for T cells and thus was excluded from the vaccine, were induced in several patients, especially in the presence of Poly-ICLC (Fig. 2B). Potentially, this epitope spreading could be induced by NY-ESO-1 protein released from NY-ESO-1-expressing residual tumors. Another possibility is that proinflammatory cytokines induced by Poly-ICLC allowed the expansion of NY-ESO-1-specific CD4⁺ T-cell precursors, by interfering with the *in vivo* suppression by Tregs (28). We are currently investigating whether qualitative differences exist between CD4⁺ T-cell responses specific for epitopes outside and within the NY-ESO-1 vaccine sequences.

Poly-ICLC is a viral mimic in man, upregulating a broad variety of immune-related genes representing multiple innate immune pathways in a pattern that closely parallels that of a live virus vaccine (29). This innate immune activation is likely playing an important role in the nature and extent of the responses that we show here with OLP. Poly-ICLC increased the kinetics and number of antigen-specific effectors (23, 30).

In summary, vaccination with OLP induced a rapid and strong integrated immune response orchestrated by both

CD4⁺ and CD8⁺ T cells as well as antibodies. In this study, Montanide was necessary to induce immune responses after peptide vaccination, and Poly-ICLC further accelerated and enhanced these responses. It remains to be determined whether adding Poly-ICLC to a vaccine formulation may render Montanide dispensable, and this question may be answered in an ongoing-related clinical trial of NY-ESO-1 protein and Poly-ICLC with or without Montanide (identifier NCT01079741). No systemic toxicity related to vaccination was seen. Local injection site reactions in some patients who received Montanide and Poly-ICLC warrant the consideration of reducing the volume of administration of Montanide, Poly-ICLC, or both. Future trials should address clinical efficacy in patients with strong NY-ESO-1 tumor expression, with biopsies before and after treatment whenever possible to assess *in situ* immune responses. Vaccine strategies may be improved further by combining OLP vaccination with modulators of immunosuppression, such as anti-CTLA-4, anti-PD-1, or anti-GITR that are integrally important for the optimization of future cancer vaccines.

Disclosure of Potential Conflicts of Interest

A.M. Salazar is the CEO and Scientific Director of Oncovir Inc, which is developing Poly-ICLC. T. Tsuji, G. Ritter, L.J. Old, and S. Gnjatic are inventors on patents/patent application regarding the NY-ESO-1 antigen, and Ludwig Institute for Cancer Research has licensed such patents. No potential conflicts of interest were disclosed by the other authors.

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Phase I Trial of Overlapping Long Peptides from a Tumor Self-Antigen and Poly-ICLC Shows Rapid Induction of Integrated Immune Response in Ovarian Cancer Patients

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