NY-ESO-1 DNA Vaccine Induces T-Cell Responses That Are Suppressed by Regulatory T Cells

Sacha Gnijatic,1 Nasser K. Altorki,2 Derek Ng Tang,3 Shi-Ming Tu,3 Vikas Kundra,4 Gerd Ritter,1 Lloyd J. Old,1 Christopher J. Logothetis,3 and Padmanee Sharma3,5

Abstract Purpose: Different vaccination strategies against the NY-ESO-1 antigen have been employed in an attempt to induce antitumor immune responses. Antigen-specific effector T-cell responses have been reported in a subset of vaccinated patients; however, these responses have not consistently correlated with disease regression. Here, we report for the first time clinical and immune responses generated by the NY-ESO-1 DNA vaccine administered by particle-mediated epidermal delivery to cancer patients.

Experimental Design: Eligible patients received treatment with the NY-ESO-1 DNA vaccine. Clinical outcomes and immune responses were assessed.

Results: The NY-ESO-1 DNA vaccine was safely administered and induced both antigen-specific effector CD4 and/or CD8 T-cell responses in 93% (14 of 15) of patients who did not have detectable pre-vaccine immune responses. Despite the induction of antigen-specific T-cell responses, clinical outcomes consisted predominantly of progressive disease. Detectable effector T-cell responses were inconsistent and did not persist in all patients after completion of the scheduled vaccinations. However, high-avidity CD4 T-cell responses that were either undetectable pre-vaccine or found to be diminished at a later time during the clinical trial were detected in certain patients’ samples after in vitro depletion of regulatory T cells.

Conclusions: Regulatory T cells play a role in diminishing vaccine-induced antigen-specific effector T-cell responses in cancer patients. The NY-ESO-1 DNA vaccine represents a feasible immunotherapeutic strategy to induce antigen-specific T-cell responses. Counteracting regulatory T-cell activity before vaccination may lead to prolonged effector T-cell responses and possibly antitumor responses in cancer patients.

Immunologic unresponsiveness to self-antigens and foreign antigens is mediated by central and peripheral mechanisms. Clonal deletion in the thymus and the induction of anergy are well-characterized mechanisms for the establishment and maintenance of tolerance (1). However, it is now clear that, in addition to these mechanisms, active suppression by regulatory T cells also allows for tolerance to both self-antigens and foreign antigens (2–9). Various subsets of regulatory T cells have been described, and much effort has been focused on understanding their ontogeny, function, and mechanisms of action. Within the CD4 T-cell subset, there is a population of naturally occurring CD4+CD25hiFOXP3+ T cells that are defined as regulatory T cells. These cells can be identified as CD4+FOXP3+ T cells by flow cytometry. FOXP3 is the forkhead box P3 transcription factor that controls regulatory T-cell development (8, 9). However, because FOXP3 is intracellular and requires permeabilization of cells for detection by flow cytometry, regulatory T cells are isolated as CD4+CD25hi regulatory T cells. These cells can be identified as CD4+FOXP3+ T cells by flow cytometry. FOXP3 is the forkhead box P3 transcription factor that controls regulatory T-cell development (8, 9). However, because FOXP3 is intracellular and requires permeabilization of cells for detection by flow cytometry, regulatory T cells are isolated as CD4+CD25hi regulatory T cells, which can be shown to have functional suppressive abilities in coculture experiments. In cancer patients, CD4+CD25hi regulatory T cells have been shown to be increased in lung, ovarian, esophageal, and prostate cancer patients (10–15). In one study with ovarian cancer patients, regulatory T cells were noted to specifically inhibit antitumor immunity and increased numbers of regulatory T cells predicted for poor survival (16). It was shown in a mouse model system that the balance between effector and regulatory T cells can influence immune responses elicited by vaccination with a tumor antigen and these regulatory T cells suppressed effector T-cell responses in vivo (17).

NY-ESO-1 is a promising target antigen in patients as a candidate for specific immune recognition of cancer because it has restricted expression in normal tissue but frequently occurs on human tumors (18). Presence of NY-ESO-1 is seen in...
approximately one third to one fourth of all lung, melanoma, ovarian, esophageal, bladder, and prostate cancers, and expression is often associated with high-grade tumors. NY-ESO-1 is spontaneously immunogenic in a proportion of these patients, eliciting both CD8 and CD4 T-cell responses that correlate with the presence of serum antibodies to NY-ESO-1 (19, 20). It was also shown that spontaneously occurring CD4 T-cell responses against NY-ESO-1 may be suppressed by CD4+CD25+ regulatory T cells (21). Clinical trials have been conducted using different NY-ESO-1 peptides and full-length protein (22–26). To deliver full-length antigen and minimize possible degradation of the antigen that can be associated with naked protein vaccines, we proposed a clinical trial with the NY-ESO-1 DNA vaccine. The effect of regulatory T-cell responses on NY-ESO-1 vaccine-induced T-cell responses has not been reported previously.

DNA immunization can be done either by skin bombardment with plasmid DNA coated onto microscopic gold particles using a gene gun or by direct intramuscular injection of plasmid DNA. The former method, also known as particle-mediated epidermal delivery, has been shown to result in higher transgene expression, compared with direct intramuscular injection, and to lead to protective immunity in a variety of animal models of infection and cancer (27–30). We relied on the Powderject particle-mediated epidermal delivery system for administration of the NY-ESO-1 DNA vaccine.

Here, we report the first NY-ESO-1 DNA vaccine clinical trial in 16 patients with advanced cancer, diagnosed as prostate adenocarcinoma (10 patients) or non-small cell lung cancer (NSCLC; 5 patients) or esophageal carcinoma (1 patient). The NY-ESO-1 DNA vaccine induced T-cell responses in treated patients, but these responses were not consistently detected throughout the vaccination schedule. Depletion of regulatory T cells from samples that previously did not reveal detectable antigen-specific T-cell responses led to identification of NY-ESO-1-specific CD4 T-cell responses, which were noted to be high-avidity T-cell responses, as defined previously by us and others using peptide titration experiments and assessing T-cell responses to naturally processed antigen (31–33). Therefore, countering regulatory T-cell activity before vaccination may prove to be an important method for induction of optimal effector T-cell responses.

Materials and Methods

Clinical trial. NY-ESO-1 plasmid DNA (pPV7611) was produced by PowderMed and Ludwig Institute for Cancer Research sponsored the clinical trial with the NY-ESO-1 DNA vaccine. All NSCLC (n = 5) and esophageal cancer (n = 1) patients were consented on an institutional review board-approved protocol at Cornell University, New York Hospital, and all prostate adenocarcinoma patients (n = 10) were consented on an institutional review board-approved protocol at The University of Texas M. D. Anderson Cancer Center.

Delayed-type hypersensitivity skin tests were done with NY-ESO-1 full-length protein in all patients and with NY-ESO-1b peptide (HLA-A2-binding motif peptide 157-165, SLMWITQFCL) in HLA-A2+ patients and NY-ESO-1 DP4 peptide (HLA-DP4-binding motif peptide 157-174, SLMWITQFCLFVLAQP) in HLA-DP4+ patients. Delayed-type hypersensitivity skin tests were placed in the forearm of each patient and read 48 to 72 h later.

Blood was drawn 1 to 4 weeks before receiving the first dose of vaccine (baseline pre-vaccine blood sample) and at weeks 3, 5, 7, 9, 11, and 13 for immunologic assessment as outlined below. Samples for immunologic monitoring were shared and analyzed at Ludwig Institute for Cancer Research at Memorial Sloan-Kettering Cancer Center and The University of Texas M. D. Anderson Cancer Center. Sufficient cells from samples obtained at week 13 for patients P002 and N07 were not available to conduct certain experiments.

Clinical responses were assessed using standard Response Evaluation Criteria In Solid Tumors and computed tomography scans with intravenous contrast at 2.5 mm reconstructions for chest computed tomography and 5 mm reconstructions for other anatomic sites. For patients with prostate cancer, change in the prostate-specific antigen (PSA) tumor marker was used to assess clinical response.

ELISA. Half-area, 96-well flat-bottomed plates (Corning) were coated with recombinant NY-ESO-1 protein solution (25 μL/well at 1 μg/mL in PBS) overnight at 4°C. Plates were washed twice with 0.1% Tween 20 in PBS and four times with PBS. Serum dilutions in blocking buffer (5% nonfat milk in PBS) at 4°C. Plates were washed four times with 0.1% Tween 20 in PBS and four times with PBS. Serum dilutions in blocking buffer (30 μL/well) were added to the plate and incubated at room temperature for 2 h. The plates were washed and 30 μL secondary antibody in blocking buffer (goat anti-human IgG-AP; Southern Biotechnology) was added per well and the plate was incubated at room temperature for 1 h. The plate was washed and 30 μL substrate was added per well (AttoPhos) for 30 min at room temperature in the dark. Stop solution was added (15 μL/well, 3 N NaOH) to wells and immediately read (SpectraMax M2; Molecular Devices). Sera were tested over a range of 4-fold serial dilution from 1:100 to 1:400,000. A positive reaction is defined as an absorbance value of a 1:40 diluted serum that exceeds the mean absorbance value of sera from normal donors by 3 SDs (23, 34, 35).

Peptides and vectors for T-cell analyses. Synthetic NY-ESO-1 20-mer overlapping peptides were produced by the Ludwig Institute for Cancer Research and designated peptide set 1: no peptides; peptide set 2: 1-20, 11-30, 21-40; peptide set 3: 31-50, 41-60, 51-70; peptide set 4: 61-80, 71-90, 81-100; peptide set 5: 91-110, 101-120, 111-130; peptide set 6: 119-143, 131-150, 139-160; and peptide set 7: 151-170, 161-180. Peptides for influenza nucleoprotein NP 206-229 (FWRGENGRK-TTRAYEMRNILKGK), tetanus toxoid TT 830-844 (QYIKANSKFIGIF), influenza hemagglutinin HA 307-319 (PKYVKQNTLKLAT), Melan A (ELAGIGILTV), and flu matrix (GILGFVFTL) were obtained from Bio-Synthesis with a purity of >95% as determined by mass
spectrometry. Fowlpox recombinant vectors encoding full-length NY-ESO-1 or irrelevant antigen were described previously and used to infect target antigen-presenting cells overnight at 37°C at 100 plaque-forming units/cell [36].

In vitro sensitization with peptides. Peripheral blood mononuclear cells were obtained from patients and CD4+ and CD8+ T cells were obtained by positive selection using antibody-coated magnetic beads (Dynabeads, Dynal). Total CD4+ or CD4+ T cells depleted of CD4+CD25+ regulatory T cells by Miltenyi regulatory T-cell depletion kit or total CD8+ T lymphocytes were seeded in 96-well round-bottomed plates (Corning) at a concentration of 5 × 10^5 per well in RPMI 1640 supplemented with 10% human AB serum (GemCell; Gemini Bioproducts), L-glutamine (2 mmol/L), penicillin (100 units/mL), streptomycin (100 μg/mL), and 1% nonessential amino acids (complete medium).

As antigen-presenting cells, autologous peripheral blood mononuclear cells depleted of CD4+ and CD8+ T cells were pulsed with 10 μmol/L or 10 μg/mL peptide overnight at 37°C, 5% CO2 at a concentration of 4 × 10^6/mL in serum-free medium (X-VIVO-15, Bio-Whittaker). Pulsed cells were washed, irradiated, and added to the plates containing CD4+ and CD8+ T cells at a concentration of 1 × 10^6 antigen-presenting cells per well. After 8 h, interleukin-2 (10 units/mL;
Table 1. Clinical and immune responses observed in patients vaccinated with the NY-ESO-1 DNA vaccine

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Patient ID</th>
<th>Disease status</th>
<th>Clinical response</th>
<th>Antibody response CD4 T-cell response</th>
<th>CD8 T-cell response</th>
<th>DTH skin response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N01*</td>
<td>Stage IIIB NSCLC (NED)</td>
<td>PD</td>
<td>Negative</td>
<td>+ (7, 9)</td>
<td>+ (5)</td>
</tr>
<tr>
<td></td>
<td>N02*</td>
<td>Stage IV NSCLC (with disease)</td>
<td>SD 23 mo</td>
<td>Negative</td>
<td>+ (9, 11)</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>N03*</td>
<td>Stage IV NSCLC (with disease)</td>
<td>SD 16 mo</td>
<td>Negative</td>
<td>+ (5, 7, 9, 11, 13)</td>
<td>+ (13)</td>
</tr>
<tr>
<td>2</td>
<td>N04*</td>
<td>Stage IV NSCLC (NED)</td>
<td>NED</td>
<td>Negative</td>
<td>+ (3, 5, 9, 11)</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>E06</td>
<td>Stage IV ESO (NED)</td>
<td>NED</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>N07</td>
<td>Stage IV NSCLC (NED)</td>
<td>NED</td>
<td>Negative</td>
<td>+ (11)</td>
<td>+ (5, 7)</td>
</tr>
<tr>
<td></td>
<td>P001*</td>
<td>Stage IV prostate cancer</td>
<td>PD</td>
<td>Negative</td>
<td>+ (5, 7, 9, 11)</td>
<td>+ (13)</td>
</tr>
<tr>
<td></td>
<td>P001</td>
<td>Stage IV prostate cancer</td>
<td>PD</td>
<td>Negative</td>
<td>+ (P, 5, 9, 11, 13)</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>P002</td>
<td>Stage IV prostate cancer</td>
<td>PD</td>
<td>Negative</td>
<td>+ (P, 3, 9)</td>
<td>Negative</td>
</tr>
<tr>
<td>3</td>
<td>P003*</td>
<td>Stage IV prostate cancer</td>
<td>PD</td>
<td>Negative</td>
<td>+ (7)</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>P004</td>
<td>Stage IV prostate cancer</td>
<td>PD</td>
<td>Negative</td>
<td>+ (7)</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>P005</td>
<td>Stage IV prostate cancer</td>
<td>PD</td>
<td>+ (P, 3, 5)</td>
<td>+ (P, 3, 5)</td>
<td>+ (P, 3)</td>
</tr>
<tr>
<td></td>
<td>P007*</td>
<td>Stage IV prostate cancer</td>
<td>PD</td>
<td>Negative</td>
<td>+ (5, 11)</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>P008*</td>
<td>Stage IV prostate cancer</td>
<td>PD</td>
<td>Negative</td>
<td>+ (3, 5, 7, 11, 13)</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>P009</td>
<td>Stage IV prostate cancer</td>
<td>PD</td>
<td>Negative</td>
<td>+ (3, 5, 7, 9, 11, 13)</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>P010</td>
<td>Stage IV prostate cancer</td>
<td>PD</td>
<td>Negative</td>
<td>+ (P, 7, 9, 11, 13)</td>
<td>Negative</td>
</tr>
</tbody>
</table>

NOTE: Disease status pre-vaccine. Numbers in parentheses indicate study week at which immune responses were detected, with P representing pre-vaccine time point.

Abbreviations: NED, no evidence of disease; SD, stable disease; PD, progressive disease; DTH, delayed-type hypersensitivity.

*Patients in whom additional CD4 T-cell responses were detected as discussed in the text and shown in Figs. 4 and 5.

Results

Clinical trial. Patients with NSCLC, esophageal carcinoma, or prostate adenocarcinoma who met all eligibility criteria as per the clinical trial protocol were enrolled sequentially into one of three cohorts (Supplementary Table S1). Cohort 1 consisted of 3 NSCLC patients who received treatment with 4 µg NY-ESO-1 DNA vaccine administered monthly at weeks 1, 5, and 9. Each 4 µg dosage of the vaccine was given via particle-mediated epidermal delivery with 1 µg vaccine administered to four separate sites of one upper arm. Cohort 2 consisted of 2 NSCLC patients, 1 esophageal carcinoma patient, and 3 prostate adenocarcinoma patients who received 8 µg vaccine administered monthly at weeks 1, 5, and 9. Each 8 µg dosage of vaccine was given via particle-mediated epidermal delivery with 1 µg vaccine administered to both upper arms and in four separate sites in each upper arm. Cohort 3 consisted of 7 prostate adenocarcinoma patients who received 8 µg vaccine administered as clustered dosing with 2 µg doses on days 1, 3, 5, and 8 of each week and given monthly at weeks 1, 5, and 9. The clustered dosing was administered via particle-mediated epidermal delivery with 1 µg vaccine given in each arm on days 1, 3, 5, and 8 for weeks 1, 5, and 9.

Eighteen patients were enrolled onto the study, but only 16 patients received at least one vaccination and 15 patients completed all scheduled vaccinations. Of the 16 vaccinated patients, 2 were diagnosed with stage IV NSCLC and treated with surgical resection of metastatic disease before enrollment onto the vaccine clinical trial. One patient was diagnosed with stage IIB NSCLC and treated with surgical resection of disease before enrollment onto the vaccine clinical trial. One patient with stage IV esophageal carcinoma was also treated with epidermal delivery with 1 µg vaccine administered monthly at weeks 1, 5, and 9.

Roche Molecular Biochemicals) and interleukin-7 (20 ng/mL; R&D Systems) were added to culture wells, and this step was repeated every 4 days until the cells were harvested for testing.

ELISPOT assays. Flat-bottomed, 96-well mixed cellulose ester membrane plates (Multiscreen; Millipore) were coated with IFN-γ monoclonal antibody (4 µg/mL; 1-D1K; Mabtech) and incubated overnight at 4°C. Plates were washed twice with RPMI 1640 supplemented with 1% nonessential amino acids and blocked with RPMI 1640 supplemented with 10% human AB serum for 2 h at 37°C. Presensitized CD8+, CD4+, or CD4+CD25+ T cells (5 x 10^4) and 1 x 10^5 target cells (antigen-presenting cells) were added to each well and incubated for 16 to 20 h in RPMI 1640 supplemented with 1% nonessential amino acids at 37°C, 5% CO2. Plates were washed thoroughly with sterile water containing 0.05% Tween 20 to remove cells, and IFN-γ monoclonal antibody (0.2 µg/mL; 7-B6-1-biotin; Mabtech) was added to each well. Plates were incubated for 2 h at 37°C, 5% CO2, washed thoroughly, and developed with streptavidin alkaline phosphatase (1:1,000 dilution; Mabtech) for 1 h at room temperature in the dark. After thoroughly washing plate, substrate (5-bromo-4-chloro-3-indolyl phosphate/nitrotetrazolium; Sigma-Aldrich) was added. After final washes, plates were dried and plate membranes displaying dark-violet spots were scanned by using CTL ImmunoSpot analyzer and software (Cellular Technologies). Responses were considered significant if the number of spots for 50,000 CD4+ T cells against NY-ESO-1 peptide-pulsed targets was >30 spots and at least three times higher than response against targets pulsed with irrelevant control peptide.

Flow cytometry. CD4+CD25hi and CD4+FOXP3+ T cells were identified by flow cytometry. Antibodies used for flow cytometry studies consisted of CD4-PerCP-Cy5.5, CD25-APC (BD Pharmingen), and FOXP3-PE (eBiosciences; clone PHC101).
of disease at the time of vaccine therapy. Two patients with stage IV NSCLC were also enrolled onto the clinical trial and had evidence of disease at the time of vaccine therapy. All 10 patients with prostate adenocarcinoma had metastatic disease as determined by increasing PSA tumor marker or computed tomography scans suggestive of metastatic disease. Patients were followed throughout the clinical trial and evaluated every 2 weeks by physical examination and routine chemistry and hematologic blood tests until week 13 to assess for safety of the vaccine treatment.

Safety of NY-ESO-1 DNA vaccine and clinical responses. All 16 patients tolerated the NY-ESO-1 DNA vaccine administered as per protocol schema with minimal side effects and toxicities related to vaccine therapy. Side effects related to vaccination consisted of grade 1 to 2 erythema at the vaccine site (Fig. 1A). One prostate adenocarcinoma patient (patient P005) on cohort 3 received only two vaccinations before being removed from the study secondary to disease progression. This patient was replaced in the study and 15 patients, consisting of 9 prostate adenocarcinoma patients, 5 NSCLC patients, and 1 esophageal carcinoma patient, completed all scheduled vaccinations.

Clinical responses (Table 1) in this small cohort of patients were limited to stable disease of 16 and 23 months for 2 patients on cohort 1 with stage IV NSCLC, who had measurable disease at the time of vaccination, and for 3 patients on cohort 2 [NSCLC (n = 2) and esophageal carcinoma (n = 1)] who had no evidence of disease at the time of vaccination. One NSCLC patient (cohort 1) who previously diagnosed stage IIIb disease did develop progressive disease. Although the prostate adenocarcinoma patients were noted to have slower increases in the PSA tumor marker during the vaccination period compared with pre-vaccine PSA levels (Fig. 1B, two representative patients), all patients, except one (patient P009), were deemed to have progressive disease as noted by PSA increase at the time of study completion at week 13. Patient P009 had stable PSA at study week 13 and repeat imaging by computed tomography scans showed a mixed tumor response, with decreased size of previously enlarged lymph nodes as well as increased size in other lymph nodes, which was considered progressive disease (Fig. 1C). Thus, clinical outcomes induced by NY-ESO-1 DNA vaccination were limited to stable disease in 2 patients, continued absence of disease in 3 patients, and progressive disease in 11 patients.

Fig. 2. CD4 T-cell responses against NY-ESO-1 representative peptides by IFN-γ ELISPOT. Results shown throughout vaccination [from pre-vaccine (Pre) to week 13 (wk 13)] following pre-sensitization and represented as the number of spots for 50,000 CD4 T cells against targets pulsed with NY-ESO-1 peptides (with median number of spots for irrelevant control target of <10 spots). Representative of at least two repeat assays. NY-ESO-1-specific CD4 T-cell responses measured throughout the clinical trial in 10 prostate cancer patients (A) and 5 NSCLC patients and 1 esophageal cancer patient (B), showing inconsistent T-cell responses with intrapatient and interpatient variabilities.
**Immune responses.** All patients who received at least one vaccination on this clinical trial developed detectable T-cell responses against NY-ESO-1 (Table 1), except for the esophageal carcinoma patient (patient E06). None of the patients on this clinical trial were noted to have a positive antibody response against NY-ESO-1 as measured by ELISA, except for patient P005, who had a baseline pre-vaccine antibody response, which remained detectable for the period that he was on the clinical trial (Table 1). All 15 patients who had measurable T-cell responses had CD4 T-cell responses against NY-ESO-1 as detected by ELISPOT assays. Figure 2A provides an overview of NY-ESO-1-specific CD4 T-cell responses induced in 10 treated prostate cancer patients during the vaccination schedule. As shown, CD4 T-cell responses were detected at different study weeks for each patient. CD4 T-cell responses fluctuated throughout the vaccination schedule. Most patients (6 of 9) who had measurable vaccine-induced CD4 T-cell responses between study weeks 7 and 11 of the clinical trial were found to have greatly diminished CD4 T-cell responses at study week 13, which was 4 weeks after the last vaccine administration. For example, patient P003 had a detectable CD4 T-cell response against NY-ESO-1 at study week 7, which was undetectable at study weeks 9 to 13. Similarly, patient P007 had detectable CD4 T-cell responses at study weeks 5 and 11 but not at weeks 7, 9, and 13 (Fig. 2A). Similar CD4 T-cell responses were seen in the 5 NSCLC patients (Fig. 2B).

CD4 T-cell responses were specific for different regions within the NY-ESO-1 protein. As shown for patient P001, CD4 T-cell responses detected at study weeks 5, 9, 11, and 13 (Fig. 3A) were predominantly directed at the overlapping NY-ESO-1 peptides found in our peptide set 6 (described in Materials and Methods; Fig. 3B), which were mapped primarily to the NY-ESO-1 peptides encompassing amino acids 119-143 and 139-160 (Fig. 3C).

In some instances, patients were noted to have CD4 T-cell responses to certain peptides, which were no longer detectable at a different time point; instead, CD4 T-cell responses against a different peptide was found. For example, patient P008 had CD4 T-cell responses directed predominantly against the NY-ESO-1 peptide encompassing amino acids 119-143 at study weeks 3, 5, and 7, but at weeks 9, 11, and 13 these responses were no longer detected; instead, CD4 T-cell responses were primarily directed against the NY-ESO-1 peptide encompassing amino acids 81-100 at study weeks 11 and 13 (Fig. 2A).

Patients N01, N03, N07, P005, and P011 had measurable CD8 T-cell responses against NY-ESO-1 as detected by ELISPOT assays (Table 1). Similar to CD4 T-cell responses, CD8 T-cell responses were also observed transiently and appeared specific for different regions of the NY-ESO-1 protein (Fig. 3D). As shown for patient P011, NY-ESO-1-specific CD8 T-cell responses were detected at study week 13, with specificity for
NY-ESO-1 peptides found in overlapping peptide sets 4 and 5 (Fig. 4D). Thus, NY-ESO-1 DNA vaccination can induce CD8 T-cell responses, but these responses were inconsistent and not detectable in blood samples from all study time points for each patient throughout the clinical trial.

**Depletion of regulatory T cells leads to detection of NY-ESO-1-specific CD4 T-cell responses.** Of the 16 cancer patients treated with the NY-ESO-1 DNA vaccine, NY-ESO-1-specific CD4 T-cell responses to certain peptides were not detected in the pre-vaccine baseline blood samples of 12 patients or in the week 13 post-vaccine blood samples of 8 patients for which samples were available (Table 1). Available samples from 7 patients (patients N02, N03, N04, P003, P007, P008, and P011) were analyzed to determine if depletion of regulatory T cells would permit detection of NY-ESO-1-specific CD4 T-cell responses. CD4⁺CD25⁺ T cells were shown to have regulatory T-cell function in standard coculture experiments (Supplementary Fig. S1). Frequency of CD4⁺FOXP3⁺ regulatory T cells did not change significantly between pre-vaccine and post-vaccine samples for 9 patients who had sufficient cells available for analyses (Supplementary Fig. S2A and B), but, as reported previously (10–15), the frequency of CD4⁺FOXP3⁺ regulatory T cells was increased in cancer patients (ranging from ~20% to 60%) compared with the average frequency of CD4⁺FOXP3⁺ regulatory T cells observed in the systemic circulation of 10 healthy donors (5 ± 2%; Supplementary Fig. S2B). For 7 patients who received the NY-ESO-1 DNA vaccine, in vitro depletion of regulatory T cells from samples that did not have detectable antigen-specific T-cell responses led to the identification of NY-ESO-1-specific CD4 T-cell responses with high avidity as evidenced by peptide titration and recognition of naturally processed antigen. As shown in Figs. 4B and 5A, patient N04 had detectable CD4 T-cell responses against NY-ESO-1 peptide 101-120 at study week 9 after vaccination, but there were no detectable CD4 T cells against this specific peptide present in the pre-vaccine or week 13 blood samples. However, after depletion of regulatory T cells from the pre-vaccine and week 13 blood samples, there were detectable CD4 T-cell responses against NY-ESO-1 peptide 101-120 (Fig. 5A, top). Interestingly, the avidity of CD4 T cells against NY-ESO-1 peptide 101-120 did not significantly differ in the presence or absence of regulatory T cells (Fig. 5A, bottom) in contrast to what has been observed for vaccine-induced NY-ESO-1 CD4 T-cell responses following peptide vaccination (26). Similarly, patients P008 and P011 were found to have detectable CD4 T-cell responses against NY-ESO-1 after depletion of regulatory T cells from their pre-vaccine blood samples (data not shown). However, as shown in Fig. 5B, patient N03 did not have CD4 T cells against NY-ESO-1 in the pre-vaccine blood sample, even when regulatory T cells were depleted (Fig. 5B).
patient N03 did have enhanced CD4 T-cell responses against the NY-ESO-1 peptide pool in the week 13 blood sample after depletion of regulatory T cells (Fig. 5B, top). The CD4 T-cell responses were specific for peptide 119-143 and the avidity of CD4 T cells remained similar in the absence and presence of regulatory T cells (Fig. 5B, bottom). Patient N02 also did not have detectable CD4 T cells against NY-ESO-1 in pre-vaccine blood sample despite regulatory T-cell depletion but did develop detectable CD4 T-cell responses in the week 13 blood sample after depletion of regulatory T cells (data not shown). Similarly, patients P003 and P007 developed CD4 T-cell responses against NY-ESO-1 in the week 13 blood samples after depletion of regulatory T cells (data not shown). Therefore, CD4 T-cell responses against all NY-ESO-1 peptides or specific NY-ESO-1 peptides may have decreased at study week 13 as a result of the presence and function of naturally occurring regulatory T cells acting to suppress antigen-specific effector T-cell responses that were induced after vaccination.

Discussion

This is the first report of the NY-ESO-1 DNA vaccine in a clinical trial with cancer patients. The vaccination method elicited CD4 T-cell responses in 14 of 15 (93%) treated patients and CD8 T-cell responses in 5 of 15 (33%) patients who did not have detectable pre-vaccine immune responses. From the three cohorts of treated patients, as little as 4 μg DNA vaccine given every 4 weeks was sufficient to elicit a measurable immune response consisting of CD4 T cells. In addition, CD8 T-cell responses were associated mostly within cohorts 1 and 2 (1 prostate cancer patient and 3 lung cancer patients), with the exception of 1 cohort 3 patient, P005, who had baseline pre-vaccine detectable CD8 T-cell responses that remained measurable post-vaccination. All of the T-cell responses against NY-ESO-1 were clustered within the carboxy-terminal region of the protein as T-cell responses were predominantly detected against peptides in this region of the protein, indicating that this region of the protein is the most immunogenic as published previously (18, 20).

Previously published clinical trials using NY-ESO-1 peptides and full-length antigen also noted induction of CD4, CD8, and/or antibody responses against NY-ESO-1 (18, 19, 22–25); however, clinical responses were limited to stable disease in most cases, which also occurred most frequently in patients who had prior surgical resection of disease before beginning vaccine therapy. In one study with melanoma patients who
predominantly had their disease surgically resected before vaccination, the authors reported that, in a cohort of 42 vaccinated patients, with a median follow-up of ~2 years, 16 patients had relapse of disease: 5 of 7 patients who received placebo vaccine had recurrent disease, 9 of 16 patients who received NY-ESO-1 protein vaccine had recurrent disease, and 2 of 19 patients who received NY-ESO-1 protein vaccine plus ISCOMATRIX adjuvant developed recurrent disease (23). The authors concluded that the vaccine appeared to be more effective when given with ISCOMATRIX adjuvant. We recently reported a study in uterine carcinosarcoma patients who had their disease surgically removed before administration of the NY-ESO-1 protein vaccine plus BCG and GM-CSF. We found that 5 of 6 vaccinated patients developed recurrent disease after a median follow-up of ~4 years (25). Our current study also reports limited clinical benefit consisting predominantly of stable disease in patients who had prior surgical resection of their disease. The role of regulatory T cells on vaccine-induced T-cell responses was not examined in previous clinical trials.

There is growing evidence that regulatory T cells, which occur naturally within the immune system, are capable of suppressing effector T-cell responses within cancer patients and may be responsible for inadequate antitumor responses (11–16). We have described previously that CD4+CD25+ regulatory T cells play an important role in the suppression of precursor CD4 T cells specific for NY-ESO-1 in healthy donors and in patients without serum antibody response to NY-ESO-1 (21). Our current data confirm that antigen-specific CD4 T-cell responses exist spontaneously in cancer patients and that these cells may be suppressed by regulatory T cells that are already present within the immune system.

We have also observed in a previous study in ovarian cancer with NY-ESO-1 peptide vaccination that these suppressing mechanisms were profound and that spontaneous CD4 T cells that were suppressed by regulatory T cells had a higher avidity for NY-ESO-1 compared with CD4 T cells that were induced after peptide vaccination (26). In the current study with NY-ESO-1 DNA immunization, our data indicate that vaccine-induced CD4 T-cell responses were diminished in some cancer patients as a result of suppression by regulatory T cells. In addition, T-cell responses to specific peptides may have been altered due to regulatory T cells acting on various subsets of effector T cells. However, contrary to what has been shown previously with peptide vaccination, DNA vaccine-induced effector CD4 T-cell responses against NY-ESO-1 appeared to be qualitatively similar to CD4 T-cell responses that were identified on depletion of regulatory T cells, consisting of high-avidity T-cell responses as measured by peptide titration and the ability to recognize naturally processed NY-ESO-1 antigen.

It is possible that regulatory T-cell suppression of effector T-cell responses tempered the potential efficacy of the NY-ESO-1 DNA vaccine immunotherapeutic approach. It is also possible that other mechanisms, including loss of tumor antigen and/or lack of presentation of tumor antigen due to loss or absence of MHC molecules within the tumor microenvironment, contributed to the lack of antitumor responses that were observed in our study. These possibilities will need to be explored in future studies. From our limited data, it does not appear that the NY-ESO-1 DNA vaccine increased the frequency of CD4+FOXP3+ regulatory T cells, but these cells exist naturally and are increased in cancer patients, thus playing a role in the suppression of vaccine-induced effector T-cell responses.

Because regulatory T cells exist naturally to play a role in controlling elicited effector immune responses, future immunotherapeutic strategies will need to consider mechanisms to overcome regulatory T-cell suppression to develop successful therapies to treat cancer patients. Depletion of regulatory T cells before vaccination may allow for enhanced effector T-cell responses with subsequent antitumor responses. Other counter-acting approaches to regulatory T-cell activity may include the use of immunostimulatory adjuvants during vaccination, including Toll-like receptor ligands, which have been shown to bypass potential suppressive activity (37, 38). It is unlikely that any single immunologic agent will be able to provide sufficient immune responses to generate clinical benefit in the majority of cancer patients, and combination therapies with multiple agents, including conventional therapies with immunotherapies, will have to be investigated for the development of clinically beneficial cancer therapeutics. In this regard, the NY-ESO-1 DNA vaccine is a tested platform on which to build. The NY-ESO-1 DNA vaccine showed an acceptable safety profile, measurable T-cell responses in 15 of 16 vaccinated patients and potential for improvement in immune responses if given in combination with regulatory T-cell depletion or in prime-boost methods, which would possibly enhance antibody and CD8 T-cell responses, or with agents such as anti-CTLA-4 antibody that would allow for an increased ratio of effector to regulatory T cells (39, 40).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Drs. Linda Pan, Eric Hoffman, Gerd Ritter, and Ralph Venhaus (Ludwig Institute for Cancer Research) for assistance in clinical trial management, Erin Horne for assistance in obtaining blood samples from patients at M. D. Anderson Cancer Center, and Marla Johnson, Jason Love, and Alan Bethea (M. D. Anderson Cancer Center) for assistance in clinical trial management and data management.

References

11. Woo EY, Chu CS, Goletz TJ, et al. Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and...


NY-ESO-1 DNA Vaccine Induces T-Cell Responses That Are Suppressed by Regulatory T Cells

Sacha Gnjatic, Nasser K. Altorki, Derek Ng Tang, et al.