Resiquimod as an Immunologic Adjuvant for NY-ESO-1 Protein Vaccination in Patients with High-Risk Melanoma

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Abstract

The Toll-like receptor (TLR) 7/8 agonist resiquimod has been used as an immune adjuvant in cancer vaccines. We evaluated the safety and immunogenicity of the cancer testis antigen NY-ESO-1 given in combination with Montanide (Seppic) with or without resiquimod in patients with high-risk melanoma. In part I of the study, patients received 100 μg of full-length NY-ESO-1 protein emulsified in 1.25 mL of Montanide (day 1) followed by topical application of 1,000 mg of 0.2% resiquimod gel on days 1 and 3 (cohort 1) versus days 1, 3, and 5 (cohort 2) of a 21-day cycle. In part II, patients were randomized to receive 100-μg NY-ESO-1 protein plus Montanide (day 1) followed by topical application of placebo gel [(arm A; n = 8) or 1,000 mg of 0.2% resiquimod gel (arm B; n = 12)] using the dosing regimen established in part I. The vaccine regimens were generally well tolerated. NY-ESO-1–specific humoral responses were induced or boosted in all patients, many of whom had high titer antibodies. In part II, 16 of 20 patients in both arms had NY-ESO-1–specific CD4+ T-cell responses. CD8+ T-cell responses were only seen in 3 of 12 patients in arm B. Patients with TLR7 SNP rs179008 had a greater likelihood of developing NY-ESO-1–specific CD8+ responses. In conclusion, NY-ESO-1 protein in combination with Montanide with or without topical resiquimod is safe and induces both antibody and CD4+ T-cell responses in the majority of patients; the small proportion of CD8+ T-cell responses suggests that the addition of topical resiquimod to Montanide is not sufficient to induce consistent NY-ESO-1–specific CD8+ T-cell responses. Cancer Immunol Res; 3(3); 278–87. ©2015 AACR.

Introduction

NY-ESO-1 is considered widely a suitable tumor antigen for vaccination due to its presence in many tumor types, its highly restricted expression in normal tissues, and the ability to induce strong spontaneous humoral and cellular immune responses (1). In vitro and in vivo studies have shown that NY-ESO-1 is immunogenic with particular regions of the protein specifically targeted by antibodies as well as CD4+ and CD8+ T cells. Although clinical trials have demonstrated that patients have immunity to NY-ESO-1, only a small number of clinical trials responses have been observed in patients with advanced disease. Induction of integrated immune responses to NY-ESO-1 consisting of humoral and CD4+ and CD8+ T-cell responses correlated with clinical benefit in patients with melanoma who received anti-CTLA4 inhibitors (2). Therefore, to achieve effective CD4+ and CD8+ T-cell priming, we vaccinated individuals with the full-length recombinant NY-ESO-1 protein and evaluated the addition of Toll-like receptor (TLR) adjuvants to the vaccine.

TLRs are a family of highly conserved transmembrane receptors that recognize specific molecular patterns in microbial components (3). Stimulation of different TLRs induces distinct patterns of gene expression, not only activating innate immunity but also directing adaptive immunity, such as the induction of a Th1 helper 1 (Th1) cell response that is necessary for antitumor immune responses (4). TLR agonists control antigen-presenting cells (APC), in particular dendritic cells (DC), by triggering their maturation program, including upregulation of the expression of human leukocyte antigen (HLA) and costimulatory molecules and secretion of cytokines such as TNFα, IL6, IL12, and IFNγ (5). In addition, animal models have shown that TLR agonists can improve the efficacy of vaccines targeting self-antigens by activation of innate immune cells and production of inflammatory cytokines (6) and alter the immunosuppressive function of regulatory T cells (Treg; ref. 7). Consequently, TLR agonists have been recognized as promising vaccine adjuvants and have been developed for use as adjuvants for cancer vaccines in clinical trials.

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doi: 10.1158/2326-6066.CIR-14-0202

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Cancer Immunol Res; 3(3); 278–87. ©2015 AACR.
exogenous antigens, resulting in the expression of TLR 7/8 agonist. Resiquimod is a TLR7/8 agonist that is chemically potent immune response than imiquimod (12).

In vitro studies using resiquimod have shown that it can activate DC maturation by increasing stimulatory molecule expression and cytokine production, and skew a Th1 cytokine profile, consequently enhancing humoral and cellular immune responses (13, 14). More recently, resiquimod has been shown to promote cross-presentation of exogenous antigens, resulting in the efficient induction of antigen-specific CD8+ T-cell responses (15). Results from animal studies have confirmed the ability of resiquimod to activate DCs (16), bias immune responses toward a predominance of Th1 cells (17), and enhance antigen-specific CD8+ T-cell responses that can inhibit tumor growth (18, 19). Therefore, the capacity of resiquimod to enhance local activation of immune cells, stimulate the production of proinflammatory cytokines, and enhance antigen-presentation by DCs leading to activation of effective cellular responses are features that support its potential as a cancer vaccine adjuvant (20).

In this randomized study, we evaluated the safety and immunogenicity of vaccination with NY-ESO-1 protein emulsified in Montanide ISA-51 VG when given with or without resiquimod in patients with resected stage IIIB–IV melanoma. We observed that NY-ESO-1 protein and Montanide, when given with or without resiquimod, induced both humoral and cellular immune responses specific for NY-ESO-1 in the majority of vaccinated patients, although the induction of significant CD8+ T-cell responses was only observed in a small subset of patients who received resiquimod.

Materials and Methods
Study design, patients, and treatment plan
This is a two-part study with resiquimod dose escalation in part I and randomization to resiquimod versus placebo in part II. The primary objectives of the study were safety of the vaccine regimen and assessment of humoral and cellular responses to NY-ESO-1. Patients with histologically confirmed resected malignant melanoma [American Joint Committee on Cancer (AJCC) stages IIIB, IIC, III, and IV] were eligible. Tumor NY-ESO-1 expression was assessed by immunohistochemistry as previously described (21), but was not required for study entry. The study (NCI00821652) was approved by the New York University School of Medicine (New York, NY) Institutional Review Board, and written informed consent was obtained from all patients before enrollment.

In part I of the study, the safety of two different dosing regimens of resiquimod was assessed in two cohorts of 3 patients each. Subjects received four 3-week cycles of subcutaneous (s.c.) injections with 100 μg of recombinant human NY-ESO-1 protein (Ludwig Institute for Cancer Research, Boston, MA) emulsified in 1.25 mL Montanide (day 1) followed by topical application of 1,000 mg of resiquimod (3M Pharmaceuticals; 0.2%) gel to the vaccination site on days 1, 13, and 25 (cohort 1) and days 1, 3, and 5 (cohort 2), respectively (Fig. 1). In part II of the study, patients were randomized in a blinded fashion to receive 100 μg NY-ESO-1 protein emulsified in 1.25 mL Montanide (day 1) s.c. followed by topical application of placebo gel (arm A; n = 8) or 1,000 mg of 0.2% resiquimod gel (arm B; n = 12) on days 1, 3, and 5 as established in part I (Fig. 1). Study arm assignments were unblinded after completion of the study and immune monitoring.

Blood samples
Blood samples were collected at baseline and day 8 of each cycle (weeks 1, 4, 7, and 10) and during the first follow-up visit at weeks 12 to 14. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll centrifugation and frozen in aliquots using pooled human serum (90%) and DMSO (10%). Plasma from each time point was also frozen.
Humoral responses

Patient plasma samples were analyzed by ELISA for seroreactivity against recombinant NY-ESO-1 protein (1 μg/mL) as well as three individual overlapping long peptides (68-mer, 1 μmol/L each) covering the NY-ESO-1 sequences as previously described (22). Synthetic long peptides were used to confirm specificity for NY-ESO-1 plasma antibodies and for approximate epitope mapping. A reciprocal titer was calculated for each plasma sample as the maximal dilution still significantly reacting to a specific antigen. This value was extrapolated by determining the intersection of a linear trend regression with a cutoff value. The cutoff was defined as ten times the average of OD values from the first four dilutions of a negative control pool comprising five healthy donor sera. In each assay, sera of patients with known presence or absence of specific reactivity were used as controls.

Titers >100 were considered reactive, and specificity was determined by comparing reactivity to control antigens and to the NY-ESO-1 peptides.

T-cell assays

T-cell responses to NY-ESO-1 were evaluated after in vitro stimulations (IVS) with overlapping pools of NY-ESO-1 peptides. T cells were restimulated with the overlapping pools of NY-ESO-1 peptides and then stained for IFNγ, TNFα, and IL2 and analyzed by flow cytometry. IVS were performed with some modifications as described previously (11). PBMCs were thawed and cultured overnight in 5% PHS (Valley Biomedicals) in RPMI (Gibco), then separated into CD4+ and CD8+ (APC) fractions using Dynal Beads (Invitrogen). For the CD25-depletion experiments, CD25+ cells were initially depleted from PBMCs before positive selection of CD4+ and CD8+ fractions. Each fraction was then washed and resuspended in 5% PHS/RPMI containing 10 U/mL IL2 (R&D Systems) and 10 ng/mL IL7 (R&D Systems). CD4+ and CD8+ cells (500,000 to 1 million cells/well) were cocultured separately for 14 to 20 days with APCs stimulated with pooled NY-ESO-1 overlapping peptides covering the NY-ESO-1 protein (1 μg/mL each) in a 96-well round-bottom plate. Medium and cytokines were replenished every 2 to 3 days.

IVS CD8+ T-cell cultures were tested on day 14 and CD4+ T-cell cultures on day 21 for reactivity to NY-ESO-1 by intracellular cytokine staining (ICS). T-cell cultures were harvested, washed, and replated in 5% PHS/RPMI medium in a 96-well V-bottom plate. A pool of all 17 NY-ESO-1 peptides (1 μg/mL each) was added to one of the wells. Control wells containing DMSO, MOG, CMV, and PMA/ionomycin were included. Epitope mapping was done using individual NY-ESO-1 peptides. For all ICS cultures, plates were incubated for 1 hour at 37°C, after which BD Golgi-Plug and GolgiStop were added to each well and the cultures were incubated for an additional 5 hours. Cells were then stained for CD4 and CD8, fixed, and permeabilized with BD Cytofix/Cytoperm solution, then washed with 1× BD Perm/Wash buffer and stained for CD3, CD4, CD8, IL2, TNF, IFNγ, and Live/Dead Violet. Cells were analyzed on a BD LSRII and BD LSRFortessa flow cytometers using FACSDivas software. Data were analyzed using FlowJo software (TreeStar).

Statistical analyses

Linear mixed-effects model analyses of repeated measures were used to compare the T-cell responses over time on the placebo (arm A) and resiquimod (arm B). Repeat measurements were obtained from blood drawn on the day of the first vaccine injection, 8 days after the first step, second, third, and fourth (final) vaccine injection, and 2 to 4 weeks after the final injection. A first-order autoregressive structure with heterogeneous variances was used for the covariance structure of the residuals. A Mann–Whitney test was used to compare plasma antibody titers at various time points between arms A and B. An exact logistic regression model was used for comparing the odds of developing NY-ESO-1–specific CD8+ T-cell responses between patients positive for the TLR7 SNP rs179008 and patients who tested negative for this SNP.

Results

Patient characteristics

A total of 6 patients were sequentially enrolled in part I of the study, 3 patients in each cohort (Fig. 1). All patients had resected cutaneous melanoma, except for 1 patient who had resected ocular melanoma. A total of 20 patients were enrolled in part II of the study. Patients were randomized into two arms—arm A (placebo) and arm B (resiquimod). All patients had resected malignant melanoma (Table 1). Expression of NY-ESO-1 in the resected tumor was not required for study entry, and specimens for immunohistochemical (IHC) analysis were available in only 4 of 6 patients in part I and 10 of 20 patients in part II. Consistent with the reported frequencies, 1 patient in part I and 3 patients in part II [arm A (placebo) = 1, arm B (resiquimod) = 3] had tumors that expressed NY-ESO-1—(23).
NY-ESO-1-specific cellular responses

Figure 3A shows representative flow cytometry results after IVS. NY-ESO-1-specific CD4⁺ T-cell responses were induced in 6 of 8 (75%) patients in arm A and 10 of 12 (83%) patients in arm B (Fig. 3B). Pretreatment NY-ESO-1–specific CD4⁺ T-cell responses were detected in 4 of 8 (50%) patients in arm A and 2 of 12 (17%) patients in arm B; vaccination increased the frequencies of these CD4⁺ T-cell responses in all patients. In responders, NY-ESO-1–specific CD4⁺ T cells were detectable after the second and third cycle of vaccination. NY-ESO-1–specific CD8⁺ T-cell responses were detected in 3 of 12 (25%) patients in arm B (Fig. 3C). Of note, 2 of the patients with NY-ESO-1–specific CD8⁺ T-cell responses also had tumors with documented NY-ESO-1 expression (Table 3). The majority of NY-ESO-1–specific CD4⁺ and CD8⁺ T cells were multifunctional as they secreted both IFNγ and TNFα, with a small proportion also secreting IL2. Results of the mixed model analyses showed that there was no significant difference between study arms for mean changes from baseline to posttreatment evaluations for NY-ESO-1–specific CD4⁺ and CD8⁺ T-cell responses.

Mapping of reactivity to vaccine-induced T-cell responses using individual peptides showed induction of NY-ESO-1–specific T-cell responses to several peptide epitopes (Fig. 3D). Vaccine-induced CD4⁺ T-cell responses mapped mostly to the central and C-terminal regions of NY-ESO-1 protein, consistent with results from previous studies (11, 24). CD4⁺ T-cell responses to peptides 81–100, 101–120, and 119–143 were detected in several patients (4 of 8 patients in arm A and 10 of 12 patients in arm B). Although not feasible in all cases due to limited cell quantities, NY-ESO-1–specific CD8⁺ T-cell responses were mapped to the central and C-terminal region of the protein. More specifically, CD8⁺ T-cell responses were mapped to peptides 81–100 and 161–180, which is consistent with results from previous studies (24, 25).

Effect of CD25 depletion on NY-ESO-1–specific cellular responses

Although designed to induce antigen-specific T-cell responses with effector function, vaccines have also been reported to induce Tregs (26, 27). We also considered that circulating Tregs might compromise the induction of antigen-specific T-cell responses as a result of vaccination. To assess these possibilities, we depleted CD25⁺ cells from bulk PBMCs before positive selection of CD4⁺ and CD8⁺ T cells and IVS. CD8⁺ T-cell responses were unmasked in an additional 2 patients (1 of 8 patients in arm A and 1 of 9 patients in arm B). CD4⁺ T-cell responses were unmasked in an additional 4 patients (2 of 2 patients in arm A and 2 of 2 patients in arm B). Figure 4A shows a representative example of NY-ESO-1–specific CD4⁺ T-cell responses in a patient before and after CD25 depletion. The effect of CD25 depletion was not consistent in all patients as some patients had no effect or only slightly ampliﬁed responses after CD25 depletion. Interestingly, we also detected NY-ESO-1–specific CD4⁺ T-cell responses in prevaccine samples in 3 of 4 patients in arm B whose responses became detectable after CD25 depletion. Although CD25 depletion resulted in the ability to detect T-cell responses in a subset of patients, CD25 expression in CD4⁺ T cells was similarly elevated pre- and posttreatment in patients with detectable NY-ESO-1–specific CD4⁺ T-cell response as compared with patients with no detectable NY-ESO-1–specific CD4⁺ T-cell response (Fig. 4B). Examination of the CD25-expressing cells revealed that CD25 was
coexpressed with CTLA-4 but not with PD-1, TIM-3, and FoxP3 in CD4^+ T cells, possibly indicative of the specific presence of inhibitory or "exhausted" cells (Fig. 4C).

**Role of TLR polymorphisms in response to vaccination**

Accumulating evidence suggests an important role for single-nucleotide polymorphisms (SNP) within TLR genes as determinants of disease susceptibility and severity (28). Furthermore, SNPs may influence response and outcome to vaccination with TLR ligands (29). We investigated the role of TLR7 and TLR8 SNPs in the ability of a patient to respond to the vaccine regimen. Two SNPs in TLR7 (rs179008 and rs864058) and six SNPs in TLR 8 (rs3764880, rs2159377, rs5744080, rs2407992, rs3747414, and rs2109135) were detected. Of these eight SNPs, TLR8 SNP rs3764880 and TLR7 SNP rs179008 were nonsynonymous.

The nonsynonymous TLR8 SNP rs3764880 was detected in 7 of the 20 (35%) patients in arms A and B (Table 3). This SNP has been shown to affect the coding region of TLR8, leading to differential activation of NF-kB (30); in addition, it was found to be associated with protection against tuberculosis (31) and increased risk for systemic lupus erythematosus (32). In our study, no association was observed between vaccine-induced immune responses and the presence of SNP rs3764880. The TLR7 SNP rs179008 has been associated with accelerated HIV-1 disease progression (33) and decreased risk for Hodgkin disease (34). SNP rs179008 was detected in the 3 patients in arm B who had detectable NY-ESO-1-specific CD8^+ T-cell responses and in 1 patient in arm A who did not have a detectable NY-ESO-1-specific CD8^+ T-cell response (Table 3). When adjusted for cohort, the estimated frequency of a CD8^+ T-cell response for patients carrying SNP rs179008 was almost 20 times that of patients who did not have this SNP (OR, 19.7; 95% CI, 1.11–999; P = 0.04).

**Clinical outcome**

The last clinical follow-up for this study was performed in January 2014. In study part I, 1 patient has died, 1 patient is alive with disease, 1 patient has undergone further resection and has remained without evidence of disease, and 3 patients have remained without evidence of disease. In part II of the study, 1 patient was lost to follow-up (arm A), 5 patients have died (arm...
A = 2; arm B = 3). 1 patient is alive with disease (arm A), 6 patients have undergone further resection and have remained without evidence of disease (arm A = 1; arm B = 5), and 7 patients have remained without evidence of disease (arm A = 3; arm B = 4; Table 3). Analysis of time to progression at the end of the study and at the last follow-up did not reveal any significant differences between study arms.

### Discussion

NY-ESO-1 is a commonly expressed tumor-specific antigen that can induce both humoral and cellular immune responses in patients with cancer. Studies have evaluated the immunogenicity of NY-ESO-1 antigens in melanoma and other solid tumors in combination with Montanide or/and TLR agonists. Montanide is considered an effective immune adjuvant because of its ability to create a depot effect, which results in slow release of antigens in combination with Montanide or/and TLR agonists. Montanide and a number of TLR agonists have been shown to induce humoral and cellular immune responses; addition of the TLR3 agonist poly-ICLC to Montanide in an NY-ESO-1 peptide vaccine was shown to induce stronger, more frequent and earlier-onset T-cell and humoral responses in patients with advanced ovarian cancer (24, 35). However, randomized studies comparing the adjuvant effect of Montanide alone versus Montanide in combination with TLR agonists have not been performed. To our knowledge, our study is the first to compare Montanide with and without the addition of a TLR agonist as adjuvant in an NY-ESO-1 protein vaccine in a randomized trial.

We show that NY-ESO-1 protein in combination with Montanide alone or Montanide plus resiquimod is both safe and immunogenic in patients with resected high-risk melanoma. All patients developed anti–NY-ESO-1 IgG antibody titers. The magnitude of antibody titers was notable: 6 of 12 (50%) patients vaccinated with NY-ESO-1 protein in Montanide plus resiquimod exhibited very high (>100,000) IgG titers, whereas 2 of 8 patients (25%) had similarly high titers in the Montanide-only cohort. No difference in the kinetics of antibody responses was seen when resiquimod was added to Montanide, in contrast with an earlier onset of IgG antibodies observed in patients immunized with NY-ESO-1 peptides with Montanide plus poly-ICLC compared with that with only Montanide (24).

Using short-term in vitro restimulation assays, CD4⁺ T-cell responses were induced in the majority of patients; no differences in the rates or magnitude of the responses were seen with the addition of resiquimod. In contrast, CD8⁺ T-cell responses, in addition to humoral and CD4⁺ T-cell responses (integrated antibody, CD4⁺, and CD8⁺ T-cell responses) had substan-

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**Table 3. Summary of NY-ESO-1 antibody titers, T-cell responses, SNPs, NY-ESO-1 expression, and clinical outcome**

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NY-ESO-1 antibody titers: 1–9,999: +; 10,000–99,999: ++; 100,000–249,999: +++; ≥250,000: ++++.

NY-ESO-1 expression in tumor: ND, not done due to insufficient tumor tissue; PID, patient identification number.

Latest clinical outcome: AWD, alive with disease; NED, no evidence of disease.

*Arm A: patients receiving NY-ESO-1 protein, Montanide, and placebo gel; Arm B: patients receiving NY-ESO-1 protein, Montanide, and resiquimod gel.

*Prevacine antibody response.

www.aacrjournals.org Cancer Immunol Res; 3(3) March 2015 283

Published OnlineFirst January 29, 2015; DOI: 10.1158/2326-6066.CIR-14-0202

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Cancer vaccines can lead to expansion of Tregs, potentially impairing the effector function of vaccine-induced Th1 responses (26, 27). Likewise, the presence of significant numbers of antigen-specific Tregs may prevent the detection of desirable antigen-specific T-cell responses. Depletion of CD25+ cells from whole T-cell populations can promote the induction of NY-ESO-1–specific T cells in vitro (43, 44). Removal of CD25+ cells from PBMCs before CD4+ and CD8+
Selection in our study resulted in detection of NY-ESO-1–specific T-cell responses in several patients; however, this effect was not consistent in all patients. Several observations therefore support an argument against a general effect of NY-ESO-1–specific Treg expansion by the vaccine: the heterogeneity of "unmasking" T-cell responses with CD25⁺ cell depletion across patients; the lack of statistically significant differences in CD25 expression between patients in whom NY-ESO-1–specific responses were unmasked by CD25⁺ depletion and those in whom they were not; the absence of an increase in CD25 expression after vaccination; and the fact that both NY-ESO-1–specific CD4⁺ and CD8⁺ T-cell responses were observed without CD25 depletion.

Using SNP analysis, we examined possible associations of TLR7 and TLR8 polymorphisms with immune response to the NY-ESO-1 vaccine. In the current study, 4 of 20 (20%) patients in part II were carriers of the nonsynonymous TLR7 SNP rs179008, which is consistent with the prevalence of this SNP in a large (n > 500) control group of predominantly male, Caucasian individuals in an HIV study (33). Notably, this SNP was detected in all 3 patients who developed CD8⁺ T-cell responses after vaccination with NY-ESO-1 and Montanide plus resiquimod. The other patient who also had this particular SNP did not develop CD8⁺ T-cell responses after vaccination with NY-ESO-1 and Montanide plus placebo. The observed statistically significant correlation between the TLR7 SNP rs179008 and CD8⁺ T-cell response induced by a vaccine containing the respective TLR agonist raises the possibility that SNP analysis may be useful to select appropriate vaccine adjuvants based on TLR polymorphisms in patients with cancer. The SNP rs179008 results in alteration of a Gln to a Leu residue in the signal peptide sequence of TLR7. In silico analysis using SignalP previously predicted that the amino acid change to Leu at position 11 extends the hydrophobic region of the signal sequence, possibly affecting the processing of TLR7 (45). It is conceivable that altered TLR7 processing could lead to enhanced sensitivity of TLR7, potentially affecting the production of mediators for inflammation and immunity.

In conclusion, vaccination with NY-ESO-1 protein given in combination with Montanide with or without topical resiquimod induces NY-ESO-1–specific humoral and CD4⁺ T-cell responses. CD8⁺ T-cell responses were only induced in a subset of patients vaccinated with NY-ESO-1 combined with Montanide and resiquimod. These immune responses were targeted at multiple epitopes of NY-ESO-1, primarily in the N-terminal portion for the antibody responses and central/C-terminal portions for the cellular responses. Recent observations of NY-ESO-1 immune responses as a predictor of objective tumor

**Figure 4.** Effect of CD25 depletion on NY-ESO-1–specific cellular responses. A, NY-ESO-1 CD4⁺ T-cell response in a patient becomes detectable after in vitro CD25 depletion. B, comparison of % CD25 expression in CD4⁺ T cells pre- and posttreatment in patients who had detectable NY-ESO-1–specific CD4⁺ T-cell responses (responders) versus patients who did not have detectable NY-ESO-1–specific CD4⁺ T-cell responses (nonresponders). C, analysis of potential coexpression of CD25 with CTLA-4, PD-1, Tim-3, and Foxp3 in CD4⁺ T cells pre- and posttreatment in all patients.
responses to CTLA-4 blockade and the finding that CTLA-4 blockade can induce or enhance polyfunctional CD4+ and CD8+ T-cell responses (2, 46) provide the rationale for concurrent therapy using NY-ESO-1 vaccines in combination with immune checkpoint blockade or other agents mediating relief from immune suppression in patients with melanoma and other cancers. An ongoing study using NY-ESO-1 vaccine and CTLA-4 blockade in melanoma (NCT01810016) is testing this hypothesis.

Disclosure of Potential Conflicts of Interest
S. Gnjatic has ownership interest in NY-ESO-1-related patents. J. Escalon is a senior nurse practitioner coordinator at Mount Sinai Hospital. P.A. Ott is a consultant/advisory board member for Amgen and Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments
The authors thank Christine Sedrak for excellent technical assistance.

Grant Support
This study was sponsored and funded by the Ludwig Institute for Cancer Research with a supplemental grant from the Cancer Research Institute as part of the Cancer Vaccine Collaborative. S. Gnjatic was supported by a grant from the Cancer Vaccine Collaborative for Immunological Monitoring. J.D. Goldberg and L. Rolnitzsky were supported by S P30 CA16087. New York University Cancer Center Support Grant S P30 CA16087.

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Received October 28, 2014; revised December 12, 2014; accepted December 16, 2014; published OnlineFirst January 29, 2015.

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NY-ESO-1 Protein and Montanide ± Resiquimod in Melanoma


Cancer Immunology Research

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