Immunization of Malignant Melanoma Patients with Full-Length NY-ESO-1 Protein Using TLR7 Agonist Imiquimod as Vaccine Adjuvant

Sylvia Adams, David W. O'Neill, Daisuke Nonaka, Elizabeth Hardin, Luis Chiriboga, Kimberly Siu, Crystal M. Cruz, Angelica Angiulli, Francesca Angiulli, Erika Ritter, Rose Marie Holman, Richard L. Shapiro, Russell S. Berman, Natalie Berner, Yongzhao Shao, Olivier Manches, Linda Pan, Ralph R. Venhaus, Eric W. Hoffman, Achim Jungbluth, Sacha Gnjatic, Lloyd Old, Anna C. Pavlick and Nina Bhardwaj

*J Immunol* 2008; 181:776-784; doi: 10.4049/jimmunol.181.1.776
http://www.jimmunol.org/content/181/1/776

**References**

This article cites 50 articles, 22 of which you can access for free at:
http://www.jimmunol.org/content/181/1/776.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Immunization of Malignant Melanoma Patients with Full-Length NY-ESO-1 Protein Using TLR7 Agonist Imiquimod as Vaccine Adjuvant


Toll-like receptors are a family of highly conserved transmembrane receptors on cells of the immune system that function to alert the host to the presence of specific molecular patterns found in microbes (1, 2). TLR ligands or agonists activate the activation of APCs such as dendritic cells (DCs) by triggering their maturation program, which involves up-regulation of the costimulatory molecules and secretion of proinflammatory cytokines such as TNF-α, IL-6, IL-12 and IFN-γ (3). In addition to activating the innate immune response, stimulation of TLRs on APCs can direct adaptive immunity, including the induction of a Th1 cell response, which is thought necessary for antitumor immunity (4–6). Incorporating TLR agonists into vaccines could be a very effective way to boost vaccine activity (7).

At least 10 members of the TLR family have been identified so far in humans. Two of these, TLR7 and TLR8, are closely related molecules that induce antiviral immune responses through the recognition of single-stranded viral RNA in the endosomal compartmen (8–11). Synthetic ligands belonging to the imidazoquinoline family have been identified that also trigger TLR7 signaling and immune activation (4, 12). Ligation of TLR7 on human myeloid and plasmacytoid DCs by these compounds induces DC maturation and the secretion of important inflammatory mediators such as TNF, IL-6, IL-12 and IFN-γ (3). In addition to activating the innate immune response, stimulation of TLRs on APCs can direct adaptive immunity, including the induction of a Th1 cell response, which is thought necessary for antitumor immunity (4–6). Incorporating TLR7 agonists into vaccines could be a very effective way to boost vaccine activity (7).

1 This work was supported by the Ludwig Institute for Cancer Research, the Cancer Vaccine Collaborative, and the Cancer Research Institute. S.A. was supported in part by an American Society of Clinical Oncology Career Development Award and National Institutes of Health Grant 5R01AI061687. Y.S. was supported in part by National Institutes of Health Grant 5P30CA016087. N.B. was supported in part by National Institutes of Health Grant 5R01AI061684, the Emerald Foundation, Center for AIDS-HIV Vaccine Immunology, the Gates Foundation, and is a recipient of the Burroughs Wellcome Fund Clinical Scientist Award and a Doris Duke Distinguished Clinical Scientist Award.

†S.A. and D.W.O. contributed equally to this work.

‡Address correspondence and reprint requests to Dr. Nina Bhardwaj, New York University Cancer Institute, Smilow 1304, 522 First Avenue, New York, NY 10016. E-mail address: Nina.Bhardwaj@nyumc.org.

© Abbreviations used in this paper: DC, dendritic cell; DTH, delayed-type hypersensitivity; ICS, Intracellular cytokine staining; IVS, in vitro T cell presensitization; LAMP, lysosome-associated membrane protein; NED, no evidence of disease; FSC, forward scatter.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/20.00

www.jimmunol.org
Table I. *Patient demographics, tumor stage and NY-ESO-1 expression, HLA typing results, and immunological and clinical summaries*

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (years)</th>
<th>Sex</th>
<th>AJCC Stage</th>
<th>Prior Therapy</th>
<th>Tumor Stage</th>
<th>NY-ESO-1 Expression</th>
<th>NY-ESO-1 CD4⁺ T Responses</th>
<th>Latest Follow-up (days)</th>
<th>Current Disease Status</th>
<th>NY-ESO-1 Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>59</td>
<td>F</td>
<td>III B</td>
<td>Surgery A1, A1, B57, B8, DR13, DR14, DQ5, DQ6, DP2, DP6</td>
<td>N/A</td>
<td>785</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>76</td>
<td>M</td>
<td>IIIB</td>
<td>Surgery A1, A42, B18, B51, Cw1, Cw5, DR3, DQ11, DQ2, DQ3, DP4, DP6</td>
<td>+ (&lt;5%) tumor cells</td>
<td>581</td>
<td>R (827 days)</td>
<td>+</td>
<td>+</td>
<td>R</td>
</tr>
<tr>
<td>3</td>
<td>38</td>
<td>M</td>
<td>III C</td>
<td>Surgery A1, A33, B51, B56, C8, C8, DR4, DQ7, DQ2, DP3, DP2, DP6</td>
<td>N/A</td>
<td>709</td>
<td>N/A</td>
<td>+</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>F</td>
<td>III B</td>
<td>Surgery A3, A11, B44, D1,C24, DR13, DQ6, DP4, DP4, DP4, DP4</td>
<td>N/A</td>
<td>869</td>
<td>N/E</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>48</td>
<td>F</td>
<td>III C</td>
<td>Surgery, radiation A24, A3, B39, B4, C7, C16, DR7, DR12, DQ3, DP3, DP6</td>
<td>N/A</td>
<td>791</td>
<td>R (123 days)</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>62</td>
<td>F</td>
<td>III B</td>
<td>Surgery A11, A25, B13, B9, C8, DR7, DP5, DP4, DP4</td>
<td>N/A</td>
<td>855</td>
<td>RDF</td>
<td>+</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>48</td>
<td>F</td>
<td>III B</td>
<td>Surgery A1, A11, B18, B51, C5, C16, DR3, DQ11, DQ2, DP3, DP4, DP4</td>
<td>N/A</td>
<td>744</td>
<td>N/E</td>
<td>+</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>51</td>
<td>F</td>
<td>III A</td>
<td>Surgery A1, A24, B13, B35, Cw4, Cw6, DR4, DQ7, DQ2, DP3, DP4, DP4</td>
<td>N/A</td>
<td>764</td>
<td>N/E</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

* R, recurrence (at x days); RDF, rendered disease-free following recurrence; LFU, lost to follow-up; +, positive; −, negative.

the treatment of anogential warts caused by human papillomavirus as well as for basal cell carcinoma and actinic keratosis (22–25). In animal models, imiquimod given either topically or systemically has demonstrated adjuvant activity in vaccines using antigenic peptides (26), proteins (27), and DNA (28, 29), as well as in vaccines using recombinant *Listeria* (30) or DCs (31). In humans, it was shown that topical imiquimod treatment may enhance the immunogenicity of a melanoma peptide vaccine when given with systemic FLT3 ligand (32). Additionally, injection of immature DCs into imiquimod-pretreated skin lead to DC activation in situ and enhanced migratory capacity to draining lymph nodes in cancer patients (33).

In this study, we test the safety and feasibility of imiquimod in a vaccine against the cancer/testis Ag NY-ESO-1, and evaluate the immunogenicity of the combination. NY-ESO-1 is detectable in ~30% of metastatic melanomas (34–36). It is expressed by a variety of cancers but not in adult somatic tissues, making it an attractive target for immunotherapies (37, 38). We show that topical imiquimod used as a vaccine adjuvant is well tolerated, and that the vaccine induces measurable NY-ESO-1-specific Ab and CD4⁺ T cell responses. Immunohistochemistry studies indicate that imiquimod induces dermal inflammatory infiltrates that are rich in APCs and T cells, which suggest a mechanism for the immunogenicity of this combination.

**Materials and Methods**

**Study design, patients, and treatment schedule**

This was a pilot, single arm study in nine patients. The primary objective was to evaluate the safety of the vaccine combination; the secondary objective was to determine the frequency of induced T and B cell immunity. Additional exploratory endpoints included the characterization of the vaccine-induced immune response and the immunostimulatory effect of imiquimod in treated skin. Patients with histologically confirmed, resected malignant melanoma (American Joint Committee on Cancer (AJCC) stages (39) IB, IIC, and II) were eligible. Additional eligibility criteria included age ≥ 18 years, ECOG performance status (40) ≤ 2, and adequate organ and marrow function. Patients were excluded for any of the following: anticitcancer therapy within 4 wk, prior NY-ESO-1 vaccination, immunodeficiency or use of immunosuppressive medications, autoimmune diseases other than vitiligo, intercurrent illnesses, pregnancy or lactation, or inflammatory skin disorders. Tumor NY-ESO-1 expression was not required for study entry, but was offered optionally as part of enrollment into the New York University Interdisciplinary Melanoma Cooperative Group (IMCG) database. Immunohistochemistry for NY-ESO-1 expression was performed as previously described (36). The clinical trial (NCT00142454, www.clinicaltrials.gov) was approved by the New York University School of Medicine Institutional Review Board and written informed consent was obtained from all patients. Nine patients were screened, all were found eligible and enrolled. All completed four vaccinations and two follow-up visits.

Imiquimod cream (5%, 250 mg) was self-applied topically by patients to a 4 × 5-cm outlined area of healthy exterior skin overnight on days 1–5 of each cycle. Application and removal times were recorded in treatment diaries. Recombinant human NY-ESO-1 protein (100 μg in 4 μl) was injected intradermally into the imiquimod-treated site on day 3. Cycles were repeated every 3 wk for a total of four injections. Imiquimod was omitted on day 5 of the last cycle to avoid biopsy site irritation.

**Blood samples and peptides**

Blood was drawn at baseline (day 1/cycle 1), on days 1 and 8 of each treatment cycle, and 3 and 6 wk following the fourth vaccine. PBMCs were isolated from heparinized blood by Ficoll centrifugation and frozen in aliquots in 90% pooled human serum/10% DMSO. Supernatant plasma from each time point was also frozen. A library of 17 overlapping peptides (20- to 22-mers, 10 aa overlap) spanning the entire NY-ESO-1 sequence was obtained from NeoMPS via the Ludwig Institute (41). The peptide sequences (starting from the NY-ESO-1 N-terminus) are: MQAGRGTGGTGSTGADDPGG, STGTDADGPPGDPGPG, PGDPDGNGAGGPGGAGAT, AGPGGEGATAGGRGPGAGA, GGRGPGRAACRASGPgpGGA, ARASGPGGPAGPBGHGGAAS, PRGPBGGAASGLNGCCRCGA, GLNGCCRCGAPGSRLEEL, RPGPSRLEFEYFLAMPFATPM, YLAMPFATPMOEALARRSLA, EAELARRSLAQADLAPP VPG, QACAPPLLPRPVGVLKKETFVSG, PVGLLKEFTVSGNILTILRTAADHRQLQQLSIS, AADHRQQLQQLSISCLQLQLSLMMWTOQCFLPFVF, WTQCFQCLVFPLAQPSGGQR. Each lyophilized peptide was resuspended in DMSO at a concentration of 2 mg/ml and stored frozen. A stock solution of 17 pooled overlapping peptides, 100 μg/ml each, was used for immune monitoring assays at a final concentration of 1 μg/ml each.

**IFN-γ ELISPOT assay**

PBMCs were thawed and cultured overnight in R-10 medium (RPMI 1640 medium with 10% heat-inactivated human AB serum, 10 mM HEPE, and 20 μg/ml gentamicin) and plated the following day at 300,000 per well in 96-well polyvinylidene difluoride filter plates (Millipore) coated with anti-IFN-γ mAb (Mabtech) in the presence or absence of pooled NY-ESO-1 peptides (1 μg/ml each) in the presence of 1 μg/ml Staphylococcus enterotoxin A (Sigma-Aldrich). Plates were cultured overnight, then washed and developed with 2 μg/ml biotinylated anti-IFN-γ Ab (Mabtech), avidin-HRP complex, and 3-amin-9-ethylcarbazole substrate (Vector Laboratories). Spots were counted with a CTL ImmunoSpot analyzer (Cellular Technology), and average values for triplicate wells multiplied by 3.33 to determine the number of spot-forming cells per million PBMCs.
In vitro T cell presensitization (IVS)

In vitro presensitizations were performed as described (41). PBMCs were thawed and cultured overnight in I-10 medium (IMDM with 10% heat-inactivated human AB serum, 1 mM HEPES, 0.1 mM MEM nonessential amino acids, and 2 mM GlutaMAX), then separated into CD4$^+$/H11001, CD8$^+$/H11001, and CD4$^+$/CD8$^+$ (APC) fractions using MACS MS columns (Miltenyi Biotec). Each fraction was then washed and resuspended in I-10 medium containing 10 U/ml IL-2 and 10 ng/ml IL-7. APCs were irradiated (3000 rads) and then cocultured with CD4$^+$ or CD8$^+$ cells (500,000 to 1 million cells per well) and pooled NY-ESO-1 peptides (1 µg/ml each) in a 96-well round-bottom plate for 7 days, replenishing medium and cytokines every 2–3 days.

Intracellular cytokine staining (ICS)

All reagents were obtained from BD Biosciences. For initial screening, 1 wk IVS cultures were harvested, washed, and replated in I-10 medium in duplicate wells of 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, with the other well being left unstimulated. An unstained control and a Staphylococcus enterotoxin A-stimulated control well were also included. For epitope mapping, IVS cultures were plated into 18 wells, 17 of which contained one of the NY-ESO-1 overlapping peptides, with the final well being unstimulated. For all ICS cultures, plates were incubated for 1 h at 37°C, after which brefeldin A (GolgiPlug) was added to each well and the cultures incubated another 5 h. Cells were then stained for CD4 and CD8, fixed and permeabilized with BD Cytofix/Cytoperm solution, then washed with 1X BD Perm/Wash buffer and stained for CD3 and IFN-γ. Cells were analyzed on a BD LSR II flow cytometer using FACS Diva software and compensated using BD CompBeads. Data were analyzed using FlowJo software (TreeStar).

Measurement of Ab responses

Patient plasma samples were analyzed by ELISA for seroreactivity against various recombinant protein Ags (NY-ESO-1, LAGE-1, MAGE-3, SSX2, p53, Melan-A, tyrosinase), as well as against pools of overlapping peptides (10 µg/ml each) covering the NY-ESO-1 or MAGE-3 sequences. Plasma was serially diluted from 1/100 to 1/100,000 and added to low-volume 96-well plates (Corning Glass) coated with 1 µg/ml Ag and blocked with PBS containing 5% nonfat milk. After incubation, plates were washed with PBS containing 0.2% Tween 20 and rinsed with PBS. Plasma IgG (total or subclasses) bound to Ags was detected with alkaline phosphatase-conjugated specific mAbs (Southern Biotechnology Associates). Following addition of ATTOPHOS substrate (Fisher Scientific), absorbance was measured using a fluorescence reader Cytofluor series 4000 (PerSeptive Biosystems). A reciprocal titer was calculated for each plasma sample as the maximal dilution still significantly reacting to a specific Ag. This value was extrapolated by determining the intersection of a linear trend regression with a cutoff value. The cutoff was defined as 10 × the average of OD values from the first four dilutions of a negative control pool made of 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 significantly reactive, and specificity was determined by comparing reactivity to control Ags and to pooled NY-ESO-1 peptides. Epitope mapping was performed using individual NY-ESO-1 peptides. For these assays, plasma samples were chosen at the peak of Ab response. Sera of three patients with known spontaneous NY-ESO-1 responses were analyzed for reactivity.

FIGURE 1. Typical local erythematous skin reaction in the area of imiquimod application (representative patient).

FIGURE 2. NY-ESO-1 Ab responses. Extrapolated reciprocal titers (y-axis) for each patient at each vaccination time point (x-axis) as measured by ELISA using NY-ESO-1 recombinant protein (circles) and NY-ESO-1 peptide pool (squares). For responders, the mean maximum titer was 1/1400 using NY-ESO-1 protein, 1/1500 using NY-ESO-1 peptides. Time points indicated by vaccination cycle (C) and day (D), or by follow-up visit (FU1, 1-mo follow-up; FU2, 2-mo follow-up).
immunity were chosen as positive control; serum from one patient with known seronegativity for NY-ESO-1 served as a negative control.

Delayed-type hypersensitivity (DTH) measurement and skin biopsies

Forty-eight hours after the final vaccination, patients were evaluated for DTH, defined as induration ≥5 mm. Punch biopsies (4 mm) were then obtained from imiquimod-treated skin, from the vaccine site within the imiquimod area, and from untreated skin. Formalin-fixed, paraffin-embedded tissue sections were stained with H&E or analyzed by immunohistochemistry with Abs to CD1a and DC-lysosome-associated membrane protein (LAMP) (Immunotech), CD3, CD4, CD8, and CD20 (Ventana Medical Systems), CD25, CD11c, CD83, and Langerin (NovoCastra), CD57 (Cell Marque), CD68 (DakoCytomation), and CD123 (BD Biosciences). Slides were developed using biotinylated secondary Abs, avidin-HRP conjugate, diaminobenzidine substrate enhanced with copper sulfate, and hematoxylin counterstain. Positive and negative controls were included with the study sections, which were evaluated by three investigators, including a pathologist blinded to the biopsied site. Positive cells were counted manually per 10 consecutive high-power fields (×400) in each of three layers: epidermis, papillary dermis and reticular dermis, and the sum of all 30 high-power fields reported.

Statistical analysis

Adverse events were graded by common toxicity criteria (CTC, version 3.0) and summarized. Positive B cell immune responses were defined as a titer ≥100 as determined by reactivity to overlapping NY-ESO-1 peptides and NY-ESO-1 protein. Positive T cell responses were defined as a 3-fold or greater increase over prevaccination levels that was also 3-fold greater than a parallel unstimulated control, with a minimum value of 10 spot-forming cells per million PBMCs for ELISPOT assays or 0.1% IFN-γ+ cells for ICS. ELISPOT, ICS, and ELISA data were summarized using descriptive statistics. The exact test ( McNemar’s) was used to compare the presence or absence of inflammatory infiltrates in biopsied skin by H&E stain. The Wilcoxon signed-rank test was used to compare immunohistochemistry results.

Results

Patient characteristics and clinical data

The patient population consisted of 7 women and 2 men, 38–76 years of age (mean age of 54 years, Table I). All patients were of white/Caucasian/non-Hispanic background. All had resected malignant melanoma (3 patients AJCC stage II, 6 patients AJCC stage III) and were disease free (no evidence of disease, NED) at the time of enrollment. All patients previously declined adjuvant IFN-α, a standard option after surgical resection for high-risk primary or regional disease. NY-ESO-1 expression in the resected tumor was not required for study entry, because we have previously shown that NY-ESO-1 expression is more prevalent in metastatic disease compared with primary tumors (36), suggestive of evolution with disease progression or recurrence. This observation provides a rationale for immunotherapeutic interventions even in patients with NY-ESO-1-negative tumors. Consistent with the reported frequencies, in our subset of patients tested, only one of three tumors expressed the Ag (Table I).

All patients remained NED at completion of the study at 4 mo. The clinical median follow-up time is 773 days (range of 581–869 days).

FIGURE 3. Intracellular cytokine staining. Following a 1-wk IVS with pooled NY-ESO-1 overlapping peptides, cells were restimulated with Ag and stained for intracellular IFN-γ. a, Gating for flow cytometry. Cell aggregates were excluded by gating for singlets (forward scatter [FSC] height [FSC-H] vs FSC area [FSC-A]), and CD4+ T cells were selected by sequentially gating for lymphocytes, CD3+, CD3− cells, and CD8+ CD4+. This gating strategy removes most autofluorescent dead or dying cells and minimizes nonspecific background. b, Quantification of IFN-γ-secreting NY-ESO-1-specific CD4+ T cells. Representative before (upper) and after (lower) vaccine samples for patient 6 are shown. Plots on the right show T cells restimulated with pooled NY-ESO-1 overlapping peptides. Plots on the left show parallel presensitized T cell controls that did not receive a second stimulation. For all plots, CD4 staining is shown on the y-axis and IFN-γ staining is shown on the x-axis. c, Summary of results for all patients. Filled bars show the percentage of CD4+ T cells secreting IFN-γ following restimulation with the NY-ESO-1 overlapping peptide pool. Open bars represent the values for parallel controls that did not receive a second stimulation. The threshold for detection for this assay, defined as 0.1% of CD4+ T cells, is indicated by a horizontal line in each graph. Time points are indicated by vaccination cycle (C1, C2, C3, or C4) and day (D01 or D10), or as follow-up visits (FU1, 1-mo follow-up; FU2, 2-mo follow-up).
days) from the start of investigational therapy. Three of the 9 patients recurred (median time to recurrence of 468 days), and 6 patients remain disease free at present (Table I).

Safety of NY-ESO-1 protein/imiquimod

NY-ESO-1/imiquimod was well tolerated, and all patients completed the study. Treatment-related adverse events were mild and transient. Local reactions at the site of imiquimod application or vaccine injection were seen in 8 of 9 patients (89%, Fig. 1). Four of 9 patients (44%) reported fatigue, and 2 of 9 patients (22%) experienced flu-like symptoms. All adverse events were grade 1 (CTC version 3.0) and were likely related to the immunomodulatory effects of imiquimod and vaccination. A hemoglobin decrease of >1 g/dl was observed in 3 patients over the course of the trial, possibly related to phlebotomy.

Humoral immune responses to NY-ESO-1

No patient had evidence of humoral immunity to NY-ESO-1 before vaccination. Ab responses to recombinant NY-ESO-1 protein were induced in 7 of 9 patients postvaccination (Fig. 2, filled circles). However, most of these patients also showed comparable seroreactivity to recombinant protein control Ags (not shown), indicating that the vaccine may have induced responses to immunogenic contaminants from the bacterial expression system. To unequivocally demonstrate NY-ESO-1 specificity, sera from the 7 protein-reactive patients were tested against a pool of NY-ESO-1 overlapping peptides, which confirmed NY-ESO-1-specificity in 4 of 7 protein-reactive patients (Fig. 2, filled squares).

T cell responses to NY-ESO-1

PBMCs for each time point were tested for the presence of NY-ESO-1-specific T cells by IFN-γ ELISPOT assay using a pool of NY-ESO-1 overlapping peptides (41), which has been shown to be able to detect both CD4+ and CD8+ T cell responses (42). By ELISPOT, no response was identified for any of the study subjects (data not shown). DTH responses to intradermally injected NY-ESO-1 protein were also not detected in any of the subjects.

To determine whether the vaccine had in fact elicited T cell responses that were not detectable by these screening methods, we analyzed PBMC samples following a 1-wk in vitro presensitization (IVS) (41) with the same pool of NY-ESO-1 overlapping peptides. Presensitized T cells were restimulated using the NY-ESO-1 peptide pool, then stained for the presence of intracellular IFN-γ and analyzed by flow cytometry (Fig. 3, a and b). Following the IVS, CD4+ T cell responses to NY-ESO-1 were detected at two or more postvaccine time points in 7 of the 9 study subjects (Fig. 3c). None of the subjects had a detectable response before vaccination, although the prevaccine sample for one subject (patient 8) was not evaluable, as the cryopreserved cells were not viable after thawing. Of the 4 subjects who had developed NY-ESO-1 Abs, 3 of these had CD4+ T responses as well (see Table I). No CD8+ T cell responses were detected following IVS for any of the study subjects, neither before or after vaccination (data not shown). Although this pilot study was not designed to determine an association between immune responses and clinical outcome, survival data in Table I do not indicate a clear correlation between induced immune responses and disease recurrence.

Epitope mapping of cellular and humoral immune responses

Mapping of the epitope reactivity of vaccine-induced T cells using individual rather than pooled peptides showed the induction of CD4+ T cells to several peptide epitopes, in particular the aa 119–143 epitope, which was mapped in all T cell responders (Fig. 4a). Additionally, one patient exhibited a particularly strong response to the aa 81–100 epitope. This reactivity, however, did not correlate with a specific HLA class II subtype (Table I). The reactivity to both of these epitopes corresponds with the results of previous studies of both vaccinated patients as well as patients with naturally occurring anti-NY-ESO-1 T cells (38, 42) (www.cancerimmunity.org/peptidedatabase/tumorspecific.htm).

Epitope mapping of humoral responses was similarly performed (Fig. 4b). Reactivity was observed toward the N-terminal region of NY-ESO-1 protein (aa 1–30 and aa 51–80), and especially to the aa 91–110 peptide epitope, which was observed in all Ab responders. As with the T cell epitope mapping studies, Ab responses to each of these epitopes have been previously described in vaccinated patients and in patients with spontaneous humoral immunity to NY-ESO-1 (38, 42).

Evaluation of skin biopsies

Skin sections showed mild to moderate perivascular and peridnexal mononuclear cell infiltrates in the papillary and reticular dermis in imiquimod-treated skin and at imiquimod-treated vaccine injection sites of all patients, but not in untreated skin (p < 0.01, Fig. 5a). Imiquimod treatment was associated with no other obvious changes except for occasional epidermal mitoses and apoptotic cells. Immunohistochemistry of the dermal infiltrates revealed that they were composed mostly of T lymphocytes (CD3+, Fig. 5b, CD4/CD8 ratio ~3:1, data not shown), CD11c+ and CD68+ cells (monocytes, macrophages, and myeloid DCs, Fig. 5b and data not shown), and NK cells (CD57+, Fig. 5b). Smaller numbers of CD123+ cells (plasmacytoid DCs) and occasional B lymphocytes (CD20+) were also identified (Fig. 5b). A minority of the cells in
the infiltrate were CD25+ (activated monocytes and T cells, T regulatory cells, data not shown). Occasional cells also expressed CD83 and DC-LAMP, markers of DC activation. All of the above immune cell types were significantly more frequent in imiquimod-treated skin compared with untreated control skin of the same patients (p < 0.05) (Fig. 5b). No statistically significant quantitative difference in immune cells was observed between imiquimod-treated skin and skin receiving imiquimod plus NY-ESO-1 (Fig. 5b). Interestingly, examination of the epidermis for Langerhans cell markers CD1a and Langerin indicated no statistically
significant difference in the number of Langerhans cells in treated and untreated skin (Fig. 5b and data not shown).

Discussion

Herein we report on the safety and feasibility of the topical TLR7 agonist imiquimod when used to condition vaccination sites for intradermal injection with recombinant cancer/testis Ag NY-ESO-1 in patients with malignant melanoma. The NY-ESO-1 protein/imiquimod vaccine regimen was well tolerated. Only mild and transient side effects were observed, and all patients completed the study.

The vaccine combination successfully elicited both humoral and cellular immune responses in a significant fraction of patients. NY-ESO-1-specific Ab responses were detected in 4 of 9 patients (44%). However, Ab titers were significantly lower than those described in a previous study using i.m. injection of NY-ESO-1 protein with the saponin-based adjuvant ISCOMATRIX (43), as well as in a more recent study by our group using Montanide ISA-51 adjuvant and TLR9 agonist CpG 7909 administered subcutaneously with recombinant NY-ESO-1 (42). In both of these studies, all seronegative patients receiving NY-ESO-1 developed Abs, and average maximum titers were 10-fold or more higher than what we observed here, suggesting that a protective carrier formulation providing a controlled release or depot effect may be required for optimal Ab responses. In the present study, imiquimod/NY-ESO-1 did, however, induce Abs in a higher percentage (44%) of patients than those in the control group of the ISCOMATRIX study who received NY-ESO-1 protein alone without any adjuvant (25% of patients) (43). Epitope mapping of the Ab responses using individual peptides confirmed that the immunogenic regions of the protein were similar to those found in patients with spontaneous NY-ESO-1 immunity, indicating that the vaccine is capable of inducing B cell responses against epitopes naturally presented by NY-ESO-1-positive tumors. We also have previously shown that these vaccine-induced NY-ESO-1 Abs facilitated cross-presentation (42).

In this study, NY-ESO-1/imiquimod vaccination induced IFN-γ-secreting NY-ESO-1-specific CD4+ T cells in 6 of 8 (75%) evaluable patients. These responses were clearly evident by intracellular cytokine staining following a 1-wk in vitro presensitization with NY-ESO-1 overlapping peptides, but were not detectable in “ex vivo” ELISPOT assays (i.e., without presensitization). In contrast, CD8+ T cell responses were not detectable using either method.

In the above-mentioned ISCOMATRIX study, both CD4+ and CD8+ T cell responses to NY-ESO-1 were detectable in a subset of patients vaccinated with the ISCOMATRIX adjuvant, although a more prolonged in vitro presensitization was used and ex vivo assays were not attempted, making a comparison with our study difficult (43). However, in our recent CpG/Montanide/NY-ESO-1 protein vaccine study almost all patients developed CD4+ T cell responses and approximately half had detectable CD8+ T cell responses as measured by the same in vitro presensitization assay described here. Additionally, ex vivo assays showed CD4+ T cell responses in 67% of subjects and CD8+ T cell responses in 39% (42). These results indicate that CpG plus Montanide is a more potent adjuvant formulation than imiquimod alone, and is capable of cross-priming CD8+ T cell responses. Of note, CpG was administered mixed with the Ag/Montanide emulsion, whereas imiquimod was applied topically before and after Ag injection.

A possible explanation for the absence of CD8+ T cell immunity observed in our study, as well as for the weaker CD4+ T cell and Ab responses, may be the timing used for the application of imiquimod. In recent experiments in mice it was observed that when TLR agonists were provided before Ag encounter, cross-presentation by APCs was impaired and cross-priming inhibited due to premature APC maturation (44). Thus, it is possible that the use of TLR agonists may be most effective at the time of, or immediately following, the delivery of Ag. The use of TLR activation stimuli may also require additional adjuvants or emulsifiers for optimal effect. In recent studies in nonhuman primates, the use of imidazoquinolines or CpG was most effective when these compounds were either covalently coupled to Ag or mixed in a water-oil emulsion (20, 21), and Montanide emulsification in our above-mentioned study might have contributed significantly to the vaccine’s strong immunogenicity (42). Additionally, it may prove that other imidazoquinolines such as resiquimod, which activates both TLR7 and TLR8, are more potent stimulators of immunity than imiquimod (45). We are currently addressing these issues in a follow-up clinical study.

One question raised by this study is whether similar results could have been obtained by vaccinating with NY-ESO-1 protein alone. Although we did not include an Ag-alone control arm, previous studies have confirmed that NY-ESO-1 or MAGE-3 proteins given alone are poorly immunogenic (43, 46, 47), and that addition of an immunological adjuvant is essential for the induction of persistent immunological memory (48). The above-mentioned NY-ESO-1 protein/ISCOMATRIX study included an NY-ESO-1 protein-alone control group of 16 patients, most of whom had resected melanoma. NY-ESO-1 protein was given i.m. at 4-wk intervals at the same dose as in our study (100 mcg). However, the frequency of induction of NY-ESO-1-specific Abs was less than we observed for NY-ESO-1/imiquimod (25% compared with 44%), and the induction of CD4+ T cells reactive to NY-ESO-1 overlapping peptides was 0% compared with 75%. As in our study, CD8+ T cells reactive to NY-ESO-1 were not induced in the absence of ISCOMATRIX. These data suggest that topical imiquimod can enhance the induction of Ab and CD4+ T cell responses, but not CD8+ T cell responses.

To address the potential mechanism of action of imiquimod in inducing vaccine responses, we show that topical application of imiquimod is associated with dermal mononuclear cell infiltrates that are composed largely of T cells, APCs (monocytes, macrophages, myeloid DCs, and plasmacytoid DCs) and NK cells. Activated DCs (CD83+ and DC-LAMP+ cells) were also evident. Only rare B cells were seen. Although we were unable to further characterize the observed CD123+ cells by double-staining, we expect most of them to be plasmacytoid DCs, as we morphologically excluded basophils (which also express CD123). Our findings are in accordance with previous observations of recruitment and activation of myeloid and plasmacytoid DCs in the skin of patients whose skin tumors were treated with imiquimod (18, 19), as well as observations of accumulation of plasmacytoid DC-like cells in the skin of mice after imiquimod treatment (17), and of “monocyte-macrophage-dendrocyte” dermal infiltrates in healthy skin chronically exposed to imiquimod (49). These observations suggest that the recruitment and activation of APCs at the vaccination site by imiquimod may lead to improved uptake and presentation of the injected protein Ag, thus promoting immunization.

In contrast to other studies, we did not observe a net loss of epidermal Langerhans cells following imiquimod application (16, 17, 49). These studies reported a decrease in CD1a+ Langerhans cells in the epidermis, suggestive of migration to draining lymph nodes, after imiquimod treatment for up to 10 days in mice and following chronic exposure over months in humans. The observed difference with our results may be due to a dose effect, as studies using the imidazoquinoline resiquimod in healthy volunteers demonstrated a decrease of CD1a+ cells only in the highest dose group.
(50). Furthermore, recent analyses have failed to detect TLR7 in Langerhans cells freshly purified from human skin, at least at the mRNA level (51).

In summary, this study demonstrates the feasibility and excellent safety profile of a topically applied TLR agonist as adjuvant for a protein vaccine in cancer patients. Although no conclusions about imiquimod’s additive effect can be drawn in the absence of a control arm, we show that the imiquimod/NY-ESO-1 combination elicits both humoral and CD4+ T cell responses in a significant fraction of patients. In light of these findings, further studies evaluating imiquimod’s relative effect on the immunogenicity of this combination, including experiments evaluating the dose and timing of its application, are warranted.

Acknowledgments
We thank Drs. Gerd Ritter, Herman Yee, Michelle Lowes, Maha Ayyoub, Danila Valmori, and Bruce Strober for their support and guidance, Dr. Thomas Del Corral for preparing the vaccines, Dr. Iman Osman and the New York University Interdisciplinary Melanoma Cooperative Group for access to tissue specimens, Dr. Vandana Mukhi for statistical support, and Juliet Escalon, Sean Lemoine, and Nadege Gilles for data management. We also thank all patients enrolled in this trial.

Disclosures

References


