

NY-ESO-1 Protein Formulated in ISCOMATRIX Adjuvant Is a Potent Anticancer Vaccine Inducing Both Humoral and CD8⁺ T-Cell-Mediated Immunity and Protection against NY-ESO-1⁺ Tumors

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ABSTRACT

NY-ESO-1 is a 180 amino-acid human tumor antigen expressed by many different tumor types and belongs to the family of “cancer-testis” antigens. In humans, NY-ESO-1 is one of the most immunogenic tumor antigens and NY-ESO-1 peptides have been shown to induce NY-ESO-1-specific CD8⁺ CTLs capable of altering the natural course of NY-ESO-1-expressing tumors in cancer patients. Here we describe the preclinical immunogenicity and efficacy of NY-ESO-1 protein formulated with the ISCOMATRIX adjuvant (NY-ESO-1 vaccine). *In vitro*, the NY-ESO-1 vaccine was readily taken up by human monocyte-derived dendritic cells, and on maturation, these human monocyte-derived dendritic cells efficiently cross-presented HLA-A2-restricted epitopes to NY-ESO-1-specific CD8⁺ T cells. In addition, epitopes of NY-ESO-1 protein were also presented on MHC

class II molecules to NY-ESO-1-specific CD4⁺ T cells. The NY-ESO-1 vaccine induced strong NY-ESO-1-specific IFN- γ and IgG2a responses in C57BL/6 mice. Furthermore, the NY-ESO-1 vaccine induced NY-ESO-1-specific CD8⁺ CTLs in HLA-A2 transgenic mice that were capable of lysing human HLA-A2⁺ NY-ESO-1⁺ tumor cells. Finally, C57BL/6 mice, immunized with the NY-ESO-1 vaccine, were protected against challenge with a B16 melanoma cell line expressing NY-ESO-1. These data illustrate that the NY-ESO-1 vaccine represents a potent therapeutic anticancer vaccine.

INTRODUCTION

The NY-ESO-1 human tumor antigen was recently identified using serological analysis of tumor antigens by recombinant cDNA expression (SEREX) cloning analysis of a human esophageal cancer (1). It is a M_r 22,000 cytoplasmic protein (180 amino acids; Ref. 2) expressed in a number of different tumor types including melanoma (reviewed in Ref. 3). NY-ESO-1 belongs to the “cancer-testis” antigens (3), whose expression is restricted to a proportion of human cancer types as well as to germ cells of the testes and/or ovaries in healthy individuals and the trophoblast (3) but is not found in other normal tissues. In contrast to other known cancer-associated antigens, humoral responses against NY-ESO-1 are frequently observed in patients with NY-ESO-1-expressing tumors (4).

CD8⁺ T-cell epitopes derived from cancer-testis antigens, have shown considerable promise in cancer immunotherapy (5). At least four CD8⁺ T-cell epitopes for NY-ESO-1 have now been described for the MHC-class I allele, HLA-A2. These include, SLLMWITQCFL_{157–167}, SLLMWITQC_{157–165}, QLSLLMWIT_{155–163}, and the recently identified cryptic epitope, LMWITQCFL_{159–167} (6–8). Although SLLMWITQCFL_{157–167}, SLLMWITQC_{157–165}, and LMWITQCFL_{159–167} exhibit good *in vitro* immunogenicity, the cryptic LMWITQCFL_{159–167} peptide does not appear to be naturally processed (9). Furthermore, QLSLLMWIT_{155–163} is poorly immunogenic and CD8⁺ CTLs recognizing this epitope are rarely detected in cancer patients (6–8). Although a poorer binder for HLA-A*0201 than the SLLMWITQCFL_{157–167} and QLSLLMWIT_{155–163} peptides, the SLLMWITQC_{157–165} peptide is very efficiently recognized by CD8⁺ T cells from HLA-A*0201 melanoma patients (9–11). Furthermore, vaccination of NY-ESO-1⁺ cancer patients with SLLMWITQCFL_{157–167} and SLLMWITQC_{157–165} peptides induced NY-ESO-1-specific CD8⁺ T cells, some stabilization of disease, and regression of individual metastases in some patients (12). The demonstration that CD8⁺ T cells can provide potent anticancer activity has resulted in a substantial number of human cancer vaccine trials aiming to induce

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anticancer CD8⁺ T cells. However, the importance of anti-tumor CD4⁺ T-cell responses for efficient activation and maintenance of anticancer CD8⁺ T cells has been established in several studies (13–15). In this regard, an NY-ESO-1 epitope restricted to the MHC class II allele, *HLA-DPB1*0401–0402* (found in 43–70% of Caucasians), has been identified (SLLMWITQCFLPVF_{157–170}) and overlaps the SLLMWITQCFL_{157–167} and SLLMWITQC_{157–165} epitopes (16). Furthermore, another *HLA-DRB1*0401*-restricted CD4 epitope has recently been identified and localizes to NY-ESO-1_{119–143} (17, 18). Thus, the major advantage of using a full-length tumor protein as a vaccine is the possibility of generating multiple MHC class I and II-restricted T-cell responses, rather than with peptide vaccines, which limit CD8⁺ T-cell responses to only one of six possible MHC class-I alleles (*i.e.*, *HLA-A2*).

Currently, there are few vaccine modalities that safely and effectively induce potent CD8⁺ T-cell responses in humans with few adjuvants approved for human use. The use of *in vitro*-generated, autologous dendritic cells (DCs) as cellular adjuvants for vaccine delivery is being widely tested in cancer patients (19, 20). However, considerable research is required to optimize the preparation, antigen loading, and route of delivery of DC-based cancer vaccines to achieve broad clinical utility (20). The ISCOMATRIX adjuvant (an immunostimulatory complex that does not contain antigen) is a cage-like structure, typically 40 nm, composed of saponin, phospholipid, and cholesterol. An ISCOMATRIX vaccine (an immunostimulatory complex that contains antigen) can be generated by associating the antigen with the ISCOMATRIX adjuvant. ISCOMATRIX-based vaccines have been used safely in humans to induce strong antibody responses as well as potent CD4⁺ T-helper-cell and CD8⁺ CTL responses to several viral and cancer antigens (21–23). ISCOMATRIX vaccines have a number of potential advantages for use in cancer immunotherapy. These include (a) the absence of potential safety issues associated with live vector systems and DNA; (b) the ability for repeated use due to the lack of induction of neutralizing antibody responses; (c) the induction of effective CD8⁺ T-cell responses, even in the presence of antibodies specific for the vaccine antigen (24); and (d) the generation of broad MHC class I and II epitopes when used with full-length protein antigen resulting in more robust and sustainable immune responses.

Here we illustrate, in human tissue culture and murine *in vivo* models, the immunogenicity of a NY-ESO-1 vaccine composed of full-length NY-ESO-1 protein combined with ISCOMATRIX adjuvant. In addition, we show that the NY-ESO-1 vaccine generates both humoral and T-cell responses and, therefore, represents a valuable therapeutic anticancer vaccine.

MATERIALS AND METHODS

Preparation of Recombinant NY-ESO-1 Protein. The expression plasmid pLICR100kan was transfected into *Escherichia coli* B strain B21, which was fermented to produce NY-ESO-1 as an insoluble inclusion body. Cells were disrupted using high-pressure homogenization, and inclusion bodies were isolated and solubilized using denaturant. The purification of NY-ESO-1 used a three-step column chromatography process of

immobilized metal affinity, anion exchange, and hydrophobic interaction chromatography using Chelating Sepharose FF, Q-Sepharose FF, and Phenyl Sepharose FF (Pharmacia Biotech, Uppsala, Sweden), respectively.⁵

Preparation of ISCOMATRIX Adjuvant. ISCOMATRIX adjuvant was prepared as described previously (25), but using ultrafiltration instead of dialysis. Briefly, a purified fraction of *Quillaia* saponin (ISCOPEP saponin; CSL Limited, Parkville, VIC, Australia) at 100 mg/ml was added to a solution of 10 mg/ml cholesterol (Sigma, St. Louis, MO) and 10 mg/ml dipalmitoylphosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) dissolved in 20% w/v MEGA-10 (Sigma) and buffered isotonic saline (pH 6.2) to give a final concentration of 4 mg/ml ISCOPEP saponin and 0.8 mg/ml of cholesterol and buffered isotonic saline. The solution was then held at 25°C for 60 min and diluted a further 1 in 4 with buffered isotonic saline (pH 6.2). During the subsequent ultrafiltration against 14 volumes of buffered isotonic saline (pH 6.2), ISCOMATRIX adjuvant containing ISCOPEP saponin, cholesterol, and buffered isotonic saline was formed.

Preparation and Characterization of NY-ESO-1 Vaccine. NY-ESO-1 vaccine was prepared by mixing recombinant NY-ESO-1 protein (0.4 mg/ml) with ISCOMATRIX adjuvant (0.4 mg/ml ISCOPEP) at a 1:1 ratio in 0.5 M phosphate buffer containing 0.75 M NaCl, 0.25 M glycine, and 0.2 M urea (pH 6.5). The concentration of ISCOPEP saponin was determined by reversed-phase high-performance liquid chromatography, and the concentration of protein was determined by amino acid analysis. Association between NY-ESO-1 and ISCOMATRIX adjuvant was confirmed by flow cytometry using a FACSCalibur equipped with CELLQuest software (Becton Dickinson, San Jose, CA). Briefly, NY-ESO-1 vaccine was double labeled using a FITC-conjugated anti-NY-ESO-1 monoclonal antibody (mAb; ES121; Ref. 26) and a biotinylated-anti-ISCOMATRIX adjuvant mAb (CSL Limited) followed by streptavidin-allophycocyanin (APC) (PharMingen/Becton Dickinson, San Jose, CA). The formulation was also analyzed by scanning electron microscopy, and the particle size was analyzed by dynamic light scattering using a Nicomp 370. The batch-to-batch variability of the NY-ESO-1 vaccine aggregate profile is relatively consistent as assessed by both screening electron microscopy and fluorescence-activated cell sorting (FACS) analysis.

IHC Analysis of NY-ESO-1 Expression in Tumor Cell Lines. Immunohistochemistry (IHC) for NY-ESO-1 expression was performed on human tumor biopsy samples and transfected tumor cell lines using the mouse-antihuman-NY-ESO-1 mAb, E978 (3 µg/ml), as described previously (26). IHC was performed using the Vectastain Elite Universal ABC kit purchased from Vector Laboratories (Burlingame, CA). All of the sections were submitted to 3% H₂O₂/PBS for 10 min to block endogenous peroxidase. Endogenous biotin activity was quenched by sequential application of egg white and skim milk.

⁵ R. Murphy *et al.*, Recombinant NY-ESO-1 cancer antigen: production and purification of a tumour specific antigen under cGMP conditions, submitted for publication.

All of the incubations were performed at room temperature using the Shandon Sequenza immunostainer. 3-Amino-9-ethyl-carbazole (Sigma A-5754) was used as the chromogen, and slides were counterstained with Mayer's hematoxylin (Amber Scientific MH-5L). Application of CrystalMount (Biomedica M03) preceded dehydration and mounting in DePeX (BDH 36125).

Quantitative Real-Time PCR Analysis of NY-ESO-1 Expression on Tumor Cell Lines. To quantify the copy number of NY-ESO-1 mRNA, quantitative real-time PCR was performed using ABI Prism 7700 Sequence Detection System Taqman (Applied Biosystems, Foster City, CA). A multiplex PCR containing 1 μ l cDNA was set up with the housekeeping gene β -actin according to the manufacturer's instructions (Applied Biosystems) to normalize the cDNA samples. Probes and primers for NY-ESO-1 were designed across intron/exon boundaries to avoid amplification of genomic DNA. The primer and probe sequences were as follows: forward, 5'-TGC TTG AGT TCT ACC TCG CCA T-3'; reverse, 5'-TAT GTT GCC GGA CAC AGT GAA-3; Probe, 6FAM-AGG ATG CCC CAC CGC TTC CC-TAMRA. The conditions used are described by Taqman protocols (Applied Biosystems) with up to 40 cycles of amplification used per sample. Results were analyzed using the SDS program version v1.7. Copy numbers of NY-ESO-1 were calculated from standard curves of the relevant plasmid and were expressed per 10^5 copies of the housekeeping gene β -actin in 100 ng of total RNA.

Generation of NY-ESO-1-Specific Human CD4⁺ and CD8⁺ T-Cell Lines. Short-term and longer-term CD8⁺ CTL cell lines specific for the NY-ESO-1 peptide SLLMWITQC₁₅₇₋₁₆₅ were established from an HLA-A2⁺ patient who had a melanoma that expressed NY-ESO-1⁺ and high-titer anti-NY-ESO-1 antibodies in serum. Similarly, short- and longer-term CD4⁺ T-cell lines specific for the NY-ESO-1 peptide SLLMWITQCFLPVF₁₅₇₋₁₇₀ were established from an HLA-DP4⁺ patient. All of the Ludwig Institute clinical trial protocols used in the present study comply with National Health and Medical Research Council guidelines and were approved by the Human Research and Ethics Committee of the Austin and Repatriation Medical Centre (A&RMC). Briefly, 1×10^6 irradiated (3000 rad) peripheral blood mononuclear cells (PBMCs) were pulsed with the NY-ESO-1 peptides SLLMWITQC₁₅₇₋₁₆₅ (HLA-A2-restricted) or SLLMWITQCFLPVF₁₅₇₋₁₇₀ (HLA-DP4-restricted); 10 μ g/ml, 2 h at room temperature), were washed twice in RPMI 1640, and were cocultured with equal numbers of nonirradiated PBMCs in 0.5 ml of Iscove's modified Dulbecco's medium supplemented with 5% human serum (CSL Limited) in 48-well plates. At day 3 of the culture, 5 units/ml interleukin (IL)-2 (Peprotech, Rocky Hill, NJ) were added. After 7 days, the T cells were restimulated with irradiated, peptide-pulsed, autologous PBMCs and were maintained in the presence of 25 units/ml IL-2 in 24-well plates. Thereafter, the CD8⁺ T-cell line (1×10^5 cells) was restimulated weekly with 1×10^4 peptide-pulsed, irradiated (10,000 rad) T2 cells and 2×10^4 HLA-A2⁺, allogeneic, irradiated (10,000 rad) EBV-transformed B cells. Similarly, the CD4⁺ T-cell lines were restimulated weekly with 1×10^4 peptide-pulsed, irradiated (10,000 rad) autologous PBMCs and 2×10^4 HLA-DP4⁺, allogeneic, irradiated (10,000 rad) EBV-transformed B cells. Both CD4⁺ and CD8⁺ T

cells were used in assays at least 1 week after the last restimulation.

Human IFN- γ Enzyme-Linked Immunospot-Forming Assay. Millipore multiscreen plates (Millipore, Molsheim, France) were coated with anti-IFN- γ antibody [5 μ g/ml, in 0.1 M NaHCO₃ buffer (pH 8.3), 2 h at room temperature] and were blocked for 1 h with PBS supplemented with 10% FCS. Peptide-sensitized (10 μ g/ml, 2 h at room temperature and washed) T2 cells or melanoma target cells (5,000/well) were cocultured with 1.5×10^3 CD8⁺ T cells specific for the NY-ESO-1 SLLMWITQC₁₅₇₋₁₆₅ peptide. T2 cells, pulsed with an irrelevant HLA-A2-restricted peptide (MAGE-3:-FLWGPRALV₂₇₁₋₂₇₉), were used as a negative control. The LAR (Ludwig Austin Repatriation) series of melanoma cell lines were derived at the Ludwig Institute from patients' biopsy samples, and consent was obtained from each patient before establishment. After overnight culture, cells were lysed with H₂O for 30 min; and horse-radish-peroxidase-labeled anti-IFN- γ antibody was added for 2 h (10 μ g/ml PBS, 3% FCS, and 0.05% Tween 20). After washing, spots were developed with 3-amino-9-ethyl-carbazole in acetate buffer for 8 min. The plates were washed with H₂O and air-dried. IFN- γ -spots were counted using a video camera (TK-1280E, Zeiss, Göttingen, Germany) and the VideoPro software (Olympus, Mount Waverly, Australia).

Uptake of NY-ESO-1 Vaccine by MoDCs and Induction of Maturation. Monocyte-derived DCs (MoDCs) were generated by affinity-purifying CD14⁺ monocytes from healthy donor PBMC buffy packs (Red Cross Blood Bank, Melbourne, VIC, Australia) using the MACS CD14 isolation kit (Miltenyi Biotech, Sunnyvale, CA). The monocytes (5×10^5 /well) were cultured in 1 ml of RPMI 1640 supplemented with 10% FCS, granulocyte macrophage colony-stimulating factor (40 ng/ml; Schering-Plough, Sydney, NSW, Australia) and IL-4 (500 units/ml; Schering-Plough, Kenilworth, NJ) in 24-well plates. On day 7, MoDCs represented >90% of cultured cells as determined by CD1a and HLA-DR expression using FACS analysis. At day 7, all of the wells were pooled and the cell concentration readjusted to 1×10^5 cells/ml. DCs were incubated with NY-ESO-1 vaccine (10 μ g/ml ISCOPREP saponin) for 1 h at 37°C, were washed thoroughly, and were recultured prior to analysis for uptake. Uptake of NY-ESO-1 vaccine by MoDCs was assessed by flow cytometry using a FITC-conjugated anti-NY-ESO-1 mAb (clone ES121) and a biotinylated-anti-ISCOMATRIX adjuvant mAb (clone 703B) followed by streptavidin-APC (PharMingen/Becton Dickinson, San Jose, CA). Isotype-matched control antibodies were used to ensure specificity of antibody binding. In a separate series of experiments, one-half of the NY-ESO-1 vaccine-pulsed MoDC cultures were matured overnight (18 h) with various classes of stimuli. These included pro-inflammatory mediators: tumor necrosis factor (TNF)- α (10 ng/ml; R&D systems, Minneapolis, MN); IFN- α 2a (1000 units/ml; Roferon-A, Roche Products Pty., Sydney, NSW, Australia); prostaglandin E₂ (PGE₂) (1 μ M final concentration; ICN Biomedicals, Aurora, OH); or CD40L-trimer (1 μ g/ml; gift from Immunex Corp, an Amgen subsidiary, Seattle, WA); or intact *E. coli* (grown at log phase and used at 5.2×10^6 *E. coli*/ml). The nonmatured MoDCs were kept in their culture medium before analysis for phenotypic maturation. Flow cytometric analysis of MoDCs was performed using the following mAbs: FITC-

conjugated IgG1 isotype control; phycoerythrin-conjugated IgG1 isotype control; anti-CD80-Phycoerythrin; anti-HLA-DR-APC; anti-HLA-A,B,C-FITC; anti-CD86-APC, (PharMingen/Becton Dickinson); anti-CD83-Phycoerythrin (Immunotech, Beckman Coulter, Gladsville, Australia).

Presentation of NY-ESO-1₁₅₇₋₁₇₀ Peptide to NY-ESO-1-Specific CD4⁺ T Cells or NY-ESO-1₁₅₆₋₁₆₅ Peptide to CD8⁺ T cells by MoDCs Pulsed with the NY-ESO-1 Vaccine. In separate experiments, MoDCs were pulsed with NY-ESO-1 vaccine (20 µg/ml ISCOPREP saponin), were washed thoroughly, and then were matured overnight by culturing in fresh medium containing granulocyte macrophage colony-stimulating factor (40 ng/ml), IL-4 (500 units/ml), TNF-α (10 ng/ml), IFN-α2a (1000 units/ml), and PGE₂ (1 µM/ml). These MoDCs were then cocultured with either an NY-ESO-1 SLLMWITQCFLPVF₁₅₇₋₁₇₀-specific CD4⁺ T-cell line (HLA-DP4-restricted) or an NY-ESO-1 SLLMWITQC₁₅₆₋₁₆₅-specific CD8⁺ T-cell line (HLA-A2-restricted) for 4 h, and peptide-induced secretion of IFN-γ by CD4⁺ or CD8⁺ T cells was assessed by intracellular cytokine secretion assay using FACS. For the CD8⁺ T-cell assays, several controls were included during the analysis. Cytokine-matured MoDCs (not pulsed with NY-ESO-1 vaccine) were pulsed with either NY-ESO-1 SLLMWITQC₁₅₇₋₁₆₅ peptide (positive control) or the MAGE-3-HLA-A2-restricted peptide (FLWGPRALV₂₇₁₋₂₇₉; negative control) and were cocultured with the NY-ESO-1 SLLMWITQC₁₅₇₋₁₆₅-specific CD8⁺ CTL cell line in parallel. As additional controls, transporter associated with antigen processing (TAP)-deficient, HLA-A2⁺, T2 cells were pulsed with NY-ESO-1 vaccine and used as APC to eliminate the possibility that NY-ESO-1 vaccine contained or generated free NY-ESO-1 SLLMWITQC₁₅₆₋₁₆₅ peptides during the assay period.

Mice. Female BALB/c and C57BL/6 mice were purchased from Animal Resource Centre (Perth, Australia) and were used at 8–12 weeks of age. Transgenic HHD mice have a transgene composed of the α1 and α2 domains of HLA-A2 linked to the α3, *trans*-membrane and cytoplasmic domains of H-2D^b, with the α1 domain linked to human β₂-microglobulin. This transgene was introduced into murine β₂-microglobulin and H-2D^b double knockout mice; thus, the only MHC expressed by the HHD mouse was the modified HLA-A2 molecule (27). HHD mice were bred at the Queensland Institute for Medical Research and were used at 8–12 weeks of age.

Immunization and CD8⁺ T-cell Assays Using HHD

Mice. Five mice/group were immunized s.c. with 100 µl into the scruff of the neck with the NY-ESO-1 vaccine (5 µg of both NY-ESO-1 and ISCOPREP saponin), or with NY-ESO-1 protein (5 µg of protein) or with the ISCOMATRIX adjuvant (5 µg of ISCOPREP saponin). Three weeks after immunization, splenocytes isolated from individual mice were separately restimulated with peptide-pulsed (10 µg/ml, 37°C, 1 h, two washes) and irradiated (3000 rad) lipopolysaccharide blasts (effector:stimulator ratio, 3:1), as described previously (28). On day 6, cultures were used as effectors in standard enzyme-linked immunospot assays (29) and/or ⁵¹Cr-release assays using EL4 HHD target cells (28) sensitized with NY-ESO-1 peptides. Some cultures were subject to two additional rounds of weekly restimulation with SLLMWITQC₁₅₇₋₁₆₅-sensitized irradiated (8000 rad) HHD EL4 cells (effector:stimulator ratio, 15:1) be-

fore use as effectors against human melanoma cell line targets. The human tumor cell lines were SK-Mel-37 (30), A11-1c, A05, A09-M, A02-Mb, and A12-M, which were derived from patients enrolled in a therapeutic clinical trial (31).

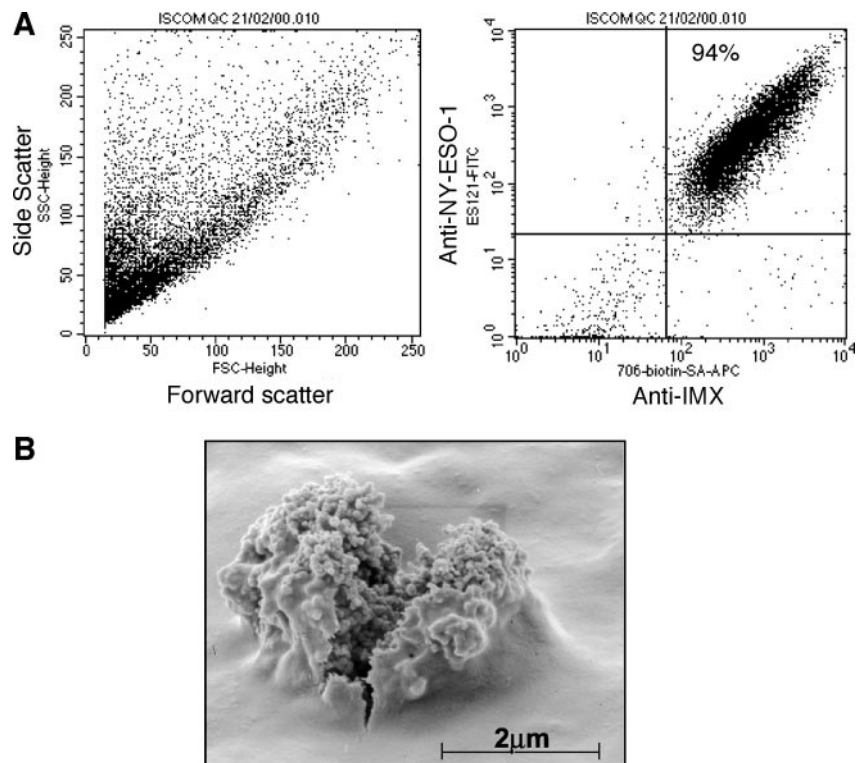
Antibody and IFN-γ Responses in BALB/C Mice.

Mice were immunized s.c. with 100 µl into the scruff of the neck, twice, 3 weeks apart, with the NY-ESO-1 vaccine (5 µg of NY-ESO-1 and ISCOPREP saponin), or with NY-ESO-1 protein (5 µg of protein), or with the ISCOMATRIX adjuvant (5 µg of ISCOPREP saponin). Immediately before, and 7 days after, the second immunization, serum was collected for antibody determination, and spleen cells were collected for the measurement of IFN-γ secretion. Antibody to recombinant NY-ESO-1 was assayed by a standard indirect enzyme immunoassay. Plates were coated by overnight incubation with 10 µg/ml NY-ESO-1 protein followed by blocking in PBS containing 1% casein. Sera were tested at 5-fold dilutions starting from 1:100. The plates were incubated with horseradish peroxidase-conjugated antimouse IgG (KPL, Gaithersburg, MD) followed by the addition of tetramethylbenzidine substrate solution (KPL). The reaction was stopped by the addition of H₂SO₄, and the absorbance was read at 450 nm. Titers were determined from a standard curve generated on each plate using four-parameter fit calculations (KCJr; Bio-Tek Instruments, Winooski, VT).

Spleen-cell-derived IFN-γ was assayed in supernatants from *in vitro*-stimulated splenocyte cultures from immunized BALB/c mice (*n* = 3/group). Splenocytes (0.5 × 10⁶/well) were cultured in 96-well plates (Costar, Cambridge, MA), together with 2.5 µg/ml of recombinant NY-ESO-1 protein for 72 h at 37°C and 5% CO₂ in RPMI 1640 supplemented with 5% v/v inactivated FCS (CSL Limited), 5 × 10⁻⁵ M 2-mercaptoethanol (Life Technologies, Inc., Inc. Rockville, MD), and 40 µg/ml gentamicin (CSL Limited Australia). Plates were coated by overnight incubation with rat-antimouse IFN-γ (Endogen, Woburn, MA) and blocked with 1% casein in PBS. Cell culture supernatants were tested in triplicate at a 1:10 dilution using recombinant purified IFN-γ (Sigma) as a standard. The plates were then incubated with biotinylated rat antimouse IFN-γ followed by streptavidin-horseradish peroxidase (Amersham Pharmacia Biotech Ltd, Little Chalfont, Bucks, United Kingdom) and were developed with tetramethylbenzidine substrate (KPL). The reaction was stopped by the addition of H₂SO₄, and the absorbance at 450 nm was determined. IFN-γ concentrations were determined from a standard curve generated on each plate using four-parameter-fit calculations (KCJr; Bio-Tek Instruments).

B16-NY-ESO-1 Tumor Challenge. B16 melanoma cells were transfected using electroporation with the mammalian expression plasmid, pCDNA3, encoding the cDNA for NY-ESO-1 (Invitrogen, Carlsbad, CA). Selection with G418 (800 µg/ml) and limit-dilution cloning yielded a clone expressing NY-ESO-1 (B16-NY-ESO-1) as determined by IHC and quantitative real-time PCR. C57BL/6 mice (*n* = 6/group) were vaccinated twice (at 0 and 4 weeks) with the NY-ESO-1 vaccine, or with the ISCOMATRIX adjuvant alone as a control. Four weeks after the second immunization, mice were challenged with B16-NY-ESO-1. The tumor cells (1 × 10⁴) were injected s.c. on the back, and tumor volume was measured over time. Mice were euthanized when tumors reached >180 mm².

Fig. 1 Recombinant NY-ESO-1 protein formulated with ISCOMATRIX adjuvant (IMX) results in association and formation of aggregates. **A**, fluorescence-activated cell sorting analysis of NY-ESO-1/IMX vaccine (ISCOM) showing size and complexity of formulation (left panel) or percentage association by dual labeling with anti-IMX adjuvant and anti-NY-ESO-1 antibodies. Isotype-matched control antibodies showed no binding to the NY-ESO-1/IMX vaccine (not shown). No gating was applied during this analysis. FSC, forward scatter; SSC, side scatter. **B**, negative contrast screening electron microscope of NY-ESO-1/IMX vaccine showing aggregate formation.



RESULTS

Characterization of the NY-ESO-1 Vaccine Formulation. Efficient induction of CD8⁺ T-cell responses by ISCOMATRIX adjuvant is believed to require association of the antigen and the adjuvant. As a result, a number of strategies to achieve this have been developed (29). In this case, simple mixing of ISCOMATRIX adjuvant with recombinant NY-ESO-1 protein appeared to be sufficient to ensure adequate association between the vaccine components, presumably because the positively charged NH₂ terminus of the NY-ESO-1 protein (1) could interact with the negatively charged ISCOMATRIX adjuvant via electrostatic interactions. Association for the two components (*i.e.*, NY-ESO-1 and ISCOMATRIX adjuvant) could be demonstrated by FACS analysis, with 94% of FACS-detectable vaccine material stained positive for both NY-ESO-1 (anti-NY-ESO-1 mAb, ES121) and ISCOMATRIX adjuvant (anti-ISCOMATRIX adjuvant mAb, 706B; Fig. 1A). ISCOMATRIX adjuvant is typically 40 nm in size and, thus, not readily detectable by FACS. However, as shown in Fig. 1B, the majority of the NY-ESO-1 vaccine formulation was composed of large aggregates of NY-ESO-1 vaccine that were ~1–2 μm in size. There were also some larger aggregates present but they represented a minority of the NY-ESO-1 vaccine and likely act in the same manner as the smaller aggregates. These data illustrate that NY-ESO-1 had effectively associated with ISCOMATRIX adjuvant and resulted in the formation of aggregates.

NY-ESO-1 Vaccine Is Taken Up by Human MoDCs but Does Not Induce DC Maturation. DCs can be efficiently generated from CD14⁺ blood monocytes (MoDCs) after 6–7 days of culture in granulocyte macrophage colony-stimulating factor and

IL-4 (19, 20). These DCs were phenotypically immature, expressing intermediate levels of MHC class II molecules and low levels of CD80, CD83, and CD86 (19, 20). We examined whether immature MoDCs could ingest the NY-ESO-1 vaccine and whether uptake of the NY-ESO-1 vaccine was sufficient to induce the phenotypic maturation of immature MoDCs *in vitro*. Immature MoDCs were incubated with the NY-ESO-1 vaccine either at 4°C or at 37°C for 1–3 h. The cells were then washed and examined by FACS for surface detection of NY-ESO-1 vaccine using either the anti-NY-ESO-1 mAb (ES121; Fig. 2B) or the anti-ISCOMATRIX mAb (A706; Fig. 2, C–D). In parallel, MoDCs were permeabilized, and surface *versus* intracellular NY-ESO-1 or ISCOMATRIX adjuvant was examined by FACS (Fig. 2, D and F). Firstly, the isotype-matched control mAb did not stain MoDCs pulsed with NY-ESO-1 vaccine (Fig. 2A). Similarly, neither the anti-NY-ESO-1 mAb (ES121) nor the anti-ISCOMATRIX adjuvant mAb bound unpulsed MoDCs (data not shown). However, the NY-ESO-1 vaccine rapidly (1 h) and efficiently bound to the surface of MoDCs as detected by either anti-NY-ESO-1 mAb (Fig. 2B) or anti-ISCOMATRIX adjuvant mAb (Fig. 2C), with between 36 and 43% of MoDCs binding detectable NY-ESO-1 or ISCOMATRIX adjuvant at the cell surface. Cell surface binding was temperature dependent, because the NY-ESO-1 vaccine was not detected when MoDCs were pulsed with the NY-ESO-1 vaccine at 4°C (data not shown). Intracellular staining after 1 h revealed that the majority of the NY-ESO-1 vaccine was bound to the cell surface; 90% of staining was detected on nonpermeabilized MoDCs (Fig. 2, B and C) with an additional 5–10% staining appearing after cell permeabilization (Fig. 2D). This suggests that, initially, the hydrophobic

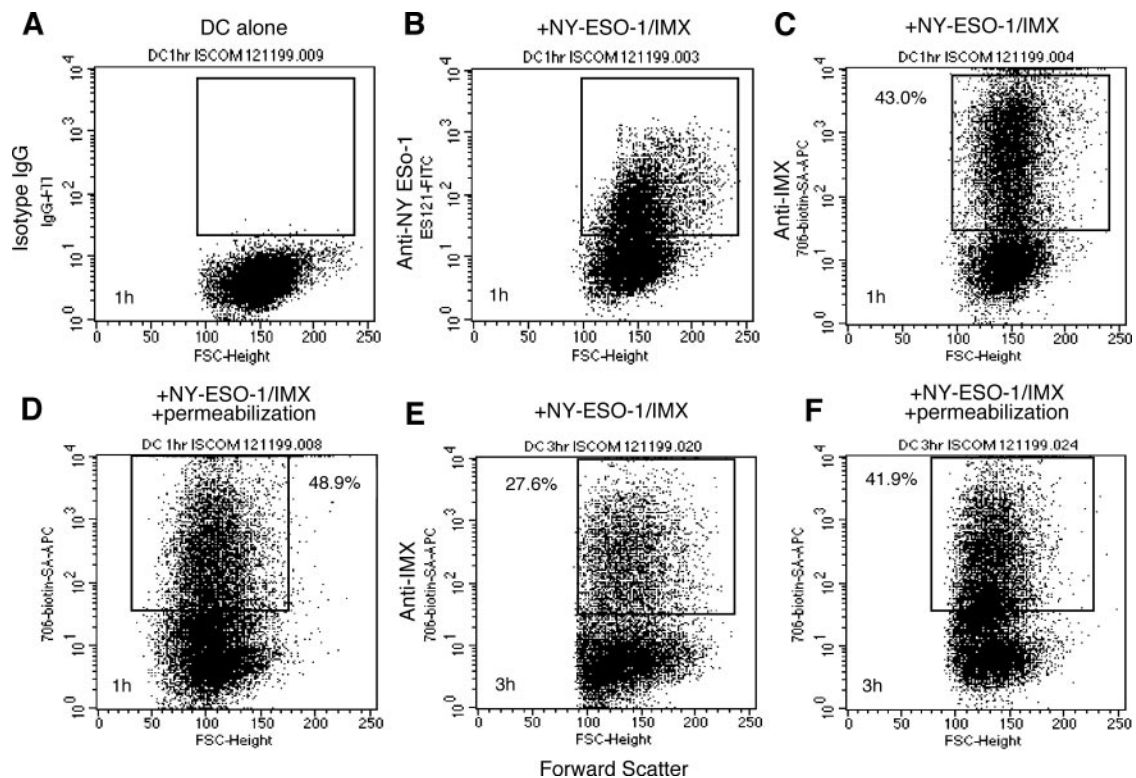


Fig. 2 Immature human monocyte-derived dendritic cells (MoDCs) efficiently take up the NY-ESO-1/ISCOMATRIX adjuvant (NY-ESO-1/IMX) vaccine *in vitro*. Immature MoDCs were generated by culturing CD14⁺ monocytes in granulocyte macrophage colony-stimulating factor and interleukin 4 for 6–7 days. Immature MoDCs were incubated with the NY-ESO-1/IMX vaccine (ISCOM) for 1 h (A–D) or 3 h (E–F) at 4°C (not shown) or at 37°C and then were stained with either anti-NY-ESO-1 monoclonal antibody (mAb; ES121) or anti-IMX adjuvant mAb (A703). Uptake of the NY-ESO-1/IMX vaccine was assessed by fluorescence-activated cell sorting analysis. A, isotype-matched control antibody. B, staining with anti-NY-ESO-1 (ES121) at 1 h. C and E, staining with anti-IMX adjuvant mAb (A703) at 1 h or 3 h. D and F, staining with anti-IMX adjuvant mAb after permeabilization with saponin at 1 h or 3 h. FSC, forward scatter; SA-APC, streptavidin-allophycocyanin.

NY-ESO-1 vaccine is predominantly incorporated into the cell surface lipid membrane with only a small proportion of the NY-ESO-1 vaccine internalized. Detection of the NY-ESO-1 vaccine decreased at the cell surface by 3 h (28% at 3 h *versus* 43% at 1 h; Fig. 2, C and E), with the majority of NY-ESO-1 vaccine detectable intracellularly at this time (42% at 3 h *versus* 49% at 1 h;

Fig. 2, D and F), suggesting progressive internalization of surface-bound NY-ESO-1 vaccine over time.

Interestingly, the NY-ESO-1 vaccine was a poor inducer of MoDC maturation *in vitro*, only marginally up-regulating HLA-DR and CD86 (<2-fold) but not CD80, CD83, or HLA-ABC (Table 1). This was in contrast to known inducers of

Table 1 Induction of phenotypic maturation of monocyte-derived dendritic cells (MoDCs) by various stimuli

Immature MoDCs were pooled and cultured (1×10^5 cells/ml) for 3 days with the indicated stimuli in their own condition medium. Data represent the means \pm SE from three separate donors.

Stimuli	Fold increase ^a in mean fluorescence intensity \pm SD				
	CD80	CD83	CD86	HLA-DR	HLA-ABC
GM + IL-4 ^b	1	1	1	1	1
+NY-ESO-1/IMX	1.1 \pm 0.2	1.2 \pm 0.3	2.3 \pm 0.2	1.1 \pm 0.3	2.0 \pm 0.5
+TNF- α	5.6 \pm 1.1	15.6 \pm 2.0	26.3 \pm 2.7	6.9 \pm 1.1	5.3 \pm 1.5
+IFN- α 2a					
+PGE ₂					
+CD40L	6.3 \pm 0.9	16.2 \pm 1.9	28.2 \pm 3.0	6.4 \pm 2.1	8.6 \pm 2.1
+ <i>Escherichia coli</i>	7.0 \pm 0.9	14.8 \pm 2.0	27.3 \pm 3.2	7.2 \pm 1.2	7.6 \pm 1.4

^a Fold increase in mean fluorescence intensity above that seen with GM + IL-4-generated immature MoDCs (expressed as 1).

^b GM + IL-4, granulocyte macrophage colony-stimulating factor and interleukin 4; IMX, ISCOMATRIX adjuvant; TNF, tumor necrosis factor; PGE₂, prostaglandin E₂.

MoDC maturation, such as CD40L, pro-inflammatory cytokines (TNF- α , IFN- α , and PGE₂), or intact *E. coli*, which all up-regulated these maturation markers (Table 1; 32).

MoDCs Pulsed with the NY-ESO-1 Vaccine Cross-Present the HLA-A2-Restricted Epitope NY-ESO-1₁₅₇₋₁₆₅ to Cytotoxic T Cells. The ability of human MoDCs to process captured NY-ESO-1 vaccine into HLA-A2-restricted peptides was next assessed using a short-term CD8⁺ CTL line specific for the HLA-A2-restricted NY-ESO-1 peptide, SLLMWITQC₁₅₇₋₁₆₅. Immature HLA-A2⁺ MoDCs were pulsed with the NY-ESO-1 vaccine for 1 h and were then either cultured for 4 h or 18 h in granulocyte macrophage colony-stimulating factor and IL-4 (immature) or matured with a cocktail of pro-inflammatory mediators (TNF- α , IFN- α , and PGE₂) for 4 h or 18 h before coculture with the CD8⁺ CTL line. As specificity controls, immature MoDCs or those matured with the above cytokine mixture were pulsed with either the NY-ESO-1 peptide, SLLMWITQC₁₅₇₋₁₆₅, or with the irrelevant HLA-A2-restricted MAGE-3 peptide, FLWGPRALV₂₇₁₋₂₇₉ (data not shown). Several observations were made. Firstly, in the absence of antigen, immature (Fig. 3A) or matured MoDCs (data not shown) were incapable of inducing IFN- γ secretion in the peptide-specific CD8⁺ CTL line, as determined by intracellular cytokine staining, but were as efficient as transporter associated with antigen processing (TAP)-deficient T2 cells at presenting pulsed-SLLMWITQC₁₅₇₋₁₆₅ peptide (Fig. 3, B and E). Second, immature MoDCs were capable of processing the NY-ESO-1 vaccine into sufficient HLA-A2/SLLMWITQC₁₅₇₋₁₆₅ peptide complexes to induce intracellular IFN- γ secretion by the CD8⁺ CTL line (Fig. 3C). Third, maturation of the NY-ESO-1 vaccine-pulsed MoDCs with pro-inflammatory cytokines resulted in a significant increase (1.1 *versus* 4.7%) in the proportion of CD8⁺ CTL secreting IFN- γ (Fig. 3D). This latter finding is consistent with the observation that the NY-ESO-1 vaccine was poor at inducing MoDC maturation *in vitro* (Table 1) and that DC activation is required for effective cross-presentation.

MoDCs, pulsed with the NY-ESO-1 vaccine, induced a lower proportion of SLLMWITQC₁₅₇₋₁₆₅-specific CD8⁺ CTLs to secrete IFN- γ as compared with SLLMWITQC₁₅₇₋₁₆₅-pulsed MoDCs. This likely reflects differences in the molar ratio of SLLMWITQC₁₅₇₋₁₆₅ peptide generated from a full-length protein that would be available for loading onto empty class I MHC in the endoplasmic reticulum (in the presence of other competing peptides), as compared with saturating levels of purified SLLMWITQC₁₅₇₋₁₆₅ peptide added externally. Furthermore, the response induced by MoDCs pulsed with the NY-ESO-1 vaccine was not due to free SLLMWITQC₁₅₇₋₁₆₅ peptide contaminating the NY-ESO-1 vaccine preparation (due to degradation products, and so forth), because the HLA-A2⁺ transporter associated with antigen processing (TAP)-deficient T2 cell line was unable to present detectable SLLMWITQC₁₅₇₋₁₆₅ peptide when pulsed with NY-ESO-1 vaccine (Fig. 3F). Finally, maturation for 4 h was as effective as for 18 h at optimizing the processing of the NY-ESO-1 vaccine into SLLMWITQC₁₅₇₋₁₆₅ peptide for detection by the CD8⁺ CTL line (Fig. 3G). These data illustrate that the NY-ESO-1 vaccine is effectively taken up and processed by human MoDCs but that, *in vitro*, these DCs require additional maturation for optimal presentation of MHC/SLLMWITQC₁₅₇₋₁₆₅ peptide complexes to CD8⁺ CTLs.

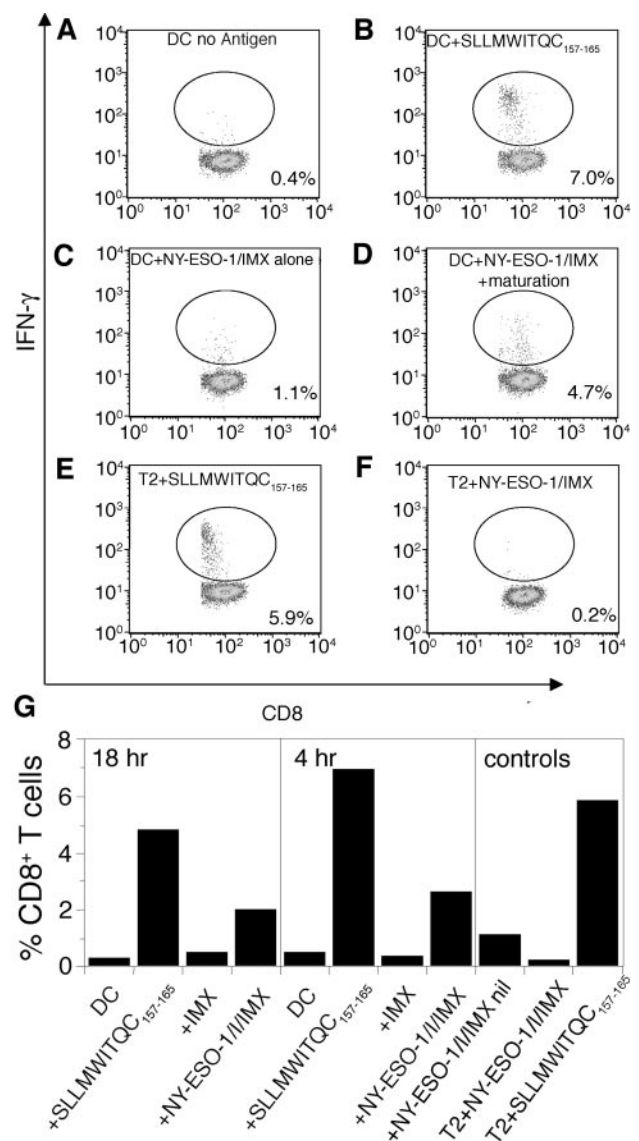


Fig. 3 Stimulation of a short-term NY-ESO-1 SLLMWITQC₁₅₇₋₁₆₅-specific CD8⁺ T-cell line using immature or matured monocyte-derived dendritic cells (MoDCs) pulsed either with the SLLMWITQC₁₅₇₋₁₆₅ peptide or with the NY-ESO-1/ISCOMATRIX adjuvant (NY-ESO-1/IMX) vaccine. **A**, unpulsed immature MoDCs cocultured with NY-ESO-1 SLLMWITQC₁₅₇₋₁₆₅-specific CD8⁺ CTL line. **B**, immature MoDCs pulsed with 1 μ g/ml SLLMWITQC₁₅₇₋₁₆₅ peptide or **C**, immature MoDCs pulsed with 10 μ g/ml NY-ESO-1/IMX vaccine and cocultured with the NY-ESO-1 SLLMWITQC₁₅₇₋₁₆₅-specific CD8⁺ CTL line. **D**, immature MoDCs pulsed with the NY-ESO-1/IMX vaccine and matured with the cytokine combination of tumor necrosis factor (TNF)- α , IFN- α , and prostaglandin E₂ for 4 h (or **G**, 18 h) before coculture with the NY-ESO-1 SLLMWITQC₁₅₇₋₁₆₅-specific CD8⁺ CTL line. **E**, T2 cells pulsed with 1 μ g/ml SLLMWITQC₁₅₇₋₁₆₅ peptide or (**F**) 10 μ g/ml NY-ESO-1/IMX vaccine. **G**, comparison between maturation of MoDCs for 4 h or 18 h on efficiency of T-cell stimulation.

MoDCs Pulsed with the NY-ESO-1 Vaccine Present T-Helper-Cell Epitopes from NY-ESO-1 via MHC class II Molecules. The NY-ESO-1 epitope, SLLMWITQCFLP-VF₁₅₇₋₁₇₀, is restricted to the MHC class II allele *HLA-*

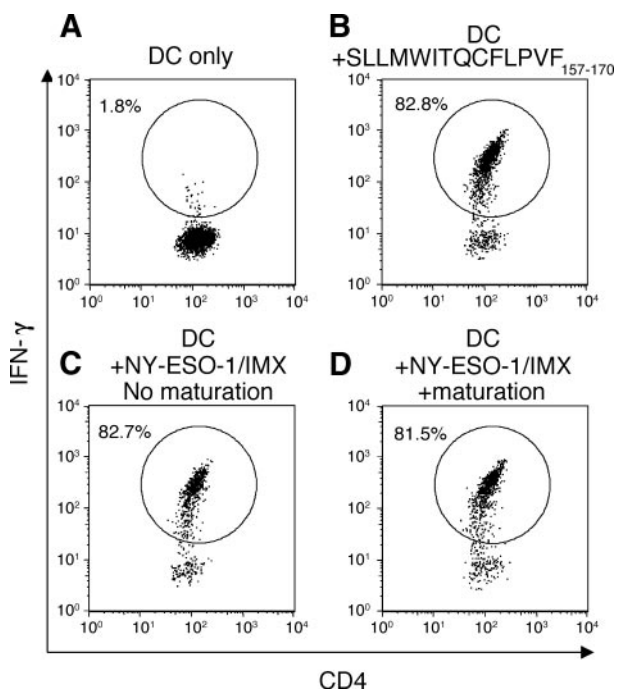


Fig. 4 Presentation of the HLA-DP4-restricted SLLMWITQCFLPVF₁₅₇₋₁₇₀ peptide to the 1F10 CD4⁺ T-cell line by monocyte-derived dendritic cells (MoDCs) pulsed with the NY-ESO-1/ISCOMATRIX adjuvant (NY-ESO-1/IMX) vaccine. **A**, unpulsed immature MoDCs cocultured with the NY-ESO-1₁₅₇₋₁₇₀-restricted 1F10 T cells. **B**, immature MoDCs pulsed with 1 μ g/ml SLLMWITQCFLPVF₁₅₇₋₁₇₀ peptide. **C**, immature MoDCs pulsed with 10 μ g/ml NY-ESO-1/IMX vaccine and cocultured with 1F10 T cells. **D**, MoDCs pulsed with the NY-ESO-1/IMX vaccine and matured with the cytokine combination of tumor necrosis factor (TNF)- α , IFN- α , and prostaglandin E₂ for 18 h before coculture with 1F10 T cells. Presentation of NY-ESO-1₁₅₇₋₁₇₀ to 1F10 CD4⁺ T cells was detected by intracellular cytokine staining for IFN- γ by fluorescence-activated cell sorting.

DPBI*0401-0402 (found in 43–70% of Caucasians). To assess presentation of the NY-ESO-1 vaccine to class II-restricted CD4⁺ T cells, immature HLA-DP4⁺ MoDCs were pulsed with the NY-ESO-1 vaccine for 1 h and then were cultured in the absence or presence of pro-inflammatory mediators (TNF- α + IFN- α + PGE₂) for 18 h before coculture with the NY-ESO-1₁₅₇₋₁₇₀ peptide-specific CD4⁺ T-cell line. As shown in Fig. 4, only low levels of IFN- γ were produced by the CD4⁺ T cells cocultured with MoDCs in the absence of antigen, whereas DCs pulsed with the NY-ESO-1 vaccine were as efficient at presenting the SLLMWITQCFLPVF₁₅₇₋₁₇₀ epitope to CD4⁺ T cells as were SLLMWITQCFLPVF₁₅₇₋₁₇₀ peptide-pulsed DCs (Fig. 4, B and C). Furthermore, DC maturation did not enhance the presentation of the SLLMWITQCFLPVF₁₅₇₋₁₇₀ epitope by the NY-ESO-1 vaccine-pulsed MoDCs; that presentation was already optimal in immature cells (Fig. 4, C and D). Therefore, presentation of MHC class II-restricted epitopes from NY-ESO-1 by MoDCs is highly efficient.

IFN- γ -Biased Responses in BALB/c Mice Vaccinated with the NY-ESO-1 Vaccine. The ability of anticancer vaccines to induce IFN- γ -secreting T cells appears to be critical to their effectiveness. Such T cells also increase the levels of

the IFN- γ -regulated IgG isotype, IgG2a, in mice. To assess whether the NY-ESO-1 vaccine induced IFN- γ -biased antibody responses, BALB/c mice were vaccinated with either NY-ESO-1 protein alone or the NY-ESO-1 vaccine, and NY-ESO-1-specific antibody responses were examined. Immunization with the NY-ESO-1 vaccine induced significantly higher serum levels of NY-ESO-1-specific IgG1 (8-fold) and dramatically increased IgG2a (300-fold; $P < 0.01$) above that seen in BALB/c mice immunized with NY-ESO-1 protein alone (Fig. 5A). The increased titer of NY-ESO-1-specific IgG2a in mice immunized with NY-ESO-1 vaccine correlated with a 3–8-fold increase in IFN- γ -secreting spleen cells *in vitro* as compared with mice immunized with NY-ESO-1 protein alone (Fig. 5B). Thus, the high level of NY-ESO-1-specific IgG2a- and IFN- γ -producing spleen cells suggested the efficient induction of IFN- γ -biased immune responses by the NY-ESO-1 vaccine.

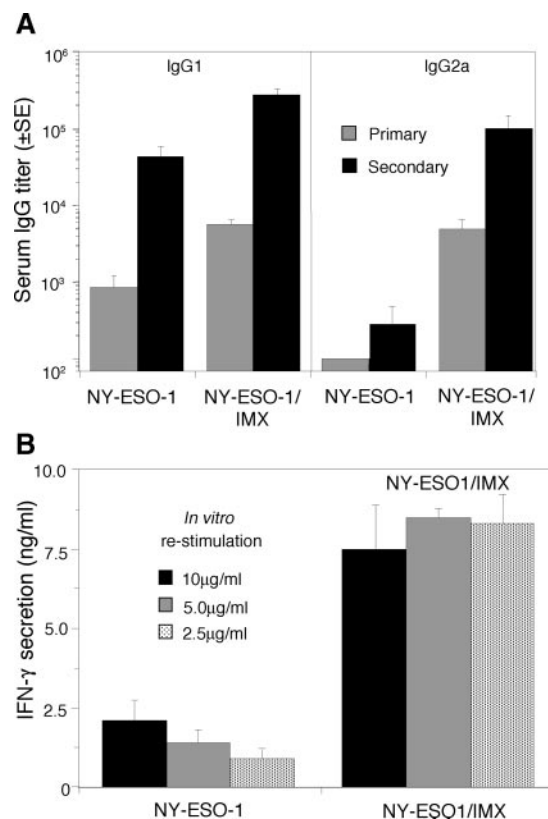


Fig. 5 Analysis of NY-ESO-1-specific antibody and IFN- γ responses in mice immunized with either the NY-ESO-1/ISCOMATRIX adjuvant (NY-ESO-1/IMX) vaccine or with nonadjuvanted NY-ESO-1 protein. **A**, detection of serum IgG1 and IgG2a specific for NY-ESO-1. NY-ESO-1-specific antibody responses were measured in the serum of mice after one (Primary) or two (Secondary) vaccinations either with the NY-ESO-1/IMX vaccine or with the nonadjuvanted NY-ESO-1 protein. Results are presented as the mean antibody titer \pm SE. **B**, detection of IFN- γ production in spleen cell cultures. IFN- γ secretion was measured in spleen cell cultures generated from mice immunized twice either with the NY-ESO-1/IMX vaccine or with nonadjuvanted NY-ESO-1 protein. Cultures were restimulated (2.5 μ g/ml to 10 μ g/ml) as indicated with the NY-ESO-1/IMX vaccine or with the nonadjuvanted NY-ESO-1 protein for 72 h, and supernatants were collected. Results are presented as the mean \pm SE of triplicate wells.

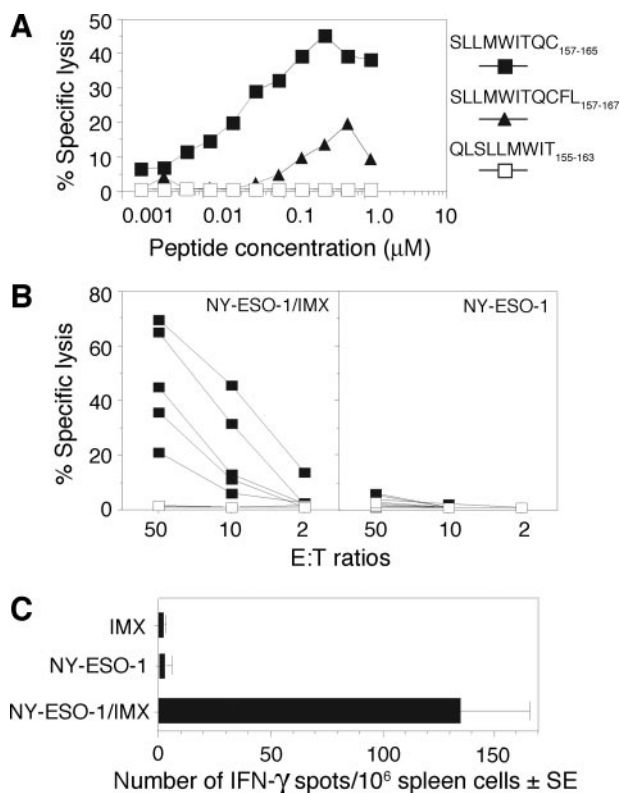


Fig. 6 Analysis of NY-ESO-1₁₅₇₋₁₆₅-specific CD8⁺ T-cell responses in HHD mice (transgenic mice with a transgene composed of *HLA-A2* α 1, *HLA-A2* α 2 linked to the α 3 domain of *H-2D^b*) immunized either with the NY-ESO-1/ISCOMATRIX adjuvant (NY-ESO-1/IMX) vaccine or with the nonadjuvanted NY-ESO-1 protein. **A**, HHD mice ($n = 5$) were immunized with the NY-ESO-1/IMX vaccine or with nonadjuvanted NY-ESO-1 protein, and spleen cell populations from each mouse were separately restimulated with SLLMWITQCFL₁₅₇₋₁₆₇ or SLLMWITQC₁₅₇₋₁₆₅ or QLSELLMWIT₁₅₅₋₁₆₃ peptides. T-cell cultures were tested in duplicate against ⁵¹Cr-labeled EL4 HHD cells sensitized with restimulating peptide at the indicated concentrations (*E:T ratio*, 50:1). Percentage of peptide-specific lysis = percentage lysis of EL4 HHD cells sensitized with peptide – percentage lysis of EL4 HHD cells without peptide. **B**, detection of SLLMWITQC₁₅₇₋₁₆₅-specific CD8⁺ CTL responses in HHD mice immunized either with the NY-ESO-1/IMX vaccine or with the nonadjuvanted NY-ESO-1 protein. HHD mice ($n = 6$ /group) were vaccinated once with either the NY-ESO-1/IMX vaccine or the nonadjuvanted NY-ESO-1 protein; and spleen cells, restimulated with SLLMWITQC₁₅₇₋₁₆₅ peptide, were used as effectors in standard ⁵¹Cr-release assays against EL4 HHD target cells (□) or against the same cells (EL4 HHD target cells) sensitized (10 μ g/ml for 1 h at 37°C and washed twice) with SLLMWITQC₁₅₇₋₁₆₅ peptide (■). The data for each individual mouse are shown. **C**, detection of SLLMWITQC₁₅₇₋₁₆₅-specific IFN- γ -secreting T cells. HHD mice ($n = 6$ /group) were vaccinated once either with the NY-ESO-1/IMX vaccine or with the IMX adjuvant alone. Spleen cells from each mouse were separately restimulated with SLLMWITQC₁₅₇₋₁₆₅ peptide and were used in an IFN- γ enzyme-linked immunospot (ELISpot) assay. Data shows the mean number of IFN- γ -secreting cells \pm SE.

HLA-A2 Transgenic (HHD) Mice Treated with the NY-ESO-1 Vaccine Generate NY-ESO-1-Specific CD8⁺ CTLs *in Vivo*. To determine whether the NY-ESO-1 vaccine could generate HLA-A2-restricted epitopes *in vivo* (epitopes relevant to the human system), HHD HLA-A2 transgenic mice were vaccinated

with the NY-ESO-1 vaccine. Spleen cells were isolated after 3 weeks and restimulated for 6 days with irradiated lipopolysaccharide blasts pulsed with HLA-A2-restricted, NY-ESO-1-specific peptides. Fig. 6A shows that HHD mice vaccinated with the NY-ESO-1 vaccine generated CD8⁺ T-cell responses that recognized the NY-ESO-1 peptide SLLMWITQC₁₅₇₋₁₆₅ and to a lesser extent the SLLMWITQCFL₁₅₇₋₁₆₇ peptide, but not the less immunogenic QLSELLMWIT₁₅₅₋₁₆₃ peptide (Fig. 6A). The results in the HHD mice parallel the epitope specificity observed in NY-ESO-1-specific CD8⁺ T cells isolated from HLA-A2⁺ individuals with NY-ESO-1-expressing tumors. Furthermore, all of the mice (five of five) immunized with the NY-ESO-1 vaccine generated CD8⁺ T cells capable of killing the SLLMWITQC₁₅₇₋₁₆₅-pulsed target cells, whereas mice vaccinated with NY-ESO-1 protein alone failed to generate such CD8⁺ T cells (Fig. 6B). Furthermore, HHD mice immunized with the NY-ESO-1 vaccine (but not NY-ESO-1 protein alone) generated SLLMWITQC₁₅₇₋₁₆₅-specific CD8⁺ T cells that secreted IFN- γ (Fig. 6C). Together, these data demonstrate that the NY-ESO-1 vaccine was a potent inducer of HLA-A2-restricted CD8⁺ T-cell responses in mice.

HHD-derived CD8⁺ T cells from mice immunized with the NY-ESO-1 vaccine (but not those immunized with NY-ESO-1 protein alone) were examined for their capacity to kill human tumor cell lines. These CD8⁺ T cells efficiently killed the HLA-A2⁺ NY-ESO-1²⁺ melanoma line SK-Mel-37, but not HLA-A2⁻ or NY-ESO-1⁻ tumor lines, or the AO2b tumor line (low NY-ESO-1 expression by IHC; Fig. 7). These results demonstrate that

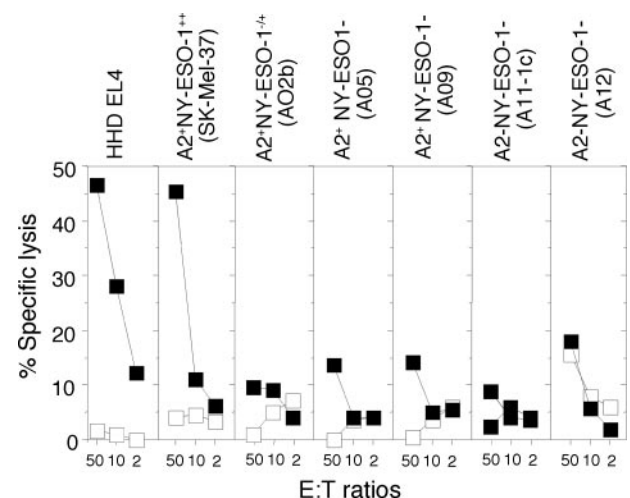


Fig. 7 NY-ESO-1/ISCOMATRIX adjuvant (NY-ESO-1/IMX) vaccine induced CD8⁺ T-cell responses in HHD mice (transgenic mice with a transgene composed of *HLA-A2* α 1, *HLA-A2* α 2 linked to the α 3 domain of *H-2D^b*) capable of killing NY-ESO-1⁺ human melanoma cells. HHD mice were immunized once with either the NY-ESO-1/IMX vaccine (■) or with IMX adjuvant alone (□). Spleen cells were harvested 3 weeks after vaccination, and SLLMWITQC₁₅₇₋₁₆₅ peptide-specific CD8⁺ CTLs were generated by restimulating weekly for 3 weeks with this NY-ESO-1-peptide-pulsed, autologous spleen stimulators. The CD8⁺ CTLs were used as effectors against SLLMWITQC₁₅₇₋₁₆₅ peptide-pulsed HHD EL4 cells (HHD EL4) or the indicated melanoma cell lines. SK-Mel-37 melanoma line strongly expressed NY-ESO-1 in \approx 100% of cells, A02-Mb showed weak expression in 5–10% of cells by immunohistochemistry (data not shown).

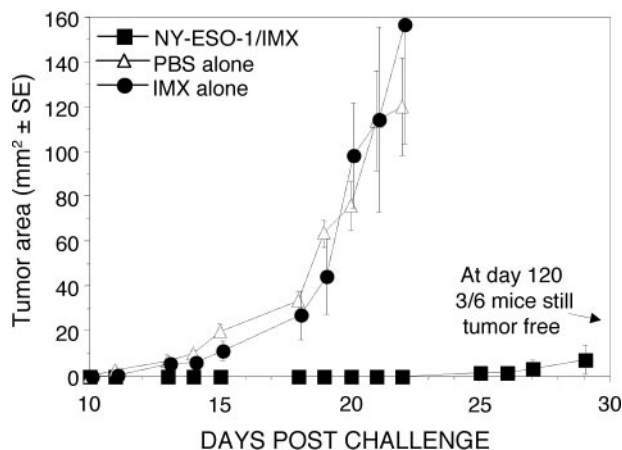


Fig. 8 The NY-ESO-1/ISCOMATRIX adjuvant (NY-ESO-1/IMX) vaccine protects mice from challenge with B16 melanoma expressing NY-ESO-1. C57BL/6 mice ($n = 6/\text{group}$) were vaccinated with the NY-ESO-1/IMX vaccine or with PBS buffer alone (buffer control group) or with IMX adjuvant alone (adjuvant control group) and were then challenged with B16 melanoma cell line transfected with NY-ESO-1. Tumor area was measured at the indicated times, and mice were sacrificed when tumors exceeded 180 mm^2 .

the NY-ESO-1 vaccine is able to induce CD8^+ T-cell effectors *in vivo* capable of lysing NY-ESO-1-expressing human tumor cells.

The Generation of NY-ESO-1-Specific CD8^+ CTL in Mice Immunized with the NY-ESO-1 Vaccine Protects Them against Tumor Challenge. The ability of C57BL/6 mice immunized with the NY-ESO-1 vaccine to reject NY-ESO-1-expressing tumors *in vivo* was next assessed. To address this question, we transfected the aggressively growing, poorly immunogenic melanoma cell line B16 with an NY-ESO-1-containing plasmid and NY-ESO-1-expressing clones identified by IHC and quantitative real-time PCR (data not shown). Fig. 8 demonstrates that B16 melanoma cells expressing NY-ESO-1 grew rapidly in mock buffer-treated and ISCOMATRIX adjuvant-treated control animals, with tumors reaching $>120 \text{ mm}^2$ by day 22, at which time mice were sacrificed (Fig. 8). In contrast, mice immunized with the NY-ESO-1 vaccine were all tumor free on day 22, and by 120 days, one-half of these mice were still without tumor (Fig. 8). This indicates that vaccination of naïve mice with the NY-ESO-1 vaccine induces a potent NY-ESO-1-specific immune response *in vivo* sufficient to regress NY-ESO-1-expressing B16 melanoma cells.

DISCUSSION

Analysis of successful immune responses against viral pathogens can provide a useful framework for designing vaccines for effective T-cell immune responses against cancer. Strategies that can convert innocuous tumor-expressed antigens into ones perceived as life threatening and, therefore, immunogenic, represent one of the many challenges facing cancer vaccine-based immunotherapy. The development of effective cancer vaccines that are not patient specific has been hampered by the paucity of vaccines that are capable of safely and effectively inducing CD8^+ T cells in humans. Furthermore, the ability to sustain anticancer CD8^+ T

cells through repeated immunizations is likely to emerge as an important feature of therapeutic anticancer vaccines (19, 20, 33). Thus, although viral vectors such as replication-defective poxvirus vaccine vectors have been tested with some success (34), these cannot be used repeatedly because of the induction of anti-vector neutralizing antibodies. DNA vaccines are also being developed as anticancer vaccine modalities but, to date, have shown limited potency; and, thus, improvements are likely to be required before this technology becomes effective against cancer (35). Adjuvant systems that are capable of inducing CD8^+ T-cell responses against tumor antigens provide an attractive alternative strategy.

The cancer-testes antigen NY-ESO-1 represents one of the more promising tumor-associated targets for vaccine-based immunotherapy of cancer. Unlike several other tumor-associated antigens, NY-ESO-1 is relatively immunogenic in cancer patients, with both humoral and T-cell responses generated either spontaneously or after vaccination with peptides (4, 12). The data presented here illustrate that associating the ISCOMATRIX adjuvant with a recombinant NY-ESO-1 protein to form an NY-ESO-1 vaccine results in a potent anticancer vaccine that induces more effective humoral and CD8^+ CTL responses *in vivo* than does non-adjuvanted NY-ESO-1 protein alone. In mice, the NY-ESO-1 vaccine induced strong IFN- γ -biased responses, resulting in both IgG1 and IgG2a antibodies and in the generation of potent CD8^+ T cells capable of killing HLA-A2 $^+$ NY-ESO-1-expressing human tumors *in vitro*, as well as protecting mice against challenge with NY-ESO-1-expressing B16 melanoma cells. The NY-ESO-1 vaccine potently induced CD8^+ T-cell responses in HLA-A2-transgenic HHD mice, similar to those seen after immunization with other CD8^+ T-cell-inducing vaccine modalities, such as live recombinant vaccinia virus (28, 36). Furthermore, the presence of the ISCOMATRIX adjuvant significantly enhanced the immunogenicity of NY-ESO-1 protein, because NY-ESO-1 protein alone generated relatively poor responses *in vivo*. The ability of ISCOMATRIX adjuvant to induce antigen-specific CD8^+ T-cell responses capable of rejecting antigen-expressing tumors was also demonstrated using the ovalbumin (OVA) antigen model. In that study, the OVA-ISCOMATRIX vaccine induced rejection of OVA-expressing EG7 tumors, OVA-expressing-B16 melanoma, and OVA-expressing Lewis Lung carcinoma in mice (24). Furthermore, in all of these tumor models, only the OVA-expressing tumors, but not the parental tumors implanted in the opposing flank, were rejected, indicating that ISCOMATRIX vaccines induce antigen-specific immunity. Finally, class II MHC $^{-/-}$ mice, immunized with the OVA-ISCOMATRIX vaccine, efficiently generated antitumor CD8^+ CTLs in the absence of CD4^+ T-cell help (24).

The NY-ESO-1 vaccine was efficiently taken up by immature human MoDCs, and this appeared to be initiated by the binding of the NY-ESO-1 vaccine onto the surface membrane of DCs. It is unclear whether this binding required the use of specific receptor-mediated mechanisms or simply binding via hydrophobic interactions between the DC lipid membrane and the lipids of the ISCOMATRIX adjuvant. Interestingly, surface-associated NY-ESO-1 vaccine steadily decreased over time, with most of the vaccine readily detectable in the cytosol by 3 h, suggesting that surface membrane turnover may represent one mechanism of vaccine incorporation into DCs for processing. NY-ESO-1 vaccine did not induce MoDC maturation, unlike known inducers of MoDC mat-

uration (e.g., pro-inflammatory mediators, CD40L, or intact bacteria). The immature state of vaccine-pulsed MoDC correlated with a low efficiency of cross-presentation of the NY-ESO-1 vaccine onto the MHC class I molecules for recognition by SLLMWITQC₁₅₇₋₁₆₅-specific CD8⁺ CTLs. However, subsequent maturation of NY-ESO-1 vaccine-pulsed MoDCs (32) resulted in efficient cross-presentation of class I-restricted peptides to antigen-specific CD8⁺ CTLs. In this regard, Nagata *et al.* (37) have recently shown that, although MoDCs were inefficient at cross-presenting free NY-ESO-1 protein to peptide-specific CD8⁺ CTLs, NY-ESO-1-IgG complexes were avidly presented, likely involving the Fcγ receptor type II. Two mechanisms could explain their finding: Fc receptor-mediated uptake of antigen is highly efficient; and the cross-linking of Fc receptors on MoDCs is a known inducer of maturation (38). However, the necessity of inducing DC maturation *in vitro* for *in vivo* vaccination is less clear because ISCOMATRIX-based vaccines are likely to create the appropriate environment for DC maturation *in situ* via the induction of cytokines by neighboring cells. In this regard, ISCOMATRIX adjuvant and ISCOMATRIX-based vaccines regulate lymphocyte trafficking into and out of lymphoid organs, resulting in dramatic lymph node activation and the release of cytokines such as IL-6, IL-8, and IFN-γ (39, 40). A major distinction must, therefore, be made between the activity of the NY-ESO-1 vaccine when used *in vitro* on purified DCs and the cytokine responses it is likely to initiate when injected *in vivo*.

Unlike with cross-presentation of NY-ESO-1 epitopes to CD8⁺ T cells, immature as well as mature MoDCs could present MHC class II-restricted epitopes to CD4⁺ T cells when pulsed with NY-ESO-1 vaccine. This ability was not only independent of the need for MoDC maturation but was also independent of the need for the NY-ESO-1 protein to be associated with ISCOMATRIX adjuvant.⁶ This highlights that the ISCOMATRIX adjuvant has a unique capacity to efficiently target full-length proteins into the MHC class I pathway for cross-presentation to CD8⁺ T cells but does not enhance the processing of proteins for MHC class II presentation. The mechanisms by which ISCOMATRIX adjuvant facilitates trafficking of proteins into the class I MHC pathway are currently the scope of investigations by our group. Interestingly, studies in mice using the ovalbumin antigen formulated in classical immune-stimulating complexes (ISCOMs) suggest that the cross-presenting capacity of ISCOMATRIX adjuvant may require DCs and may use mechanisms independent of the proteasome (41, 42).

The HHD mouse system represents an ideal model for evaluating the potency of NY-ESO-1 vaccine formulations, because HLA-A2-restricted NY-ESO-1-specific CD8⁺ T cells, generated from both human and mouse, recognized the NY-ESO-1 peptides SLLMWITQC₁₅₇₋₁₆₅ and SLLMWITQCFL₁₅₇₋₁₆₇ but not the upstream QLSLLMWIT₁₅₅₋₁₆₃ peptide. Whether SLLMWITQCFL₁₅₇₋₁₆₇ simply represents a longer and, therefore, less potent version of the SLLMWITQC₁₅₇₋₁₆₅ epitope is unclear. However, a recent report demonstrated that not all CD8⁺ T-cell

lines restimulated with SLLMWITQCFL₁₅₇₋₁₆₇ recognize SLLMWITQC₁₅₇₋₁₆₅ and have identified the highly immunogenic, cryptic, NY-ESO-1 epitope LMWITQCFL₁₅₉₋₁₆₇, which also resides within the SLLMWITQCFL₁₅₇₋₁₆₇ sequence (8). Similarly, given the ability of the NY-ESO-1 vaccine to effectively protect C57BL/6 mice from challenge with NY-ESO-1-expressing B16 tumors, there are likely to be several NY-ESO-1-specific CD8⁺ and CD4⁺ T-cell epitopes presented on the relevant C57BL/6 MHC class I and class II alleles.

Finally, NY-ESO-1 has recently been found to share 94% identity at the nucleotide level with another independently identified cancer-testis antigen, LAGE-1. Although the two gene products are frequently coexpressed, a significant proportion of melanoma patients express only one or the other protein. In a recent study of melanoma patients, 45% of patients had tumors expressing either one or the other antigen in at least one lesion (43). The sequence homology and the fact that SLLMWITQC₁₅₇₋₁₆₅-specific CD8⁺ T cells also recognize the LAGE-1 epitope indicate that an NY-ESO-1 vaccine could also generate CD8⁺ T cells capable of killing tumor cells expressing LAGE-1. Thus the NY-ESO-1 vaccine may serve as a bivalent vaccine targeting two independently expressed tumor antigens.

The present study represents a preclinical strategy for evaluating vaccine formulations via the use of both human and murine model systems. The results of the present study show that ISCOMATRIX-based vaccines are ideally suited for cancer immunotherapy and set the stage for evaluating the NY-ESO-1 vaccine formulation in cancer patients. Indeed, the first Phase I trial of the NY-ESO-1 vaccine in NY-ESO-1⁺ cancer patients with minimal residual disease (LUD99-008) has demonstrated the NY-ESO-1 vaccine to be safe and highly immunogenic, paralleling the types of immune responses generated in the mouse models reported here.⁷ It will be of interest to determine whether the types of immune responses generated in human patients with the NY-ESO-1 vaccine are sufficient to impact disease progression.

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