NY-ESO-1 Encodes DRB1*0401-restricted Epitopes Recognized by Melanoma-reactive CD4+ T Cells[1]

Hassane M. Zarour,[2] Walter J. Storkus, Vladimir Brusic, Eileen Williams, and John M. Kirkwood

Department of Medicine and Melanoma Center, University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania 15213 [H. M. Z., J. M. K., E. W.]; Department of Surgery and Molecular Genetics and Biochemistry, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania 15261[W. J. S.]; and Kent Ridge Digital Labs, Singapore 119613 [V. B.]

ABSTRACT

The *NY-ESO-1* gene is expressed by a range of human tumors and encodes HLA-A2-restricted melanoma peptides recognized by CD8+ CTLs. Here we report that the *NY-ESO-1* gene also encodes two overlapping, but non-cross-reactive, HLA-DRB1*0401-presented peptides that are recognized by CD4+ T cells. The NY-ESO-1$_{119-143}$ peptide was able to induce specific CD4+ T cells *in vitro* from both an HLA-DRB1*0401$^+$ normal donor and an HLA-DRB1*0401$^+$ patient with melanoma. Bulk and cloned CD4+ T cells produced IFN-γ specifically in response to, and also lysed, T2.DR4 cells pulsed with peptide NY-ESO-1$_{119-143}$ and the autologous tumor cell line, but not a DRB1*0401$^+$ melanoma cell line that does not express NY-ESO-1. Interestingly, the NY-ESO$_{119-143}$ peptide contains two overlapping putative "core" epitopes recognized by non-cross-reactive anti-NY-ESO-1$_{119-143}$ CD4+ T-cell clones. Taken together, these data support the use of this novel DR4-restricted tumor peptide, NY-ESO-1$_{119-143}$, or its two "sub-epitopes" in immunotherapeutic trials designed to generate or enhance specific CD4+ T-cell responses against tumors expressing NY-ESO-1 *in vivo*.

INTRODUCTION

A large number of class I-restricted tumor-associated epitopes recognized by CD8+ T cells have been identified, but few class II-restricted melanoma epitopes recognized by CD4+ T cells have been reported to date (1–8). The search for class II-restricted epitopes in cancer has a compelling rationale, given accumulating evidence that CD4+ T cells play critical roles in the induction and maintenance of antitumor responses (9, 10). Here we focused our studies on an evaluation of antitumor CD4+ T-cell responses in a long-lived patient who has remained disease-free for more than a decade after therapy for stage IV metastatic melanoma. This individual's serum contains a high titer of IgG2a (a T-dependent isotype) antibodies directed against the NY-ESO-1 tumor antigen, suggesting the involvement of CD4+ T helper in their humoral immune response. The NY-ESO-1 antigen was initially identified by SEREX analysis of a human esophageal cancer and has subsequently been shown to encode class I-restricted peptides expressed by a diverse range of cancers, including melanoma (11–13).

We have recently designed a strategy to identify a novel DR4-restricted Melan-A/MART-1-derived peptide recognized by CD4+ T cells (14). A peptide-binding algorithm (15, 16) was used to select candidate DR4-binding epitopes, which were then screened for recognition by melanoma-reactive CD4+ T cells derived from normal donors or long-lived patients with melanoma. This strategy has now been applied to demonstrate that the NY-ESO-1 protein encodes at

least two distinct, but overlapping, DR4-restricted epitopes capable of stimulating CD4+ T-cell responses *in vitro*. Both bulk and cloned CD4+ T cells recognized DR4$^+$ antigen-presenting cells pulsed with the NY-ESO-1$_{119-143}$ peptide as well as DR4$^+$, NY-ESO-1$^+$ melanoma cell lines. These findings support the use of the NY-ESO-1$_{119-143}$ peptide or analogues derived from this sequence as a cancer vaccine for DR4+ patients with NY-ESO-1$^+$ tumors.

MATERIALS AND METHODS

Cell Lines, Media, and Antibodies. Patient UPCI-MEL 527.1 remains disease-free for >10 years after successful therapy for distant metastasis of melanoma. High-titer anti-NY-ESO-1 antibodies have been consistently observed in the serum of patient UPCI-MEL 527. The UPCI-MEL 527.1 cell line was derived from an axillary metastatic lymph node in April 1993. Patient UPCI-MEL 527 has been genotyped HLA-A0201$^+$, -DRB1*0401$^+$. HLA-DR4 genotyping was performed using a commercial DR4 typing panel of PCR primers according to the manufacturer's instructions (Dynal, Oslo, Norway). The T2.DR4 cell line (kindly provided by Dr. Janice Blum, University of Indiana, IN) was generated through transfection of HLA-DRB1*0401 cDNA into T2 cells (17). The T2.DR4 cell line is HLA-DM deficient, making its cell surface DRB1*0401 complexes receptive to loading by exogenous peptides. All cell lines were cultured in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FCS, L-arginine (116 mg/l), L-asparagine (36 mg/l), and L-glutamine (216 mg/l). The HB55 and HB95 hybridomas, secreting the L243 anti-HLA-DR (class II) mAb[3] and the W6/32 anti-HLA-A,B,C (class I) mAb, respectively, were purchased from the American Type Culture Collection (Rockville, MD).

Synthesis of NY-ESO-1 HLA-DRB1*0401 Binding Peptides. Peptides were synthesized using standard Fmoc chemistry by the University of Pittsburgh Peptide Synthesis Facility (Shared Resource), were >90% pure as indicated by analytical high-performance liquid chromatography, and were validated for identity by mass spectrometry. Lyophilized peptides were dissolved in PBS/10% DMSO or 100% DMSO at a concentration of 2 mg/ml and stored at −20°C until use. Synthesis of NY-ESO-1 peptides was based on the sequence of a *NY-ESO-1* gene published by Chen *et al.* (Ref. 11; GenBank accession no. U87459).

The full-length NY-ESO-1 and SSX recombinant proteins were produced in baculovirus and were kindly provided by Drs Lloyd J. Old and Gerd Ritter (Ludwig Institute for Cancer Research, New York Branch, New York, NY).

Induction of CD4+ T Cells with Peptides. PBMCs were isolated by density centrifugation on Ficoll-Hypaque gradients (LSM; Organon-Teknika, Durham, NC) and used to prepare mature DCs using the procedure of Jonuleit *et al.* (18) with minor modifications. PBMCs were resuspended at 10^7/ml in AIM-V medium (Life Technologies, Inc., Grand Island, NY) and were incubated for 90 min in 75-cm^2 tissue culture flasks or six-well plates (37°C; 5% CO$_2$). Nonadherent (T cell-enriched) cells were gently washed out with HBSS and subsequently frozen. The plastic adherent cells were cultured in AIM-V medium supplemented with 1000 units/ml rhGM-CSF and 1000 units/ml rhIL-4 (Schering-Plough, Kenilworth, NJ). Six days later, the culture medium was removed, and the immature DCs were cultured in AIM-V supplemented with 1000 units/ml rhGM-CSF and 1000 units/ml rhIL-4, 1000 units/ml rhIL-6 (Sandoz), 10 ng/ml recombinant human tumor necrosis factor-α (R & D), and 10 ng/ml IL-1β (R & D). Mature DCs were harvested on day 8, centrifuged,

[3] The abbreviations used are: mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; DC, dendritic cell; rhGM-CSF, recombinant human granulocyte/macrophage-colony stimulating factor; rhIL, recombinant human interleukin; PBL, peripheral blood lymphocyte; APC, antigen-presenting cell.

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frozen, or used to stimulate autologous T cells. The stimulator cells were resuspended in AIM-V at 10^6/ml supplemented with each peptide (10 μg/ml) and incubated for 4 h at 37°C. The peptide-pulsed DCs were then irradiated (50 Gy) and washed and resuspended in culture medium (Iscove's medium supplemented with 10% human serum, L-arginine, L-asparagine, and L-glutamine). Autologous CD4+ T cells were positively isolated from PBMCs with immunomagnetic beads (Miltenyi Biotech, Germany) and added (10^6) to the peptide-pulsed DCs (10^5) in a final volume of 2 ml of culture medium (24-well tissue culture plate) along with 1000 units/ml IL-6 and 10 ng/ml IL-12 (Genetics Institute, Cambridge, MA). On day 7 and weekly thereafter, lymphocytes were restimulated with autologous irradiated DCs pulsed with peptide in culture medium supplemented with 10 units/ml IL-2 and 5 ng/ml IL-7 (Genzyme). The stimulated CD4+ T cells were analyzed for specificity in ELISPOT assays at day 18 and then every 10 days after the most recent stimulation.

Induction of CD4+ T Cells with Protein. Immature DCs were prepared as indicated previously and incubated at 37°C, 5% CO_2 for 2 days in AIM-V supplemented with 1000 units/ml rhGM-CSF and 1000 units/ml rhIL-4, 1000 units/ml rhIL-6 (Sandoz), 10 ng/ml recombinant human tumor necrosis factor-α (R & D), and 10 ng/ml IL-1β (R & D) in the presence of the recombinant NY-ESO-1 protein (30 μg/ml). DCs were harvested on day 8, centrifuged, and frozen or used to stimulate autologous PBMCs. The protein-loaded DCs were then irradiated at 50 Gy, washed, and resuspended in culture medium (Iscove's medium supplemented with 10% human serum, L-arginine, L-asparagine, and L-glutamine). Autologous PBMCs were added (3×10^6) to the protein-loaded DCs (10^5) in a final volume of 2 ml of culture medium (24-well tissue culture plate) along with 1000 units/ml IL-6 and 10 ng/ml IL-12 (Genetics Institute, Cambridge, MA). On days 7 and 14, the lymphocytes were restimulated with autologous irradiated protein-pulsed DCs in culture medium supplemented with 10 units/ml IL-2 and 5 ng/ml IL-7 (Genzyme). The stimulated CD4+ T cells were analyzed for specificity in ELISPOT assays at day 21.

The CD4+ T cells were cloned by limiting dilution using allogeneic PBLs and EBV-B cells as feeders in the presence of IL-2 (100 units/ml) and phytohemagglutinin (0.5 μg/ml) and subsequently tested for specificity in IFN-γ ELISPOT and IFN-γ ELISA assays. The CD4+ T-cell clones were maintained by restimulation every 2 weeks by alternating irradiated allogenic PBL and EBV-B cells or autologous peptide-pulsed DCs as stimulators.

Assessment of T-Cell Responses to Peptides and Tumor Cells. The recognition of APCs pulsed with peptides and tumor cells was assessed by ELISPOT and ELISA assays specific for hu-IFN-γ. For IFN-γ ELISPOT assay, multiscreen hemagglutinin antigen plates (Millipore, Bedford, MA) were coated with 10 μg/ml of antihuman IFN-γ mAb (1-D1K; Mabtech, Stockholm, Sweden) in PBS (Life Technologies) overnight at 4°C. Unbound antibody was removed by four successive washings with PBS. After blocking the plates with RPMI/10% human serum (1 h at 37°C), CD4+ T cells were seeded in triplicates (10^4 for bulk CD4+ T cells and 10^3 for CD4+ T-cell clones) in multiscreen hemagglutinin antigen plates. Nonirradiated T2.DR4

cells (7.5×10^4) or melanoma cells (25×10^3) were added. Synthetic peptides were then added at indicated concentrations into ELISPOT assays after seeding APCs. Control wells contained unstimulated T cells, T cells in the presence of unloaded T2.DR4 cells, or tumor cells alone. Alternatively, 25×10^3 protein-loaded DCs were added to 5×10^3 CD4+ T-cell clones/well. Culture medium was AIM-V at a final volume of 200 μl/well. After incubation at 37°C in 5% CO_2 for 20 h, cells were removed by washings with PBS/0.05% Tween 20 (PBS/T). Captured cytokine was detected at sites of its secretion by incubation for 2 h with biotinylated mAb anti-hu-IFN-γ (7-B6-1; Mabtech) at 2 μg/ml in PBS/0.5% BSA. Plates were washed six times with PBS/T, and avidin-peroxidase complex (diluted 1:100; Vectastain Elite kit; Vector, Burlingame, CA) was added for 1 h at room temperature. Unbound complex was removed by three successive washings with PBS/T and three with PBS alone. Peroxidase staining was performed with 3-amino-9-ethyl-carbazole (Sigma) for 4 min and stopped by rinsing the plates under running tap water. Spot numbers and spot sizes were automatically determined with the use of computer-assisted video image analysis as described previously (19).

Alternatively, the IFN-γ secretion was assessed by ELISA. T2.DR4 cells were incubated for 6 h at 7.5×10^4 per flat-bottomed microwell with peptides at indicated concentrations, and CD4+ T-cell clones at 2×10^3 cells/well were added to the assay for 20 h. Culture supernatants were then harvested, and IFN-γ secretion by CD4+ T cells was measured using a commercial ELISA kit (R & D systems; detection limit, 7 pg/ml).

RESULTS

Selection and Identification of Putative NY-ESO-1-encoded Peptides Binding to HLA-DRB1*0401. Predicated on the high serum antibody titer against the NY-ESO-1 protein, we focused our attention on the NY-ESO-1 protein as a potential source of CD4+ T-cell epitopes using the long-lived, disease-free melanoma patient UPCI-MEL 527 as a responder. As expected, the autologous UPCI-MEL 527.1 melanoma cell line expresses the *NY-ESO-1* gene products (as assessed by both PCR and Western blot). Because a genotypic analysis indicated that this patient expressed the *DRB1*0401* allele, we targeted our analysis of CD4+ T-cell reactivity against potential DR4-binding peptides derived from the NY-ESO-1 tumor antigen.

The NY-ESO-1 protein sequence was obtained from the GenBank and analyzed for HLA-DR4 binding peptides using a neural network algorithm (15, 16). High-scoring, nine amino acid-long "core" peptide sequences were typically extended by three amino acids on either flank using the corresponding genomic sequences and synthesized. Alternatively, if multiple high-scoring sequences overlapped, a single extended sequence encompassing the overlaps and two to three terminal amino acid extensions was synthesized. Overall, three peptides of 23–26 amino acids in length were chosen for subsequent analyses (Table 1).

The IFN-γ ELISPOT assay was used to analyze the reactivity of freshly isolated peripheral blood CD4+ T cells derived from patient UPCI-MEL 527 against T2.DR4 cells pulsed with each of the three potential DR4-restricted NY-ESO-1 melanoma peptides. No immunoreactivity against any of these peptides was observed in these responder cells that had not been subjected to *in vitro* stimulation (data not shown).

CD4+ Bulk T Cells Recognize Peptide NY-ESO-1$_{119-143}$ Presented on HLA-DR4. In an independent series of *in vitro* experiments, we "primed" CD4+ T cells from a DRB1*0401$^+$ normal donor and from patient UPCI-MEL 527 against the potential DR4-binding peptides predicted by the MHC binding algorithm (Table 1). "Mature" DCs were incubated with each of the three different peptides (10 μg/ml), irradiated, and used to stimulate autologous CD4+ T cells (previously isolated from the peripheral blood, as described in "Materials and Methods"). The individual responder cell cultures were restimulated on a weekly basis with irradiated autologous mature DCs loaded with the corresponding peptide used in the primary

Table 1 *HLA-DRB1*0401-binding NY-ESO-1 peptides predicted by the peptide binding algorithm*

Peptide binding sequences are provided using single-letter amino acid designation. Peptide binding algorithm (11, 12) scores for the highest-scoring embedded 9-mer sequences (underlined) are indicated for each peptide evaluated.

| NY-ESO-1 peptides: amino acid residues	Sequences	Algorithm score, 0–10	Peptides tested in the study
119–143	PGVLLKEFTVSGNILTIRLTAADHR		+
119–133	PGVLLKEFTVSGNIL		+
123–137	LKEFTVSGNILTIRL		+
129–143	SGNILTIRLTAADHR		+
122–130	LLKEFTVSG	9	
126–134	FTVSGNILT	4	
132–140	ILTIRLTAA	4	
144–169	QLQLSISSCLQQLSLLMWITQCFLPV		+
147–155	LSISSCLQQ	3	
153–161	LQQLSLLMW	3	
158–166	LLMWITQCF	6	
161–170	WITQCFLPV	3	
158–180	LLMWITQCFLPVFLAQPPSGQRR		+
161–170	WITQCFLPV	3	
162–170	FLPVFLAQP	5	
166–174	VFLAQPPSG	6	
169–177	FLAQPPSGQ	4	

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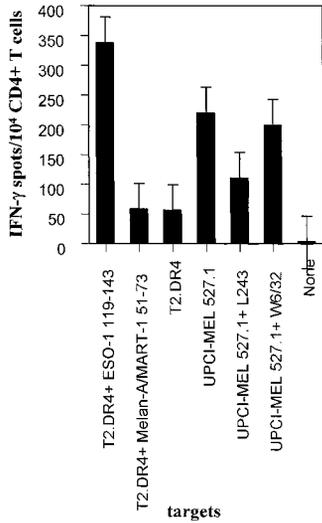
Fig. 1. Recognition of the NY-ESO-1$_{119-143}$ peptide and UPCI-MEL 527.1 cell line by CD4+ T cells of an HLA-DRB1*0401 normal donor. CD4+ T cells from an HLA-DRB1*0401 donor underwent three rounds of *in vitro* stimulation with autologous DCs pulsed with the NY-ESO-1$_{119-143}$ peptide, as described in "Materials and Methods." Ten thousand of the resulting responder CD4+ T cells were incubated in a 20-h IFN-γ ELISPOT assay in the presence of T2. DR4 cells were pulsed with the NY-ESO-1$_{119-143}$ peptide or Melan-A/MART-1 $_{51-73}$ peptide (10 μg/ml), UPCI-MEL 527.1 cells $\pm$ anti-HLA-DR antibodies (L243), or UPCI-MEL 527.1 cells $\pm$ anti-HLA-A,B,C antibodies (W6/32). IFN-γ spots were developed and counted by computer-assisted video image analysis. *Columns,* mean spot numbers of triplicates with 10^4 CD4+ T cells initially seeded/well; *bars,* SD. Data from one representative experiment of three performed are depicted.

line UPCI-MEL 527.1, which was inhibited by addition of anti-HLA-DR mAb (L243) but not anti-HLA-A,B,C mAb (W6/32) to ELISPOT. No IFN-γ spots were produced by the CD4+ T cells cultured with T2.DR4 cells alone or pulsed with an irrelevant DR4-restricted peptide (*i.e.,* Melan-A/MART-1$_{51-73}$; Ref. 14) or with the NY-ESO-1$^-$ cell line, UPCI-MEL 136.1. No IFN-γ spots developed in wells containing UPCI-MEL 136.1 cells or T2.DR4 cells in the absence of added CD4+ T cells (data not shown). No immunoreactivity against the two other potential DR4-binding peptides, NY-ESO-1$_{149-168}$ and NY-ESO-1$_{158-180}$, was observed in CD4+ T-cell cultures stimulated with autologous DCs pulsed with either NY-ESO-1$_{119-143}$, NY-ESO-1$_{149-168}$, or NY-ESO-1$_{158-180}$.

CD4+ T-Cell Clone 10/4 (Normal Donor) Recognizes the NY-ESO-1$_{119-133}$ Core Epitope within the NY-ESO-1$_{119-143}$ Sequence. Clone 10/4 was obtained by limiting dilution from the CD4+ bulk T cells of an HLA-DR4$^+$ normal donor stimulated *in vitro* with the NY-ESO-1$_{119-143}$ peptide. Clone 10/4 specifically recognized T2.DR4 cells pulsed with the NY-ESO-1$_{119-143}$ peptide and the HLA-DR4-matched melanoma cell line UPCI-MEL 527.1 (Fig. 2). The recognition of UPCI-MEL 527.1 was inhibited by the addition of anti-HLA-DR mAb (L243) but not anti-HLA-A,B,C mAb (W6/32) to ELISPOT or ELISA wells. No IFN-γ spots were produced by clone 10/4 cultured with T2.DR4 cells alone or pulsed with an irrelevant DR4-restricted peptide (*i.e.,* Melan-A/MART-1$_{51-73}$) or with the NY-ESO-1$^-$ UPCI-MEL 136.1 cell line. However clone 10/4 produced IFN-γ in the presence of the UPCI-MEL 136.1 cell-pulsed with NY-ESO-1$_{119-143}$ peptide. No IFN-γ spots were produced by UPCI-MEL 136.1 cells or T2.DR4 cells in the absence of added CD4+ T cells (data not shown).

The ability of CD4+ T cell clone 10/4 to produce IFN-γ in the presence T2.DR4 cells, preincubated with the NY-ESO-1$_{119-143}$ peptide at various concentrations, was evaluated to determine the peptide-dose "threshold" for effector T-cell recognition. Both ELISPOT and ELISA assays provided similar information (Fig. 3), suggesting that
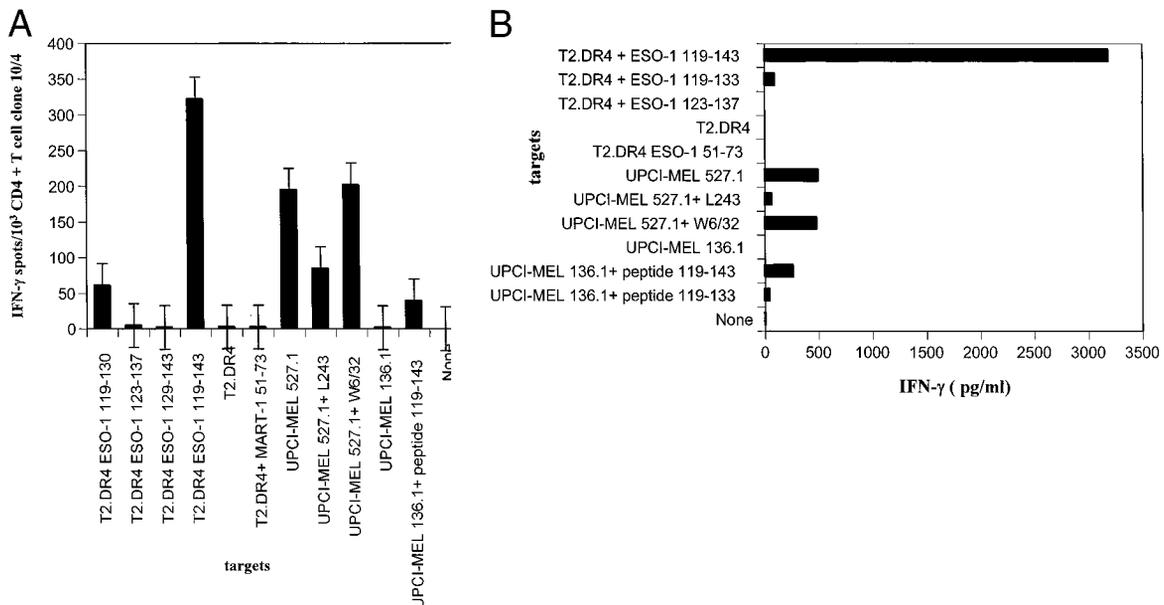
stimulation. After at least three restimulations, the immunoreactivity of the CD4+ T-cell cultures were analyzed in IFN-γ ELISPOT assays. Bulk CD4+ T cells that were stimulated with the NY-ESO-1$_{119-143}$ peptide specifically recognized T2.DR4 cells pulsed with the immunogenic peptide (Fig. 1). These CD4+ T cells also displayed reactivity against the HLA-DR4-matched NY-ESO-1$^+$ melanoma cell

Fig. 2. Recognition of the NY-ESO-1$_{119-143}$ and NY-ESO-1$_{119-133}$ peptides and the NY-ESO-1$^+$, DR4$^+$ cell line, UPCI-MEL 527.1 cell line by the CD4+ T-cell clone 10/4. CD4+ T cells were isolated from the peripheral blood of patient UPCI-MEL 527 and stimulated *in vitro* with autologous DCs pulsed with the NY-ESO-1$_{119-143}$ peptide. The CD4+ T cells that recognize not only APCs pulsed with the NY-ESO-1$_{119-143}$ peptide but also the DR4$^+$, NY-ESO-1$^+$, melanoma cell line UPCI-MEL 527 were cloned by limiting dilution as described in "Materials and Methods." One thousand CD4+ T cells from clone 10/4 were incubated in a 20-h IFN-γ ELISPOT assay (*A*) or in a 20-h IFN-γ ELISA (*B*) in the presence of T2. DR4 cells pulsed with peptides (10 μg/ml), UPCI-MEL 527.1 cells $\pm$ anti-HLA-DR antibodies (L243), UPCI-MEL 527.1 cells $\pm$ anti-HLA-A,B,C antibodies (W6/32) are shown. The DR4$^+$, NY-ESO-1$^-$ melanoma cell line UPCI-MEL 136.1 unpulsed or pulsed with the NY-ESO-1$_{119-143}$ peptide were added as targets in the assays. Data from one representative experiment of three performed are depicted. *Bars,* SD.

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Fig. 3. Lysis of the NY-ESO-1^{+}, DR4^{+} cell line UPCI-MEL 527.1 by clone 10/4. The melanoma cells were preincubated for 48 h with IFN-γ prior to the lysis assay to up-regulate HLA-DR4 expression. Unlabeled T2.DR4 cells or NY-ESO-1$_{119-143}$-pulsed T2.DR4 (50,000/well) were added as cold-target inhibitors to suppress lysis. Chromium release was measured after 4 h. We show the data from one representative experiment of two performed; *bars,* SD.
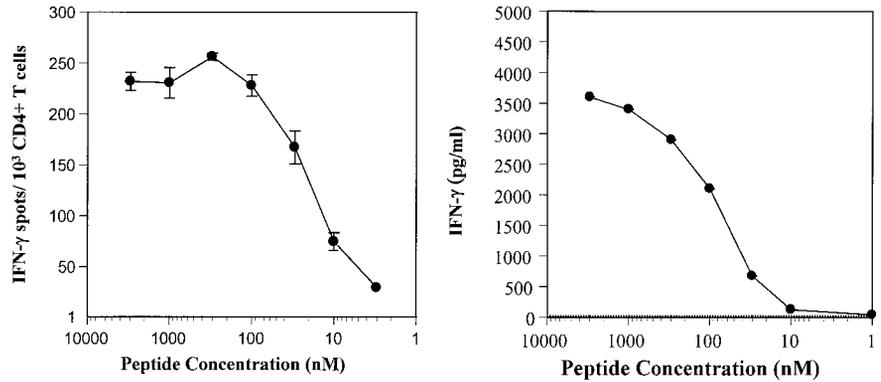
half-maximal stimulation of NY-ESO-1$_{119-143}$ peptide-reactive CD4+ T cells required peptide "loading" concentrations between 20 and 60 nM.

Clone 10/4 also exhibited modest lytic activity against the DR4^{+} NY-ESO-1^{+} melanoma cell line, UPCI-MEL 527.1. Cytolysis was inhibited by addition of anti-HLA-DR mAb (L243) but not anti-HLA-A,B,C mAb. Although specific lysis of UPCI-MEL 527.1 cells was not inhibited significantly by the addition of cold T2.DR4 target cells (cold:hot target ratio, 50:1), the addition of T2.DR4 target cells preloaded with the NY-ESO-1$_{119-143}$ peptide (10 μg/ml) blocked lysis of UPCI-MEL 527.1 cells by 68% (Fig. 4).

The NY-ESO-1$_{119-143}$ peptide is 25 amino acids long and encompasses three (see Table 1; *i.e.,* 122–130, 126–134, and 132–140) potential immunogenic "core epitopes," according to the algorithm. We synthesized a series of peptides (*i.e.,* 119–133, 123–137, and 129–143) from the internal sequence of the immunogenic NY-ESO-1$_{119-143}$ peptide. Peptide NY-ESO-1$_{119-133}$, but not peptides NY-ESO-1$_{123-137}$ or NY-ESO-1$_{129-143}$, were able to sensitize T2.DR4 cells for recognition by the NY-ESO-1$_{119-143}$-reactive CD4+ T cell clone 10/4 as monitored by either IFN-γ ELISPOT and ELISA assays (Fig. 2). Although full-recognition NY-ESO-1$_{119-133}$ peptide was only a fraction of that observed against the NY-ESO-1$_{119-143}$ peptide, these data suggest that one immunogenic epitope encoded by NY-ESO-1 is located between residues 119 and 133, likely encompassing the NY-ESO-1$_{122-133}$ core epitope identified by the peptide-binding algorithm.

CD4+ T-Cell Clone 11/4 (Melanoma Patient) Recognizes the NY-ESO-1$_{123-137}$ Core Epitope within the NY-ESO-1$_{119-143}$ Sequence. Clone 11/4 was obtained by limiting dilution from the CD4+ bulk T cells of patient 527 that recognized the NY-ESO-1$_{119-143}$ peptide. Clone 11/4 specifically recognized T2.DR4 cells pulsed with either the NY-ESO-1$_{119-143}$ peptide or the NY-ESO-1$_{123-137}$ peptide and the autologous melanoma cell line UPCI-MEL 527.1 (Fig. 5). The recognition of the UPCI-MEL 527.1 was inhibited by the addition of anti-HLA-DR mAb (L243) but not anti-HLA-A,B,C mAb (W6/32) to ELISPOT or ELISA wells. No IFN-γ spots were produced by clone 11/4 cultured with T2.DR4 cells alone or pulsed with an irrelevant DR4-restricted peptide (*i.e.,* Melan-A/MART-1$_{51-73}$) or with the UPCI-MEL 136.1 cell line that fails to express NY-ESO-1 T cells. The ability of clone 11/4 to produce IFN-γ in the presence T2.DR4 cells, preincubated with various concentrations of either the NY-ESO-1$_{119-143}$ peptide or the NY-ESO-1$_{123-137}$ peptide, was evaluated. Half-maximal stimulation of clone 11/4 required peptide "loading" concentrations between 1 and 10 nM (Fig. 6).

Clone 11/4 was also capable of lysis of the autologous melanoma cell line (UPCI-MEL 527.1) and T2.DR4 cells pulsed with the NY-ESO-1$_{119-143}$ peptide (10 μg/ml) but not the DR4^{+}, NY-ESO-1^{−}, UPCI-MEL 136.1 melanoma cells (Fig. 7). This lysis was inhibited by

the addition of anti-HLA-DR mAb (L243) but not anti-HLA-A,B,C mAb.

Peptide NY-ESO-1$_{123-137}$ but not peptides NY-ESO-1$_{119-133}$ (preferentially recognized by clone 10/4) or NY-ESO-1$_{129-143}$ was able to sensitize T2.DR4 cells for recognition by clone 11/4 in IFN-γ ELISPOT and chromium release assays (Figs. 5 and 7). These data indicate that a second immunogenic epitope encoded by NY-ESO-1 is located between residues 123 and 137 and is likely to contain the NY-ESO-1$_{126-134}$ core epitope identified by the peptide-binding algorithm.

DCs Loaded with Recombinant NY-ESO-1 Protein Present the NY-ESO-1 Epitope Recognized by Clone 11/4 and Stimulate CD4+ T Cells That Recognize the NY-ESO-1$_{119-143}$ Peptide from a DRB1*0401[+] Normal Donor. Autologous DCs were loaded with 30 μg/ml of either the NY-ESO-1 protein or the SSX protein (as indicated in "Materials and Methods"). Twenty-five thousand protein-loaded DCs were added to 5 × 10^3 CD4+ T cells per triplicate well in a 24-h ELISPOT assay. Only clone 11/4 but not clone 10/4 was able to recognize autologous DCs loaded with the NY-ESO-1 protein in ELISPOT
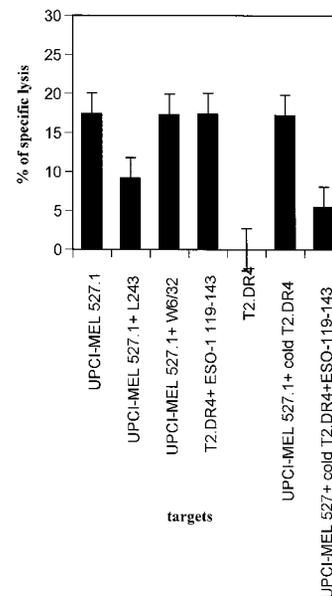

Fig. 4. Reactivity of CD4+ T-cell clone 10/4 to titered doses of peptide NY-ESO-1$_{119-143}$. T2.DR4 cells were pulsed with different concentrations of the NY-ESO-1$_{119-143}$ peptide and used as target cells in a 20-h IFN-γ ELISPOT assay (*A*). Alternatively, T2.DR4 cells were similarly pulsed with NY-ESO-1$_{119-143}$ peptide and incubated in the presence of the CD4+ T cell clone 10/20 in a 20-h IFN-γ ELISA assay (*B*). The data represent the average of triplicate cultures; *bars,* SD. The data were obtained from one representative experiment of three performed, with comparable results observed in each assay.
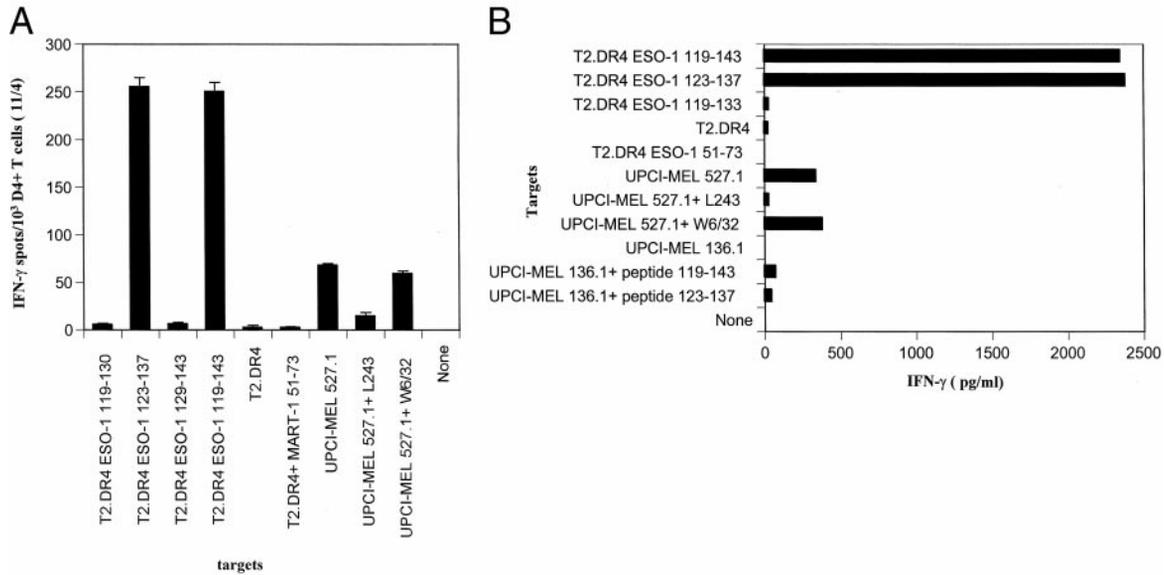
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Fig. 5. Recognition of the NY-ESO-1$_{119-143}$ and NY-ESO-1$_{123-137}$ peptides and the autologous melanoma cell line, UPCI-MEL 527.1, by the CD4+ T-cell clone 11/4. CD4+ T cells were isolated from the peripheral blood of patient UPCI-MEL 527 and stimulated *in vitro* with autologous DCs pulsed with the NY-ESO-1$_{119-143}$ peptide. The CD4+ T cells that recognize not only APCs pulsed with the NY-ESO-1$_{119-143}$ peptide but also the DR4+, NY-ESO-1+ autologous melanoma cell line UPCI-MEL 527 were cloned by limiting dilution as described in "Materials and Methods." One thousand CD4+ T cells from clone 11/4 were incubated in a 20-h IFN-γ ELISPOT assay (*A*) or in a 20-h IFN-γ ELISA (*B*) in the presence of T2.DR4 cells pulsed with peptides (10 μg/ml), UPCI-MEL 527.1 cells ± anti-HLA-DR antibodies (L243), UPCI-MEL 527.1 cells ± anti-HLA-A,B,C antibodies (W6/32). The DR4+, NY-ESO-1− melanoma cell line UPCI-MEL 136.1 unpulsed or pulsed with the NY-ESO-1$_{119-143}$ peptide were added as targets in the assays. Data from one representative experiment of three performed are depicted. *Bars,* SD.

assays (Fig. 8). Unloaded DCs or the DCs fed with the recombinant SSX protein served as baseline and peptide controls, respectively.

PBMCs from a DRB1*0401+ normal donor were stimulated with DCs loaded with protein NY-ESO-1 (as indicated in "Materials and Methods"). The responder cell cultures were then restimulated on a weekly basis with irradiated autologous protein DCs used in the primary stimulation. After three restimulations, the immunoreactivity of the T-cell cultures was analyzed in IFN-γ ELISPOT assays. T cells that were stimulated with the protein-pulsed DCs specifically recognized T2.DR4 cells pulsed with NY-ESO-1$_{119-143}$ peptide (Fig. 9). These CD4+ T cells also displayed reactivity against the HLA-DR4-matched melanoma cell line UPCI-MEL 527.1, which was inhibited by the addition of anti-HLA-DR mAb (L243) but not anti-HLA-A,B,C mAb. No significant immunoreactivity could be observed against the other potential DR4-binding NY-ESO-1 peptide (*i.e.,* NY-ESO-1$_{144-168}$ and NY-ESO-1$_{158-180}$).

DISCUSSION

Our strategy for the identification of novel class II melanoma epitopes is based on the selection of putative DR4-binding peptides using a neural network algorithm and evaluation of the ability of these peptides to activate melanoma-specific CD4+ precursors T cell*s* from the blood of normal individuals or long-lived patients with melanoma *in vitro*. Using this approach, we obtained CD4+ T-cell clones that recognize either of two MHC class II-presented epitopes, NY-ESO$_{119-133}$ and NY-ESO$_{123-137}$, respectively, derived from the NY-ESO-1 antigen. These epitopes are immunogenic *in vitro* and promoted the expansion of CD4+ T cells that specifically secreted IFN-γ in response to, and lysed, peptide-pulsed DR4+ target cells, as well as HLA-DR4+ melanoma cells constitutively expressing the NY-ESO-1 protein. However, in patient 527 and two other DR4+ melanoma patients that were analyzed in this study, we could not detect any elevated "memory/effector " CD4+ T cell frequency against the NY-ESO-1$_{119-143}$ peptide in PBLs that were freshly isolated from the peripheral blood and analyzed using the ELISPOT assay.

CD4+ T-cell clones recognizing the NY-ESO-1$_{119-143}$ peptide specifically secreted the Th1-type cytokine IFN-γ in response to peptide-pulsed DR4+ nonmelanoma target cells and HLA-DR4+ melanoma cells that constitutively expressed the NY-ESO-1 gene product. Specific recognition of melanoma targets could be effectively

Fig. 6. Reactivity of CD4+ T-cell clone 11/4 to titered doses of the NY-ESO-1$_{119-143}$ and NY-ESO-1$_{123-137}$ peptides. T2.DR4 cells were pulsed with different concentrations of either the NY-ESO-1$_{119-143}$ peptide (●) or the NY-ESO-1$_{123-137}$ peptide (○) and used as target cells in a 20-h IFN-γ ELISPOT assay (*A*). Alternatively, T2.DR4 cells were similarly pulsed with each peptide and incubated in the presence of the CD4+ T-cell clone 10/20 in a 20-h IFN-γ ELISA assay (*B*). The data represent the average of triplicate cultures; *bars,* SD. The data were obtained from one representative experiment of three performed, with comparable results observed in each assay.
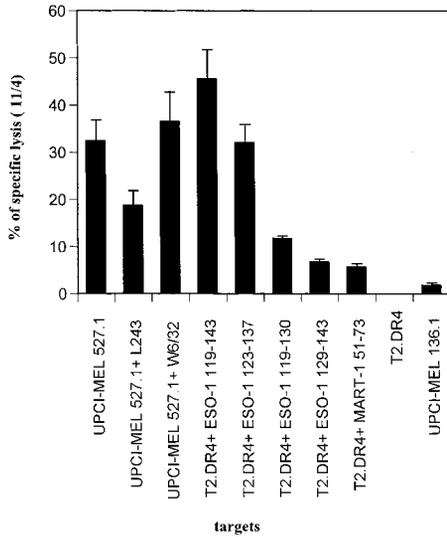
4950
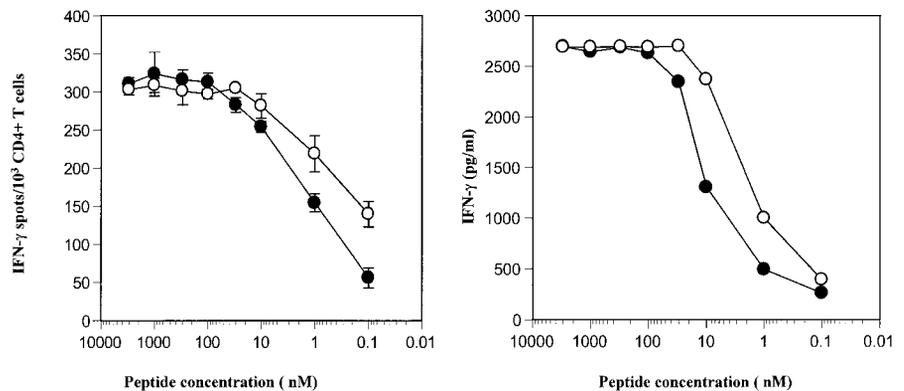
Fig. 7. Lysis of the autologous melanoma cell line UPCI-MEL 527.1 by clone 11/4. CD4+ T cells from clone 11/4 were incubated in the presence of autologous melanoma cells, UPCI-MEL 527.1, ± anti-HLA-DR antibodies (L243), ± anti-HLA-A,B,C antibodies (W6/32). The melanoma cells were preincubated for 48 h with IFN-γ prior to the lysis assay to up-regulate HLA-DR4 expression. Unlabeled T2.DR4 cells or NY-ESO-1$_{119-143}$-pulsed T2.DR4 (50,000/well) were added as cold-target inhibitors to suppress antigen-specific lysis. Chromium release was measured after 4 h. We show the data from one representative experiment of two performed. *Bars,* SD.
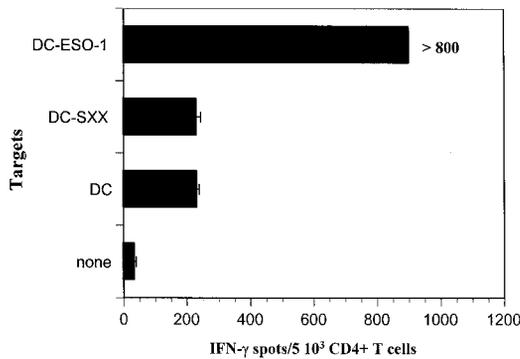

Fig. 8. Clone 11/4 recognizes autologous DCs loaded with the ESO-1 protein. Five thousand cells of T-cell clone 11/4 were incubated in a 20-h IFN-γ ELISPOT assay in the presence of DCs loaded either with the ESO-1 protein or the SSX protein (30 μg/ml) and unloaded DCs. IFN-γ spots were developed and counted by computer-assisted video image analysis. *Columns,* mean spot numbers of triplicates; *bars,* SD.

blocked by anti-HLA-DR antibody. Furthermore, HLA-DR4+ target cells that lacked expression of the *NY-ESO-1* gene product were not recognized unless prepulsed with the NY-ESO-1$_{119-143}$ peptide.

These results may suggest a direct antitumor effector role for such CD4+ T cells against autologous NY-ESO-1$^+$ melanoma cells that express HLA-DR4 class II molecules at their surface. Interestingly, the ESO-1 protein, like other MAGE proteins, is localized in the cytosol and nucleus, yet appears to be processed naturally via the endogenous class II pathway, giving rise to class II-restricted peptides presented at the surface of the tumor cells as recognized by our specific CD4+ T-cell clones (20). Thus, our data are consistent with results reported for CD4+ T-cell recognition of alternate cancer-testis (CT) epitopes, such as the MAGE-3$_{281-295}$ peptide presented by DR11 (3). Despite the possibility of direct tumor presentation of these class II-binding epitopes, antitumor immunity may often be optimally promoted by "cross-priming" or "cross-presentation" of both CD8 and CD4 epitopes of tumor antigens by professional APCs (including DCs; Ref. 21). APCs may acquire tumor antigens from necrotic or apoptotic bodies in the tumor microenvironment (22). This observa-

tion is consistent with other recent studies (23) that have documented the role of the CD4+ T cells in antitumor immunity of class II-negative tumors. This mechanism of immune-directed recognition of tumors would allow specific antitumor CD4+ T-cell responses to be beneficial in patients, irrespective of the MHC class II expression status of their tumors. Interestingly, we have also demonstrated that HLA-DR4$^+$ DCs can process the NY-ESO-1 protein and present NY-ESO-1-derived epitopes able to stimulate *in vitro* CD4+ T cells that specifically recognize peptide NY-ESO-1$_{119-143}$ and HLA-DR4$^+$ tumor cells that naturally express the NY-ESO-1 protein.

Half-maximal CD4+ T effector cell stimulation required peptide loading concentrations as low as 1–60 nM. These results are in the range of values reported recently for CD4+ T-cell recognition of the HLA-DR13-restricted MAGE-3$_{114-127}$ and HLA-DRB1*0101-restricted mutated TPI epitopes (2, 5).

We demonstrated that the original peptide NY-ESO-1$_{119-143}$, used to generate the specific CD4+ T cells *in vitro,* encompasses two overlapping but non-cross-reactive epitopes (NY-ESO$_{119-133}$ and NY-ESO-1$_{123-137}$) recognized by distinct CD4 T-cell clones. Although the sensitivity of the different clones to the longer NY-ESO-1$_{119-143}$ peptide seems to be similar for all of the ESO-1-reactive CD4+ T-cell clones evaluated, the reactivity of CD4+ T-cell clones to the shorter peptide sequences (*i.e.,* NY-ESO$_{119-133}$) may be inferior to that observed against the longer peptide (*i.e.,* NY-ESO$_{119-143}$). Although, we do not have a clear explanation for this phenomenon, one may hypothesize that although containing the DR4-binding core epitopes, the NY-ESO$_{119-133}$ peptide in particular may exhibit a lower affinity for DR4 or a poorer solubility index than the NY-ESO$_{119-143}$ peptide, perhaps because of the nature of its flanking sequences. We are currently evaluating this possibility. Alternatively, the shorter sequence may be subject to enhanced proteolytic degradation of the core epitope and hence neutralization of its "bioactivity" (24).
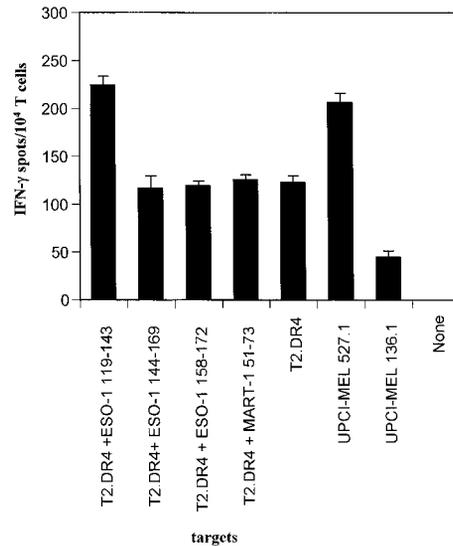

Fig. 9. CD4+ T cells stimulated with autologous DCs loaded with the NY-ESO-1 protein recognize the NY-ESO-1$_{119-143}$ peptide and the autologous melanoma cell line UPCI-MEL527.1. Total PBMCs from an HLA-DRB1*0401 donor underwent three rounds of *in vitro* stimulation with autologous DCs loaded with the NY-ESO-1 protein as described in "Materials and Methods." Ten thousand of the resulting responder T cells were incubated in a 20-h IFN-γ ELISPOT assay in the presence of T2.DR4 cells pulsed with the potential NY-ESO-1 DR4-binding peptides including NY-ESO-1$_{119-143}$ peptide or Melan-A/MART-1$_{51-73}$ peptide (10 μg/ml). The DR4$^+$, NY-ESO-1$^+$ UPCI-MEL 527.1 cells and the DR4$^+$, NY-ESO-1$^-$ UPCI-MEL 136.1 cells were included as targets in the assays. IFN-γ spots were developed and counted by computer-assisted video image analysis. *Columns,* mean spot numbers of triplicates with 10^4 CD4+ T cells initially seeded per well; *bars,* SD.

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Our findings add two new candidate CD4 "helper T cell" epitopes for future peptide-vaccine trials. Vaccination to both epitopes might be best accomplished using peptide NY-ESO-1$_{119-143}$ that encompasses the two overlapping sequences. Given the role of the CD4+ T cells in maintaining CD8+ T-cell responses and the potential direct antitumor effector function of CD4+ T cells, it will be important to broaden clinical strategies to target the *in vivo* induction of both tumor-specific CD4+ T cells and CD8+ T cells. This might be most readily accomplished by vaccination with protein or multivalent peptides including class I and class II-restricted epitopes encoded by cancer-testis (*i.e.,* the NY-ESO-1$_{157-165}$ and NY-ESO-1$_{119-143}$) and/or melanocyte-specific genes (*i.e.,* the Melan-A/MART-1$_{27-35}$ and Melan-A/MART-1$_{51-73}$ peptides) in HLA-A2$^+$, DR4$^+$ patients with melanoma.

NY-ESO-1 is expressed by 30% of metastatic melanoma and by non-small cell lung cancer, bladder, prostate, and head and neck tumors (25). Although nearly 18% of American Caucasians express the HLA-DR4 allele (26), we have observed an HLA-DR4 allele frequency of 34% (15 of 44) in a recent serological assessment of melanoma patients treated at the University of Pittsburgh Cancer Institute.[4] Because the DRB1*0401, DRB1*0404, and DRB1*0408 subtypes are expressed by ~80% of the DR4+ population (27–29), NY-ESO-1$_{119-143}$ peptide will likely prove to be clinically relevant in 4.4–8.2% of Caucasian melanoma patients. Recent studies also demonstrate that HLA-DR4 molecules are part of a larger HLA class II supertype (30), including several other common DR types. Because the identification of a broadly cross-presented epitope would clearly expand the potential population coverage, we are currently determining the ability of the NY-ESO-1$_{119-143}$ peptide to bind to a broader range of HLA-DR alleles. Interestingly, in a recent study by Jager *et al.* (31), three DRB4*0101–0103 (DR53)-restricted epitopes recognized by melanoma reactive CD4+ T cells have been identified. Two of these had sequences similar to the two epitopes reported in our study, NY-ESO-1$_{115-132}$ and NY-ESO-1$_{121-138}$, respectively. This supports the relevance of the NY-ESO-1$_{119-143}$ peptide and its two "sub epitopes" as potential immunogens for cancer vaccines designed to treat HLA-DR4$^+$ and HLA-DR53$^+$ patients with NY-ESO-1$^+$ tumors.

ACKNOWLEDGMENTS

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NY-ESO-1 Encodes DRB1*0401-restricted Epitopes Recognized by Melanoma-reactive CD4+ T Cells

Hassane M. Zarour, Walter J. Storkus, Vladimir Brusic, et al.

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