

NY-ESO-1 119–143 Is a Promiscuous Major Histocompatibility Complex Class II T-Helper Epitope Recognized by Th1- and Th2-Type Tumor-reactive CD4+ T Cells¹

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ABSTRACT

The NY-ESO-1 gene product is expressed by a range of human tumors and is recognized by antibodies from sera of cancer patients with NY-ESO-1-expressing tumors. The NY-ESO-1 gene also encodes several MHC class I- and MHC class II-restricted tumor epitopes recognized by T lymphocytes. In particular, we previously reported that the NY-ESO-1 119–143 peptide contains at least two HLA-DRB1*0401-presented epitopes that are recognized by melanoma-reactive CD4+ T cells. Here we report that the NY-ESO-1 119–143 peptide can be presented in the context of multiple HLA-DR alleles to stimulate tumor-reactive CD4+ T cells. The NY-ESO-1 119–143 peptide is able to bind to several DR molecules. The NY-ESO-1 119–143 peptide is also capable of inducing specific CD4+ T cells *in vitro* from peripheral blood lymphocytes of normal donors and patients with melanoma who express these HLA-DR alleles. These CD4+ T cells recognize NY-ESO-1⁺, HLA-matched or autologous melanoma cell lines, as well as autologous antigen-presenting cells fed with the NY-ESO-1 protein. We also demonstrate that the NY-ESO-1 119–143 peptide stimulates *in vitro* both Th1-type and Th2-type CD4+ T-cell responses from peripheral blood lymphocytes of normal donors and melanoma patients. Taken together, these data suggest a key role of the NY-ESO-1 119–143 peptide sequence in the induction of cellular and humoral responses against NY-ESO-1-expressing tumors. They support the relevance of cancer vaccine trials with the NY-ESO-1 119–143 peptide in the large number of cancer patients with NY-ESO-1-expressing tumors.

INTRODUCTION

The NY-ESO-1 antigen is expressed by many tumors of different histological types, including breast, prostate, lung, and melanoma, and by male germ-line cells, but it is silent in normal tissues. Because of this expression pattern, NY-ESO-1 is classified in the group of TAA^s alternatively designated cancer-testis antigens (1), cancer-germ line antigens (2), or tumor-specific shared antigens (3). The NY-ESO-1 antigen was initially identified by serological analysis of a recombinant cDNA library from a human esophageal cancer (4). NY-ESO-1 has subsequently been shown to encode class I-restricted peptides expressed by a diverse range of cancers (1, 5). More recently, multiple

NY-ESO-1-derived epitopes presented by HLA-DRB1*0401, HLA-DRB4*0101, and HLA-DRB1*0401 and capable of stimulating CD4+ T cells, have been reported (6–8). NY-ESO-1 appears to be very immunogenic, inducing both spontaneous cellular and humoral responses in ~50% of patients with NY-ESO-1⁺ tumors (1, 9). The induction of primary NY-ESO-1-specific CD8+ T-cell responses has been reported after intradermal peptide vaccination in patients with NY-ESO-1⁺ tumors (10).

We have previously reported a peptide-based strategy to identify novel DR4-restricted tumor-derived peptides recognized by CD4+ T cells (11). In doing so, we have already identified several DR4-restricted peptide sequences, including peptide NY-ESO-1 119–143 (7). Here we develop a strategy to identify those sequences that can be broadly presented by multiple DR alleles among peptide sequences initially identified as DR4-restricted. In particular, we demonstrate that peptide NY-ESO-1 119–143 not only binds to multiple DR alleles, but also stimulates CD4+ T-cell responses when presented at the surface of these molecules. We also observed that peptide NY-ESO-1 119–143, which stimulated *in vitro* strong Th1-type T-cell responses, could also induce Th2-type CD4+ T-cell responses from PBLs of normal donors and melanoma patients.

These findings support the use of the NY-ESO-1 119–143 peptide as a cancer vaccine for a large number of patients with NY-ESO-1-expressing tumors.

MATERIALS AND METHODS

Cell Lines, Media, and Antibodies. Tissues and blood samples used for all studies reported here were obtained under UPCI Institutional Review Board-approved protocol 96-99. Patients UPCI-MEL 527, UPCI-MEL 285, and UPCI-MEL 598 are long-lived patients who have remained disease free several years after successful therapy for distant NY-ESO-1-expressing metastatic melanoma. The UPCI-MEL 527.1, UPCI-MEL 285.1, and UPCI-MEL 591.8 cell lines were derived from metastatic lesions of these patients. Patients UPCI-MEL 527, UPCI-MEL 285, and UPCI-MEL 598 have been genotyped as HLA-DRB1*0401, HLA-DRB1*0101/DRB1*0401, and HLA-DRB1*0701/DRB1*1101, respectively. HLA-DR genotyping was performed with a commercial DR typing panel of PCR primers, according to the manufacturer's instructions (Dynal, Oslo, Norway). High-titer anti-NY-ESO-1 antibodies have been consistently observed in the serum of patient UPCI-MEL 527.

The T2.DR4 cell line was generated through transfection of HLA-DRB1*0401 cDNA into T2 cells (18). The T2.DR4 cell line is HLA-DM deficient, making its cell surface DRB1*0401 complexes receptive to loading by exogenous peptides. The homozygous B-EBV-transformed cell lines MGAR and POER were provided by Dr. Penelope Morel (UPCI, Pittsburgh, PA). DR-transfected mouse cells, *i.e.*, L.DR1, L.DR3, L.DR7, and L.DR53 were a gift of Dr. Robert Karr (Searle, Inc., St. Louis, MO). All cell lines were cultured in RPMI 1640 (Life Technologies) supplemented with 10% FCS, L-arginine (116 mg/liter), L-asparagine (36 mg/liter), and L-glutamine (216 mg/liter). The HB55 and HB95 hybridomas, which secrete the L243 anti-HLA-DR (class II) mAb and the W6/32 anti-HLA-A, B, C (class I) mAb,

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³ The abbreviations used are: TAA, tumor-associated antigen; Th, T-helper; PBL, peripheral blood lymphocyte; UPCI, University of Pittsburgh Cancer Institute; EBV, Epstein Barr virus; mAb, monoclonal antibody; HPLC, high-performance liquid chromatography; ELISPOT, enzyme-linked immunospot; IL, interleukin; DC, dendritic cell; APC, antigen-presenting cell.

respectively, were purchased from the American Type Culture Collection (Rockville, MD).

Peptide Synthesis. The NY-ESO-1-derived peptides were synthesized using standard Fmoc chemistry by the University of Pittsburgh Peptide Synthesis Facility (Shared Resource), were >90% pure as indicated by analytical HPLC, 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. 4; GenBank accession no. U87459). The peptides used in the binding assays were synthesized using Fmoc chemistry as described previously (13). Biotinylated peptides were obtained by reaction with biotinyl-6-aminocaproic acid (Fluka Chimie, St. Quentin Fallavier, France) on their NH₂ termini. All of these peptides were purified by reverse-phase HPLC on a C18 Vydac column, and their quality was assessed by electrospray mass spectrometry and analytical HPLC.

Recombinant Proteins. 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).

Purification of HLA-DR Molecules. HLA-DR molecules were purified from homozygous EBV cell lines by affinity chromatography using the monomeric mAb L243 coupled to protein A-Sepharose CL 4B gel (Amersham Pharmacia Biotech, Orsay, France) as described previously (13, 14).

HLA-DR Peptide-binding Assays. HLA-DR molecules were diluted in 10 mM phosphate, 150 mM NaCl, 1 mM DM, 10 mM citrate, 0.003% thimerosal buffer with an appropriate biotinylated peptide and serial dilutions of competitor peptides. Samples (100 μl/well) were incubated in 96-well polypropylene plates (Nunc, Roskilde, Denmark) at 37°C for 24 h, except for the peptides produced by the DRB1*0301, DRB1*1501, DRB1*1301, and DRB4*0101 alleles, which were incubated 72 h. After pH neutralization, samples were applied to 96-well Maxisorp ELISA plates (Nunc) previously coated with 10 μg/ml mAb L243. They were incubated on the antibody-coated plates for 2 h at room temperature. Bound biotinylated peptides were detected by a streptavidin-alkaline phosphatase conjugate (Amersham, Little Chalfont, United Kingdom) and 4-methylumbelliferyl phosphate as substrate (Sigma Chemical Co., St. Quentin Fallavier, France). Fluorescence (emission at 450 nm; excitation at 365 nm) was measured by a Fluorolite 1000 fluorometer (Dynex, Issy les Moulineaux, France). Maximal binding was determined by incubating the biotinylated peptide with the MHC II molecule in the absence of competitor. Binding specificity for each HLA-DR was ensured by the choice of the biotinylated peptides as described previously (14). Data were expressed as the concentration of peptide that prevented binding of 50% of the labeled peptide (IC₅₀).

Induction of CD4+ T Cells with Peptides. The induction of CD4+ T cells *in vitro* with the NY-ESO-1-derived peptides was performed as reported previously (7, 11). The CD4+ T cells were cloned by limiting dilution using allogeneic PBL and EBV-B cells as feeders in the presence of IL-2 and phytohemagglutinin and were subsequently tested for specificity in IFN-γ ELISPOT assays. The CD4+ T-cell clones were maintained by restimulation every 2 weeks, by alternating irradiated allogeneic PBL and EBV-B cells or autologous peptide-pulsed DCs as stimulators.

Induction of CD4+ T Cells with Protein. The induction of CD4+ T cells with proteins was performed as described previously (7). The stimulated CD4+ T cells were analyzed for specificity in ELISPOT assays at day 21.

Assessment of T-Cell Responses to Peptides and Tumor Cells. The recognition of APCs pulsed with peptides and tumor cells was assessed by ELISPOT assays specific for hu-IFN-γ and IL-5. For the ELISPOT assays, multiscreen HA plates (Millipore, Bedford, MA) were coated with 10 μg/ml of either mAb antihuman IFN-γ (1-D1K; Mabtech, Stockholm, Sweden) or mAb antihuman IL-5 (BD-PharMingen) in PBS (Life Technologies, Inc.) overnight at 4°C. Unbound mAb was removed by four successive washings with PBS. After the plates were blocked with RPMI-10% human serum (1 h at 37°C), CD4+ T cells were seeded in triplicate (10⁴ for bulk CD4+ T cells and 10³ for CD4+ T cell clones) in multiscreen HA plates. Nonirradiated T2.DR4 cells or L cells (7.5 × 10⁴) or melanoma cells (25 × 10³) were added. Synthetic peptides were then added at indicated concentrations into ELISPOT assays after APCs were seeded. Control wells contained unstimulated T cells, T cells in the presence of unloaded T2.DR4 cells, L cells, or tumor cells alone. Alternatively, 25 × 10³ protein-loaded DCs were added to 5 × 10³ CD4+ T-cell clones per well. Culture medium was AIM-V at a final volume of 200 μl/well. After incubation at 37°C in 5% CO₂ for either 40 h (IFN-γ ELISPOT assays) or 20 h (IL-5 ELISPOT assays), 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 4 h with either biotinylated mAb anti-hIFN-γ (7-B6-1; Mabtech) at 2 μg/ml or mAb anti-hIL-5 (JES1-5A10; BD-PharMingen) 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) or alternatively with 3,3',5'-tetramethylbenzidine (Vector Laboratories) 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 (15).

Statistical Analysis. For statistical analysis of the ELISPOT data, comparisons of CD4+ T-cell immunoreactivity were performed with one-sided Wilcoxon rank-sum tests (the Wilcoxon test is preferred to the *t* test for these comparisons because of the small sample sizes; Refs. 16, 17). For the comparison of one triplicate to another, where all of the counts in the first triplicate are larger than all of the counts in the second, the *P* from the Wilcoxon test is 0.05. *P* = 0.05 was considered significant and is indicated with an asterisk (*) in relevant figures.

RESULTS

NY-ESO-1 119–143 Peptide Binds to Multiple HLA-DR Molecules. We evaluated the binding capacities of multiple NY-ESO-1-derived peptides to 10 different HLA-DR molecules, including the 7 molecules encoded by the *HLA-DRB1* genes (*i.e.*, DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*1101, DRB1*1301, and DRB1*1501) and three molecules encoded by the *DRB3*, *DRB4*, and *DRB5* genes. All of these molecules are present in high frequency in the Caucasian population and hence encompass the majority of

Table 1 Binding capacities to immunopurified HLA-DR molecules NY-ESO-1 peptides

IC₅₀s are expressed in nm and have been evaluated from at least three independent experiments. The biotinylated peptides used at indicated concentrations in these assays are the following: HA 306–318 (PKYVKQNTLKLAT) for DR1 (1 nm), DR4 (30 nm), DR11 (40 nm), and DRB5 (10 nm); MT 2–16 (AKTIAYDEEARRGLE) for DR3 (200 nm); YKL (AAYAAKAAALAA) for DR7 (5 nm); B1 21–36 (TERVRLVTRHIYNREE) for DR13 (500 nm); A3 152–166 (EAEQLRRAYLDGTGVE) for DR15 (30 nm); Lo1 191–120 (ESWGAVWRIDTPDKLTGPFT) for DRB3 (10 nm); and E2/E168 (AGDLLAIETDKATI) for DRB4 (3 nm). IC₅₀s for the active peptides are <1000 nm and are indicated in *bold* (19).

Allele frequency	Alleles									
	DR1	DR3	DR4	DR7	DR11	DR13	DR15	DRB3	DRB5	DRB4
Peptides										
NY-ESO-1 119–143	20	>10,000	12	49	5	>10,000	40	800	43	125
NY-ESO-1 119–133	220	>10,000	13	63	1630	>10,000	400	1,830	8,330	1,830
NY-ESO-1 121–138	65	>10,000	20	13	38	>10,000	>10,000	3,000	600	>10,000
NY-ESO-123–137	18	>10,000	27	11	11	>10,000	>10,000	1,250	775	>10,000
NY-ESO-1 129–143	1,670	>10,000	1050	333	550	>10,000	8,000	>10,000	550	175

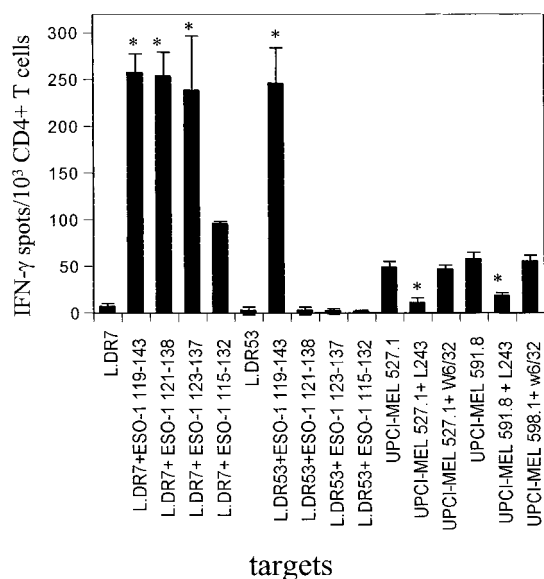


Fig. 1. Recognition of the NY-ESO-1 119–143 peptide and NY-ESO-1⁺ melanoma cell lines by CD4⁺ T cells of an HLA-DRB1*0701⁺/DRB4*0101⁺ normal donor. CD4⁺ T cells from an HLA-DRB1*0701⁺/DRB4*0101⁺ normal donor underwent three rounds of *in vitro* stimulation with autologous DCs pulsed with the NY-ESO-1 119–143 peptide as described in “Materials and Methods.” One thousand of the resulting responder CD4⁺ T cells were incubated in a 20-h IFN-γ ELISPOT assay in the presence of L.DR7 or L.DR53 cells pulsed with peptides NY-ESO-1 119–143, NY-ESO-1 121–138, NY-ESO-1 123–137, or NY-ESO-1 115–132 (10 μg/ml). The CD4⁺ T cells were also incubated in the presence of the HLA-DRB1*0701⁺/DRB4*0101⁺ melanoma cell line, MEL 591.8, and the HLA-DRB1*0701⁺/DRB4*0101⁺ melanoma cell line, MEL 527.1, +/- anti-HLA-DR (L243) antibodies or +/- anti-HLA-A, B, C (W6/32) antibodies. IFN-γ spots were developed and counted by computer-assisted video image analysis. Each column represents the mean spot number of triplicates ± SD (bars) with 10³ CD4⁺ T cells initially seeded per well (P values of 0.05 were considered significant and are indicated by *). Data from one representative experiment of three performed are depicted.

European and American individuals. Peptide sequences from NY-ESO-1 (*i.e.*, NY-ESO-1 119–143, NY-ESO-1 119–133, NY-ESO-1 121–138, and NY-ESO-1 123–137) were chosen as they are recognized by DR4-restricted melanoma-reactive T cells as reported previously (Table 1; Ref. 7). In agreement with these data, NY-ESO-1 119–143 binds well to DRB1*0401 (Table 1). Interestingly, peptide NY-ESO-1 119–143 was also able to bind very efficiently to multiple peptides produced by DR alleles, including DRB1*0101, DRB1*0701, DRB1*1101, DRB1*1501, DRB3*0101 (also known as DR52), DRB4*0101 (DR53), and DRB5*0101 (DR51). The binding activities of the shortest peptide sequences revealed that peptide NY-ESO-1 119–143 contains a number of binding determinants. For DR4 and DR7, peptides NY-ESO-1 121–138, NY-ESO-1 119–133, and NY-ESO-1 123–137 exhibit equivalent activity to peptide NY-ESO-1 119–143, whereas peptide NY-ESO-1 129–143 is less active. DR1 and DR11 accommodate mainly peptides NY-ESO-1 121–138 and NY-ESO-1 123–137, and only peptide NY-ESO-1 129–143 binds to DR53. Moreover, it was not possible to delineate the binding determinants with this set of short peptides for the DR15 and DRB5 molecules because none of them is as active as peptide NY-ESO-1 119–143. Altogether, peptide NY-ESO-1 119–143 appears extremely rich in HLA-DR-restricted determinants.

Peptide NY-ESO-119–143 Is Presented by Multiple HLA-DR Molecules to Stimulate Tumor-reactive CD4⁺ T Cells. In an independent series of *in vitro* experiments, we “primed” CD4⁺ T cells from three normal donors and one melanoma patient against peptides NY-ESO-1 119–143, NY-ESO-1 121–138, NY-ESO-1 123–137, and NY-ESO-1 115–132. Two were DRB1*0701⁺/DRB4*0101⁺ (donors 1 and 2), one was DRB1*1101/DRB1*1501⁺ (donor 3), and one was DRB1*0101/DRB1*0401⁺ (patient 4). “Mature” DCs were incubated

with each of the four 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 stimulation. After at least three restimulations, the immunoreactivities of the CD4⁺ T-cell cultures were analyzed in IFN-γ ELISPOT assays. As APCs in the IFN-γ ELISPOT assays, we used L cells, which have genetically been engineered to express either DR1 (L.DR1) or DR3 (L.DR3) or DR4 (L.DR4), DR7 (L.DR7), or DR53 (L.DR53). Alternatively, we also used the DRB1*1101⁺ homozygous EBV lymphoblastoid cell line, POER.

Bulk CD4⁺ T cells from donors 1 and 2 that were stimulated with the NY-ESO-1 119–143 peptide specifically recognized L.DR7 cells pulsed with NY-ESO-1 peptides 119–143, 121–138, 123–137, or 115–132 (the data obtained with the cells of donor 1 are presented in Fig. 1). These CD4⁺ T cells also displayed reactivity against the L.DR53 cells pulsed with peptide NY-ESO-1 119–143, but not with peptides NY-ESO-1 121–138, 123–137, or 115–132. These CD4⁺ T cells also displayed reactivity against the DR7-matched and the DR53-matched NY-ESO-1⁺ melanoma cell lines UPCI-MEL 591.8 and UPCI-MEL 527.1, respectively. This tumor reactivity was partially inhibited by the addition of anti-HLA-DR mAb (L243) but not by anti-HLA-A, B, C mAb. No immunoreactivity was observed in the presence of the NY-ESO-1⁻, HLA-DR53-matched tumor cell line, UPCI-136.1 (data not shown). Moreover, the CD4⁺ T cells pulsed with NY-ESO-1 peptides 121–138, 123–137, or 115–132 did not exhibit significant reactivity against L.DR53 cells pulsed with the relevant peptide. No IFN-γ ELISPOT reactivity developed in wells containing L.DR7 or L.DR53 cells in the absence of added CD4⁺ T cells (data not shown).

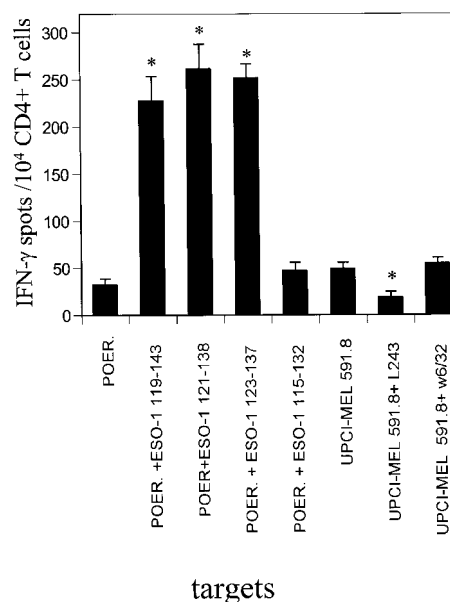


Fig. 2. Recognition of the NY-ESO-1 119–143 peptide and NY-ESO-1⁺ melanoma cell lines by CD4⁺ T cells of an HLA-DRB1*1101⁺ normal donor. CD4⁺ T cells from an HLA-DRB1*1101⁺ normal donor underwent three rounds of *in vitro* stimulation with autologous DCs pulsed with the NY-ESO-1 119–143 peptide, as described 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 DRB1*1101⁺ B-lymphoblastoid cells pulsed with peptides NY-ESO-1 119–143, NY-ESO-1 121–138, NY-ESO-1 123–137, or NY-ESO-1 115–132 peptide (10 μg/ml) and the NY-ESO-1⁺, DR11⁺ melanoma cell line, MEL 591.8, +/- anti-HLA-DR antibodies (L243) or +/- anti-HLA-A, B, C antibodies (w6/32). IFN-γ spots were developed and counted by computer-assisted video image analysis. Each column represents the mean spot number of triplicates ± SD (bars) with 10⁴ CD4⁺ T cells initially seeded per well (P = 0.05 was considered significant and is indicated by *). Data from one representative experiment of two performed are depicted.

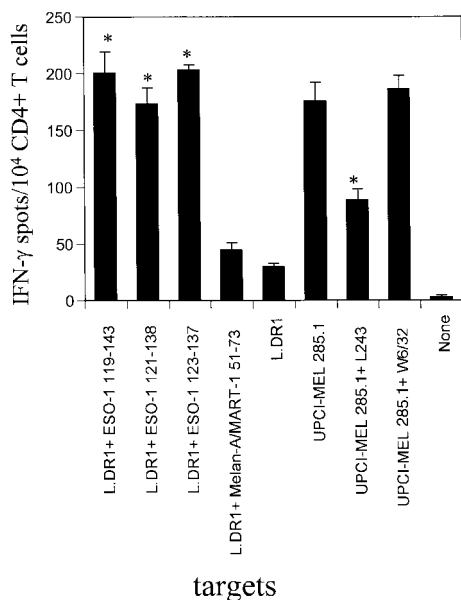


Fig. 3. Recognition of the NY-ESO-1 119–143 peptide and the autologous NY-ESO-1⁺ melanoma cell line by CD4⁺ T cells of an HLA-DRB1*0101⁺ melanoma patient. CD4⁺ T cells from a long-lived HLA-DRB1*0101 patient with melanoma, UPCI 285, underwent three rounds of *in vitro* stimulation with autologous DCs 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 L.DR1 cells pulsed with peptides NY-ESO-1 119–143, NY-ESO-1 121–138, NY-ESO-1 123–137, or Melan-A/MART-1 51–73 NY-ESO-1 123–137 or Melan-A/MART-1 51–73 (10 μg/ml) and the autologous melanoma cell line, MEL 285.1, +/- anti-HLA-DR antibodies (L243) or +/- anti-HLA-A, B, C antibodies (W6/32). IFN-γ spots were developed and counted by computer-assisted video image analysis. Each column represents the mean spot number of triplicates ± SD (bars) with 10⁴ CD4⁺ T cells initially seeded per well ($P = 0.05$ was considered significant and is indicated by *). Data from one representative experiment of two performed are depicted.

Bulk CD4⁺ T cells from donor 3 stimulated with the NY-ESO-1 119–143 peptide specifically recognized the DRB1*1101⁺ POER cells pulsed with peptides NY-ESO-1 119–143, 121–138, or 123–137, but not with peptide NY-ESO-1 115–132 (Fig. 2). These CD4⁺ T cells also recognized the DR11-matched NY-ESO-1⁺ melanoma cell line, UPCI-MEL 591.8.

Bulk CD4⁺ T cells from patient 4 stimulated with the NY-ESO-1 119–143 peptide specifically recognized L.DR1 cells pulsed with peptides NY-ESO-1 119–143, 121–138, or 123–137 (Fig. 3). These CD4⁺ T cells also displayed reactivity against the NY-ESO-1⁺ autologous melanoma cell line, UPCI-MEL 285.1; this reactivity was partially inhibited by the addition of anti-HLA-DR mAb (L243) but not anti-HLA-A, B, C mAb.

These data corroborate our binding data and demonstrate the ability of peptide NY-ESO-1 119–143 to stimulate tumor-reactive CD4⁺ T cells when presented at the surface of the DR1, DR7, DR11, and DR53 molecules.

DCs Loaded with Recombinant NY-ESO-1 Protein Stimulate CD4⁺ T Cells That Recognize the NY-ESO-1 119–143 Peptide from a DRB1*0701/DRB4*0101⁺ Normal Donor. Peripheral blood mononuclear cells from a DRB1*0701⁺/DRB4*0101⁺ normal donor (patient 2) were stimulated with DCs loaded with protein NY-ESO-1 (as indicated in “Material and Methods”). The responder cell cultures were then restimulated on a weekly basis with the irradiated autologous protein-fed DCs used in the primary stimulation. After three restimulations, the immunoreactivities of the T-cell cultures were analyzed in IFN-γ ELISPOT assays. T cells that were stimulated with the protein-pulsed DCs specifically recognized L.DR7 pulsed with peptide NY-ESO-1 119–143 (Fig. 4). These CD4⁺ T cells also displayed reactivity against L.DR7 cells pulsed with peptide NY-

ESO-1 121–138 or peptide NY-ESO-1 123–137 and against the HLA-DR53-matched melanoma cell line, UPCI-MEL 285.1; this reactivity was inhibited by the addition of anti-HLA-DR mAb (L243) but not anti-HLA-A, B, C mAb.

Bulk CD4⁺ T cells from donor 1 were obtained after three rounds of *in vitro* stimulation with autologous DCs pulsed with the NY-ESO-1 119–143 peptide. NY-ESO-1 protein-loaded DCs (1×10^5) were added to 5×10^3 CD4⁺ T cells per triplicate well in a 24-h IFN-γ ELISPOT assay. These CD4⁺ T cells recognized autologous DCs previously loaded with the NY-ESO-1 protein (Fig. 5). Unloaded DCs and the DCs fed with the recombinant SSX protein served as baseline and peptide controls, respectively.

Peptide NY-ESO-1 119–143 Stimulates Th1- and Th2-Type Tumor-reactive CD4⁺ T Cells. Patient UPCI-MEL 527 is a long-lived patient with melanoma with a high IgG serum antibody titer against the NY-ESO-1 protein. As reported previously, we have successfully derived both CD4⁺ T-cell clones and bulk CD4⁺ T cells from PBLs of patient UPCI-MEL 527 that produced IFN-γ in the presence of APCs pulsed with the NY-ESO-1 119–143 peptide as well as DR4⁺, NY-ESO-1⁺ melanoma cell lines. We tested the ability of these CD4⁺ T cells to also exhibit a Th2-type response. Clones 10/4 and 11/4 did not produce IL-5 in the presence of T2.DR4 pulsed with peptide NY-ESO-1 119–143 or the autologous melanoma cell line 527.1 and thus appeared to be Th1-type clones. However, bulk CD4⁺ T cells previously shown to specifically produce IFN-γ spots in the presence of NY-ESO-1 119–143 peptide-pulsed APCs or the autologous melanoma cells also produced IL-5 spots (Fig. 6). These data indicate the ability of peptide NY-ESO-1 119–143 to stimulate both Th1- and Th2-type CD4⁺ T-cell responses. Of note, peptide NY-ESO-1 119–143 was not recognized by anti-NY-ESO-1 serum antibodies; we failed to detect any reactivity in ELISA assays with serum of patient UPCI-MEL 527 (data not shown). However, we have detected a high level of antibody against the NY-ESO-1 protein

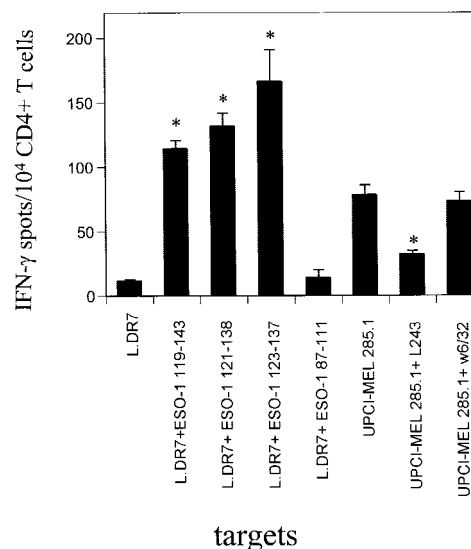


Fig. 4. CD4⁺ T cells from an HLA-DRB1*0701⁺/DRB4*0101⁺ normal donor stimulated with autologous DCs loaded with the NY-ESO-1 protein recognize the NY-ESO-1 119–143 peptide and the DRB4*0101-matched melanoma cell line, MEL 285.1. Total peripheral blood mononuclear cells from an HLA-DRB1*0701⁺/DRB4*0101⁺ normal 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 L.DR7 pulsed with peptides (10 μg/ml) and the DR53⁺, NY-ESO-1⁺ UPCI-MEL 285.1 cells. IFN-γ spots were developed and counted by computer-assisted video image analysis. Each column represents the mean spot number of triplicates ± SD (bars) with 10⁴ CD4⁺ T cells initially seeded per well ($P = 0.05$ was considered significant and is indicated by *).

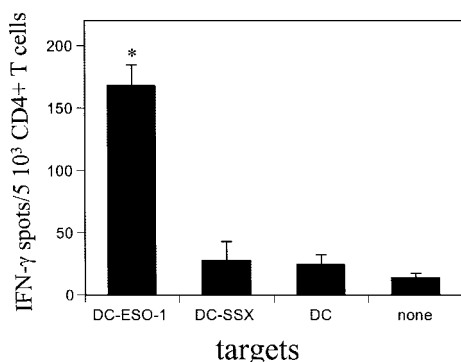


Fig. 5. CD4⁺ T cells from an HLA-DRB1*0701⁺/DRB4*0101⁺ normal donor recognize autologous DCs loaded with the ESO-1 protein. CD4⁺ T cells were isolated from the peripheral blood of an HLA-DRB1*0701⁺/DRB4*0101⁺ normal donor and stimulated *in vitro* with autologous DCs pulsed with the NY-ESO-1 119–143 peptide. Five thousand CD4⁺ T cells 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) as well as unloaded DCs. IFN-γ spots were developed and counted by computer-assisted video image analysis. Each column represents the mean spot number of triplicates ± SD (bars). $P = 0.05$ was considered significant and is indicated by *.

in the serum of this patient. Similar data were observed in a series of experiments done with PBLs of an HLA-DRB1*0101/DRB1*0401 patient with melanoma (UPCI-MEL 285) and two HLA-DRB1*0701/DRB1*1101 normal donors (data not shown).

DISCUSSION

Our strategy for the identification of promiscuous class II melanoma epitopes is based on extensive studies of binding to multiple DR molecules of peptides recognized by melanoma-reactive CD4⁺ T cells from the blood of normal individuals or long-lived patients with melanoma *in vitro*.

We demonstrated the good binding capabilities of peptide NY-ESO-1 119–143 to five molecules of the most commonly expressed alleles encoded by the *HLA-DRB1* genes (*i.e.*, DRB1*0101, DRB1*0401, DRB1*0701, DRB1*1101, and DRB1*1501). These molecules are expressed by nearly 46.1% of the American-Caucasian population. We have also shown that NY-ESO-1 119–143 binds well to the HLA allele molecules DRB3*0101, DRB4*0101, and DRB5*0101. These molecules have the advantage of being less polymorphic and occurring with high frequency in the Caucasian population (Table 1). Unlike peptide NY-ESO-1 119–143, the shortest sequences that we and others have reported previously (*i.e.*, NY-ESO-1 119–133, 121–138, and 123–137) exhibit little, if any, reactivity for the DR53 molecule and exhibit intermediate binding properties with DR15 and the B3 and B5 molecules. This may be explained by the existence of a peptide-binding sequence in the COOH-terminal amino acid portion of peptide NY-ESO-1 119–143 that is able to bind to DR53 and the B5 molecules. In this regard, our binding data show that the NY-ESO-1 129–143 peptide sequence has the capability to bind to the B3 and DR53 molecules. Several peptides capable of broadly binding to many class II allele molecules have been reported (13, 18–22). In the field of cancer immunology, HER2/neu and MAGE-3 have been shown to give rise to promiscuous DR epitopes (23, 24).

In a series of *in vitro* experiments with DCs and PBLs from melanoma patients and normal donors, we have confirmed the implications of the binding data and demonstrated the immunogenicity of peptide NY-ESO-1 119–143 at the surface of several DR molecules. In particular, we demonstrated the capability of peptide NY-ESO-1 119–143 to stimulate melanoma-reactive CD4⁺ T cells that recognized this peptide in the context of the HLA-DR4, -DR7, -DR11, and

-DR53 molecules. We also demonstrated that the CD4⁺ T cells from a DRB1*0701/DRB4*0101⁺ (DR53) normal donor, stimulated *in vitro* with DCs fed with the NY-ESO-1 protein, recognized NY-ESO-1 peptide at the surface of the DR7 and DR53 molecules. This indicates that peptide NY-ESO-1 119–143 contains epitopes that are processed and presented at the surface of multiple DR molecules. This set of experiments also confirmed the ability of these epitopes to stimulate melanoma-reactive CD4⁺ T cells when presented at the surface of these DR molecules.

We demonstrated the ability of the NY-ESO-1 119–143 peptide sequence to stimulate both Th1- and Th2-type CD4⁺ T cells. Both cell types have been shown to play an antitumor role *in vivo*. Adoptive transfer of either Th1 or Th2 T cells from Ovalbumin-specific T-cell receptor-transgenic mice induced tumor regression (25). Two different mechanisms may pertain to these subsets because Th1 cells were able to stimulate antitumor CTL responses, whereas Th2 cells induced tumor necrosis. In a recent report, the administration of Th1 and Th2 cell clones to a TAA (P 815 AB) induced tumor rejection, with quantitative rather than qualitative differences in the antitumor activities of both types of cells (26). In this study, both types of cells initiated CTL responses and required the presence of CD8⁺ T cells. The rejection of P815 tumors following adoptive transfer with either type of clone (Th1 or Th2) induced a strong immunological memory. Of note, tumor-specific shared antigens or cancer-testis antigens, such as NY-ESO-1, display an expression pattern very similar to the P815 AB antigen, which is an immunogenic TAA of murine mastocytoma cells. This may suggest that both Th1 and Th2 responses directed against NY-ESO-1-derived epitopes could be important in eliciting antitumor responses *in vivo*.

Both the immunogenicity of the NY-ESO-1 gene products and the pan-DR promiscuity of peptide NY-ESO-1 119–143 support its rel-

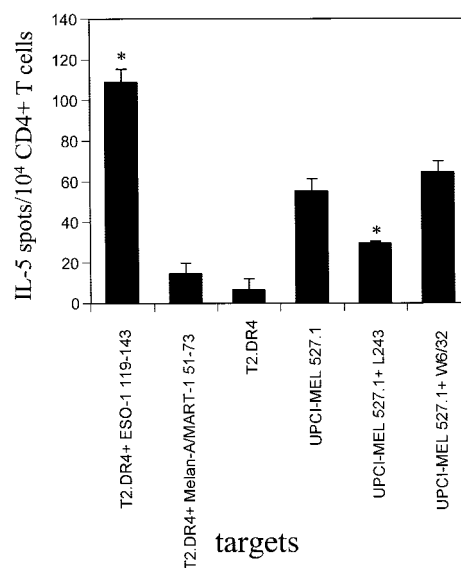


Fig. 6. Peptide NY-ESO-1 119–143 stimulates Th2-type CD4⁺ T cells of an HLA-DRB1*0401/DRB4*0101⁺ melanoma patient. CD4⁺ T cells from an HLA-DRB1*0401⁺/DRB4*0101⁺ melanoma patient underwent at least three rounds of *in vitro* stimulation with autologous DCs pulsed with the NY-ESO-1 119–143 peptide. Ten thousand of the resulting responder CD4⁺ T cells were incubated in a 48-h IL-5 ELISPOT assay in the presence of T2.DR4 cells pulsed with peptides NY-ESO-1 119–143 or Melan-A/MART-1 51–73 (10 μg/ml) and the autologous melanoma cells, MEL 527.1; MEL 527.1 cells +/- anti-HLA-DR antibodies (L243), or UPCI-MEL 527.1 cells +/- anti-HLA-A, B, C antibodies (W6/32). IL-5 spots were developed and counted by computer-assisted video image analysis. Each column represents the mean spot number of triplicates ± SD (bars) with 10⁴ CD4⁺ T cells initially seeded per well. Data from one representative experiment of three performed are depicted. $P = 0.05$ was considered significant and is indicated by *.

evance as a potential immunogen for cancer vaccines designed to treat a large number of patients with NY-ESO-1⁺ tumors. NY-ESO-1 is expressed by 50% of breast and prostate carcinomas and 30% of metastatic melanoma, non-small cell lung cancer, bladder, and head and neck tumors (27). Here, we demonstrated the ability of peptide NY-ESO-1 119–143 to be presented at the surface of multiple DR molecules expressed altogether by 91.6% of the Caucasian-American population. Therefore, peptide NY-ESO-1 119–143 will be clinically relevant in 45.8% of Caucasian patients with breast or prostate cancers and 27.5% of Caucasian patients with melanoma or lung and head and neck cancers.

Given the role of CD4⁺ T cells in maintaining CD8⁺ T-cell responses and the potential direct antitumor effector function of CD4⁺ T cells, peptide NY-ESO-1 119–143 is a reasonable vaccine candidate for the *in vivo* induction of both tumor-reactive CD4⁺ and CD8⁺ T cells.

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