

# One NY-ESO-1-Derived Epitope That Promiscuously Binds to Multiple HLA-DR and HLA-DP4 Molecules and Stimulates Autologous CD4<sup>+</sup> T Cells from Patients with NY-ESO-1-Expressing Melanoma<sup>1</sup>

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NY-ESO-1 is expressed by a broad range of human tumors and is often recognized by Abs in the sera of cancer patients with NY-ESO-1-expressing tumors. The NY-ESO-1 gene also encodes several MHC class I- and class II-restricted tumor epitopes recognized by T lymphocytes. In this study we report one novel pan-MHC class II-restricted peptide sequence, NY-ESO-1 87–111, that is capable of binding to multiple HLA-DR and HLA-DP4 molecules, including HLA-DRB1\*0101, 0401, 0701, and 1101 and HLA-DPB1\*0401 and 0402 molecules. We also demonstrate that peptide NY-ESO-1 87–111 stimulates Th1-type and Th-2/Th0-type CD4<sup>+</sup> T cells and clones when presented in the context of these HLA-DR and HLA-DP4 molecules. Both bulk CD4<sup>+</sup> T cells and CD4<sup>+</sup> T cell clones were capable of recognizing not only peptide-pulsed APCs, but also autologous dendritic cells, either loaded with the NY-ESO-1 protein or transfected with NY-ESO-1 cDNAs. Using IFN- $\gamma$  and IL-5 ELISPOT assays and PBL from patients with NY-ESO-1-expressing tumors, we observed the existence of Th1-type circulating CD4<sup>+</sup> T cells recognizing peptide NY-ESO-1 87–111 in the context of HLA-DP4 molecules. Taken together, these data represent the first report of an HLA-DR- and HLA-DP-restricted epitope from a tumor Ag. They also support the relevance of cancer vaccine trials with peptides NY-ESO-1 87–111 in the large number of cancer patients with NY-ESO-1-expressing tumors. *The Journal of Immunology*, 2005, 174: 1751–1759.

The NY-ESO-1 Ag is expressed by many tumors of different histological types (including breast, prostate, lung, and melanoma) and by male germline cells, but not by normal tissues (1). NY-ESO-1 encodes MHC class I- and class II-restricted peptides expressed by a diverse range of cancers and recognized by T cells (2–7). More recently, we have reported NY-ESO-1-derived peptides capable of binding to multiple HLA-DR molecules and of stimulating CD4<sup>+</sup> T cells (5). NY-ESO-1 appears to be very immunogenic, inducing both spontaneous CD8<sup>+</sup> T cell, CD4<sup>+</sup> T cell, and humoral responses in a large number of patients with NY-ESO-1<sup>+</sup> tumor (7, 8).

In this study we develop a strategy to identify pan-MHC class II peptide sequences, which can be broadly presented by multiple

HLA-DR alleles and HLA-DP4 to stimulate CD4<sup>+</sup> T cells. Our strategy is based on the combination of binding assays to multiple HLA-DR and HLA-DP4 molecules with in vitro stimulations with T cells and APCs from normal donors and melanoma patients. These pan-MHC class II peptides may be useful in vaccine trials for a large number of patients without prior HLA typing, because any patient will most likely express one or more allele molecules capable of presenting the peptide. In this report we apply this strategy to peptide NY-ESO-1 87–111. We demonstrate that peptide NY-ESO-1 87–111 not only binds to multiple HLA-DR and HLA-DP4 alleles, but also stimulates CD4<sup>+</sup> T cell responses when presented in the context of these molecules. We also observed that this peptide sequence stimulated circulating Th1-type CD4<sup>+</sup> T cells from PBL of melanoma patients. These findings support the use of the NY-ESO-1 87–111 peptide as a cancer vaccine for a large number of patients with NY-ESO-1-expressing tumors.

## Materials and Methods

### Cell lines, media, and Abs

The tissues and blood samples used for all studies reported in this manuscript were obtained under University of Pittsburgh Cancer Institute (UPCI) institutional review board approval. Patients UPCI-MEL 527 and UPCI-MEL 285 are long-lived patients who remain disease-free several years after successful therapy for disseminated NY-ESO-1-expressing metastatic melanoma. UPCI-MEL 527.1 and UPCI-MEL 285.1 cell lines were derived from one metastatic lesion of each patient. Patients UPCI-MEL 527 and UPCI-MEL 285 have been genotyped as HLA-DRB1\*0401<sup>+</sup>/DRB1\*1401<sup>+</sup>/DRB4\*0101<sup>+</sup>/DPB1\*0402<sup>+</sup> and HLA-DRB1\*0401<sup>+</sup>/DRB1\*0101<sup>+</sup>/DRB4\*0101<sup>+</sup>/DPB1\*0401<sup>+</sup>, respectively. HLA-DR and HLA-DP genotyping of melanoma patients and normal donors was performed using commercial typing panels of PCR primers according to the manufacturer's instructions (Dynal Biotech). Normal donor 1 (ND1) has

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<sup>3</sup> Abbreviations used in this paper: ORF, open reading frame; DC, dendritic cell.

been genotyped as HLA-DRB1\*0701<sup>+</sup>/DRB1\*1101<sup>+</sup>/DRB4\*0101<sup>+</sup>/DPB1\*0401<sup>+</sup>, respectively. HLA-DRB1\*0401-transfected T2 cells, i.e., T2.DR4 cells, and HLA-DR-transfected mouse cells, i.e., L.DR cells, were previously described (5, 9). All cell lines were cultured in RPMI 1640 medium (Invitrogen Life Technologies) 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 L243 anti-HLA-DR (class II) mAb and W6/32 anti-HLA-A, -B, and -C (class I) mAb, respectively, were purchased from American Type Culture Collection. The B7/21 anti-HLA-DP (class II) mAb used in our blocking experiments was previously described (10).

#### Peptide synthesis

The NY-ESO-1-derived peptides were synthesized and stored as previously reported (5, 6). Synthesis of NY-ESO-1 peptides was based on the sequence of a NY-ESO-1 gene reported by Chen et al. (1) (GenBank accession no. U87459).

#### Recombinant proteins

The full-length NY-ESO-1 recombinant protein was produced in *Escherichia coli* and was provided by Drs. L. J. Old and G. Ritter (Ludwig Institute for Cancer Research, New York, NY). The LAGE-1 open reading frame 2 (ORF2) protein was also produced in *E. coli* as previously described (11).

#### Purification of HLA-DR molecules

HLA-DR molecules were purified from homozygous EBV cell lines by affinity chromatography using the monomorphic mAb L 243 coupled to protein A-Sepharose CL 4B gel (Amersham Biosciences) as previously described (12, 13).

#### HLA-DR peptide binding assays

Binding to multiple HLA-DR and HLA-DP4 molecules was performed as previously reported (12–14). Maximal binding was determined by incu-

bating the biotinylated peptide with the MHC class II molecule in the absence of competitor. Binding specificity for each HLA-DR and HLA-DP4 molecule was ensured by the choice of biotinylated peptides as described previously (13, 14). Data were expressed as the concentration of peptide that prevented binding of 50% of the labeled peptide (IC<sub>50</sub>).

#### Induction of CD4<sup>+</sup> T cells with peptides

The induction of CD4<sup>+</sup> T cells and clones in vitro with the NY-ESO-1-derived peptides was performed as previously reported (6, 15).

#### In vitro sensitization with peptides

PBL from patients with active melanoma, long-term survivors, and normal donors (Table I) were used to purify CD8 and CD4<sup>+</sup> T cells with immunomagnetic beads (Miltenyi Biotec). Two million non-CD8/CD4<sup>+</sup> T cells were pulsed with 10 μg of peptide (4 h, 37°C), irradiated (3000 rad), washed, and used to stimulate 2 × 10<sup>6</sup> autologous CD4<sup>+</sup> T cells in Iscove's medium supplemented with 10% human AB serum (Sigma-Aldrich) without any cytokine. On day 7 of in vitro stimulation, the responder CD4<sup>+</sup> T cells were harvested and analyzed in ELISPOT assays.

#### IFN-γ and IL-5 ELISPOT assays

The recognition of APC pulsed with peptides, proteins, and tumor cells was assessed by ELISPOT assays specific for human IFN-γ and IL-5 as previously reported (5, 6). Alternatively, 5000 protein-loaded or cDNA-transfected dendritic cells (DCs) were added to 10<sup>3</sup> CD4<sup>+</sup> T cell clones/well. The protein-loaded DCs were prepared as previously reported in the presence of the recombinant NY-ESO-1 ORF1 or LAGE-1 ORF2 proteins (30 μg/ml). Mature DCs were transfected with either pcDNA3-NY-ESO-1 (provided by Drs. E. Jager and A. Knuth (Department of Oncology, Krankenhaus Nordwest, Frankfurt, Germany)) or pcDNA3-MART-1 using human Nucleofactor (Amaxa). Shortly, 1 × 10<sup>6</sup> DCs were transfected with 5 μg of plasmid DNA in 100 μl of human DC Nucleofector solution. Electroporation was performed using the Nucleofector device (Amaxa). DCs were incubated, then collected and used for ELISPOT assays. In the blocking experiments, 3 μg of each purified anti-HLA mAb was added to

Table I. HLA-DP4 typing, NY-ESO1 Ab status, and stage of disease of the patients evaluated in this study<sup>a</sup>

Patients number	Sex	HLA-DPB1*0401/0402	Primary Melanoma	Stage of Disease	NY-ESO-1 Ab
<b>Melanoma patients</b>					
1	M	+	Unknown	IV	+++
2	F	+	Unknown	III C	+++
3	F	–	Mucosal	NED	–
4	F	+	Unknown	IV	–
5	M	+	IV	IV	–
6	M	+	Ocular Melanoma	IV	–
7	F	+	Mucosal	IV	+
8	F	+	Unknown	IV	–
9	F	–	III A	IV	–
10	F	–	III	III	–
11	M	+	IV	IV	–
12	M	–	III A	IV	–
13	F	+	Ocular Melanoma	IV	–
14	M	–	II B	IV	–
15	M	+	II A	IV	++
16	M	–	I B	NED	–
17	M	+	IV	IV	–
18	M	–	III A	IV	+++
19	M	–	I B	NED	–
20	F	–	I B	NED	–
21	F	+	III	NED	–
<b>Long-term survivors</b>					
22	F	–	IV	NED	++
23	M	+	IV	NED	–
24	F	+	IV	NED	++
25	F	–	IV	NED	–
<b>Normal donors</b>					
26	NA	+			–
27	NA	+			–
28	NA	+			–
29	NA	+			–

<sup>a</sup> NED, no evidence of disease; NA, not available.

each ELISPOT well. Spot numbers and spot sizes were determined with the use of computer-assisted video image analysis as previously described (16). For statistical evaluation, a *t* test for unpaired samples was used. A value of *p* < 0.05 was considered significant.

#### IFN- $\gamma$ and IL-4 cytokine secretion assays

The recognition of autologous DCs pulsed with peptides (10  $\mu$ g/ml) or proteins (30  $\mu$ g/ml) was also assessed by MACS secretion assays for IFN- $\gamma$  and IL-4 (Miltenyi Biotec) as previously described (11).

## Results

### Peptide NY-ESO-1 87–111 binds to multiple HLA-DR and HLA-DP4 molecules

We evaluated the binding capacities of peptide NY-ESO-1 87–111 (LLEFYLAMPEATPMEAEELARRSLAQ) as well as peptide NY-ESO-1 158–180 (LLMWITQCFLPVFLAQPPSGQRR) to 10 different HLA-DR molecules, including seven molecules encoded by the HLA-DRB1 genes (i.e., HLA-DRB1\*0101, -DRB1\*0301, -DRB1\*0401, -DRB1\*0701, -DRB1\*1101, -DRB1\*1301, and -DRB1\*1501); three molecules encoded by the DRB3, DRB4, and DRB5 genes; and two molecules encoded by HLA-DPB1\*0401 and 0402 (Table II). All these molecules are present at high frequencies in the Caucasian population. These peptide sequences have been chosen for analysis because they were previously predicted to contain HLA-DRB1\*0401-binding sequences (6), as well as anchor residues to bind to the HLA-DPB1\*0401 and 0402 molecules (residues are underlined).

We observed that the HLA-DPB1\*0401-restricted sequence, NY-ESO-1 158–180, which includes the HLA-DP4-presented epitope NY-ESO-1 157–170 (4), binds efficiently to HLA-DRB1\*0401 and HLA-DRB4\*0101. However, it poorly binds to other HLA-DR molecules.

In sharp contrast, peptide NY-ESO-1 87–111 binds not only to HLA-DRB1\*0401, but also to HLA-DRB1\*0101, -DRB1\*0701, -DRB1\*1101, -DRB1\*1501, and -DRB5\*0101. Importantly, peptide NY-ESO-1 87–111 also binds well to HLA-DPB1\*0401 and -0402. NY-ESO-1 87–111 contains at least one HLA-DPB1\*0401/0402-binding motif, i.e., NY-ESO-1 91–99 (YLAMPFATP), that is most likely responsible for the DP4-binding activity of the peptide. This is confirmed by our binding data, which show that peptide sequence NY-ESO-1 87–101, but not 97–111 or 92–105, was capable of binding to HLA-DPB1\*0401 and -0402. Moreover, peptide NY-ESO-1 87–101, but not 97–111 or 92–105, appears to encompass the promiscuous HLA-DR-binding region contained in the peptide NY-ESO-1 87–111. The IC<sub>50</sub> values of peptide NY-ESO-1 87–101 were very similar to those of peptide NY-ESO-1 87–111, except for HLA-DR15. Overall, the NY-ESO-1 87–111 peptide exhibits broad binding capacity to multiple HLA-DR molecules as well as to the HLA-DPB1\*0401 and -0402 molecules.

### Peptide NY-ESO-1 87–111 is presented by multiple HLA-DR and HLA-DPB1\*0401 and -0402 molecules and stimulates tumor-reactive CD4<sup>+</sup> T cells

In an independent series of in vitro experiments, we primed CD4<sup>+</sup> T cells from one normal donor and two melanoma patients against peptide NY-ESO-1 87–111. Melanoma patients and normal donor were typed as HLA-DRB1\*0401<sup>+</sup>/DRB1\*1401<sup>+</sup>/DRB4\*0101<sup>+</sup>/DPB1\*0402<sup>+</sup> (patient 1), DRB1\*0101<sup>+</sup>/DRB1\*0401<sup>+</sup>/DRB4\*0101<sup>+</sup>/DPB1\*0401<sup>+</sup> (patient 2), and DRB1\*0701<sup>+</sup>/DRB1\*1101<sup>+</sup>/DRB4\*0101<sup>+</sup>/DPB1\*0401<sup>+</sup> (donor 1), respectively. Mature DCs were incubated with peptide NY-ESO-1 87–111 (10  $\mu$ g/ml), irradiated, and used to stimulate autologous CD4<sup>+</sup> T cells, as previously described (6, 15). The CD4<sup>+</sup> T cells were restimulated on a weekly basis with irradiated autologous mature DCs pulsed with peptide. After at least three restimulations, the immunoreactivity of the CD4<sup>+</sup> T cell cultures was analyzed in IFN- $\gamma$  ELISPOT assays. We used as APCs in IFN- $\gamma$  ELISPOT assays, L cells that have been genetically engineered to express HLA-DR1 (L.DR1), HLA-DR4 (L.DR4), HLA-DR7 (L.DR7), HLA-DR 53 (L.DR53), or HLA-DP4 (L.DP4).

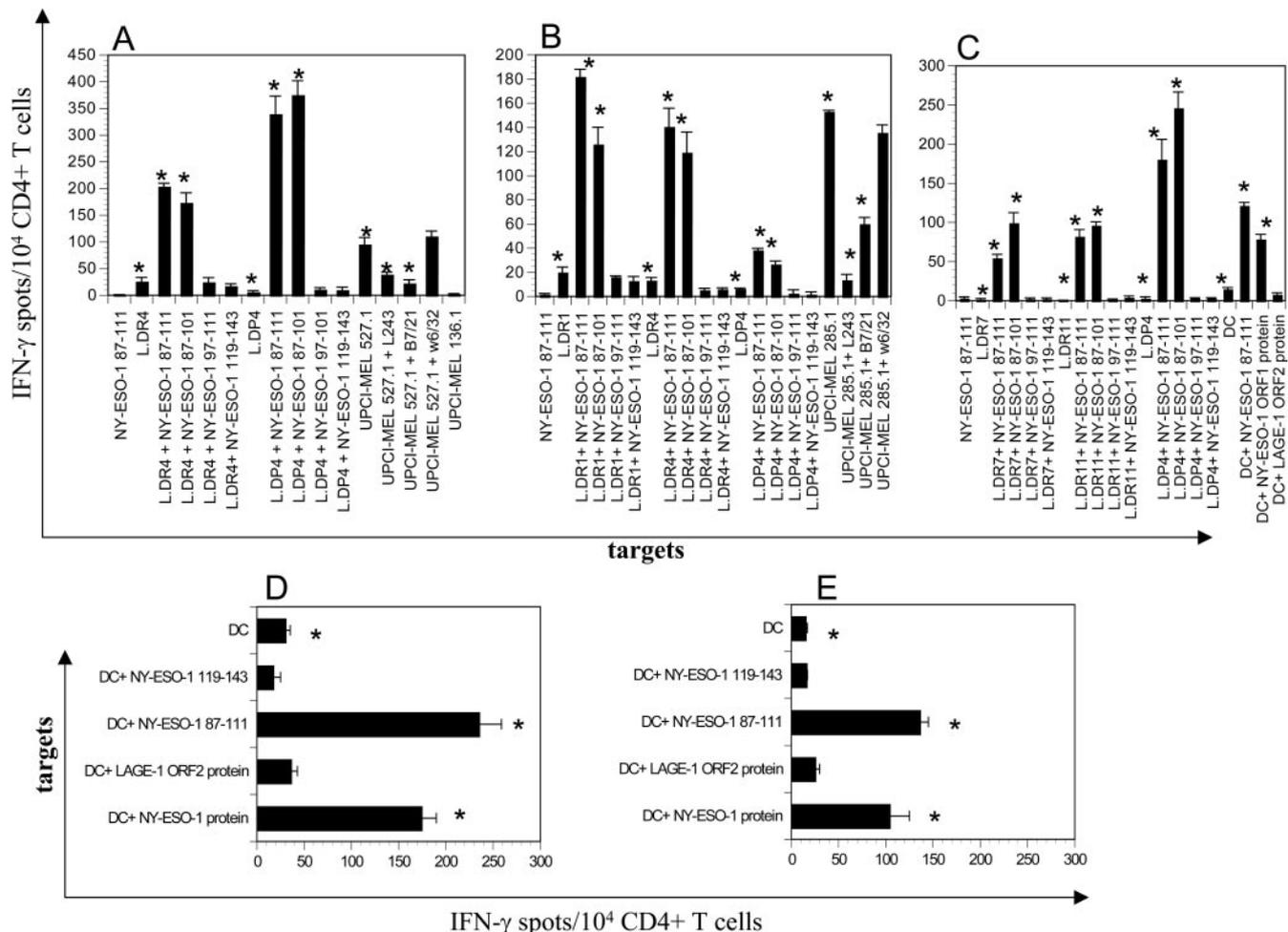
Bulk CD4<sup>+</sup> T cells isolated from patient 1 stimulated with NY-ESO-1 87–111 peptide specifically recognized L.DR4 cells and L.DP4 cells pulsed with peptides NY-ESO-1 87–111 or NY-ESO-1 87–101, but not NY-ESO-1 97–111 (Fig. 1A). These CD4<sup>+</sup> T cells also displayed reactivity against the NY-ESO-1<sup>+</sup> autologous melanoma cell line, UPCI-MEL527.1, that was partially inhibited by addition of the anti-HLA-DR mAb (L243) or anti-HLA-DP mAb (B7/21), but not the anti-HLA-A, -B, or -C mAb. No significant reactivity was noted against an HLA-DRB1\*0401<sup>+</sup>, NY-ESO-1<sup>-</sup> melanoma cell line, UPCI-MEL 136.1. Of note, the CD4<sup>+</sup> T cells produced IFN- $\gamma$  not only in the presence of NY-ESO-1 87–111 peptide-pulsed DCs, but also in the presence of autologous DCs loaded with the NY-ESO-1 protein (Fig. 1B). No significant amount of IFN- $\gamma$  was produced by CD4<sup>+</sup> T cells in the presence of peptide NY-ESO-1 87–111 alone. The autologous DCs pulsed with an irrelevant peptide or protein were not recognized. L.DR cells expressing allogenic HLA-DR molecules (i.e., L.DR1, L.DR7, and L.DR11) and pulsed with peptide NY-ESO-1 87–111 were not recognized (data not shown).

Bulk CD4<sup>+</sup> T cells isolated from patient 2 stimulated with peptide NY-ESO-1 87–111 peptide specifically recognized L.DR1 cells, L.DR4 cells, or L.DP4 cells pulsed with peptide NY-ESO-1 87–111 or NY-ESO-1 87–101, but not peptide NY-ESO-1 97–111 (Fig. 1C). These CD4<sup>+</sup> T cells also recognized the autologous NY-ESO-1<sup>+</sup> melanoma cell line, UPCI-MEL 285.1, that was partially inhibited by addition of anti-HLA-DR mAb (L243) or anti-HLA-DP mAb (B7/21), but not anti-HLA-A, B, C mAb.

Table II. Binding capacities to immunopurified HLA II molecules<sup>a</sup>

Peptides	HLA II Alleles											
	HLA-DR B1*0101	HLA-DR B1*0301	HLA-DR B1*0401	HLA-DR B1*0701	HLA-DR B1*1101	HLA-DR B1*1301	HLA-DR B1*1501	HLA-DR B3*0101	HLA-DR B5*0101	HLA-DR B4*0101	HLA-DP B1*0401	HLA-DP B1*0402
Frequency	17.7	20.6	10.9	26	17.6	11.6	15.4	17.6	15.2	48.2	64	20.8
Reference	3	43	32	28	15	1400	33	8	12	4	13	13
NY-ESO-1 158–180	>10,000	>10,000	333	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	35	25	64
NY-ESO-1 87–111	9	>10,000	85	15	60	>10,000	275	4,000	7	1250	186	87
NY-ESO-1 87–101	1	ND	98	25	130	ND	2,687	ND	10	ND	219	66
NY-ESO-1 92–105	251	ND	6,000	>10,000	7,000	ND	17,321	ND	9,000	ND	7,483	2,468
NY-ESO-1 97–111	593	ND	15,000	>10,000	600	ND	16,733	ND	700	ND	>100,000	26,000

<sup>a</sup> ND, not done. Peptide binding capacities were evaluated in competitive ELISA and were expressed as IC<sub>50</sub> in nanomoles. A reference peptide was used to validate each assay. This peptide is the nonbiotinylated form of the biotinylated probe used in the assay. IC<sub>50</sub> values did not vary by more than a factor of 3. Active peptides have an IC<sub>50</sub> inferior to 1000 nM.



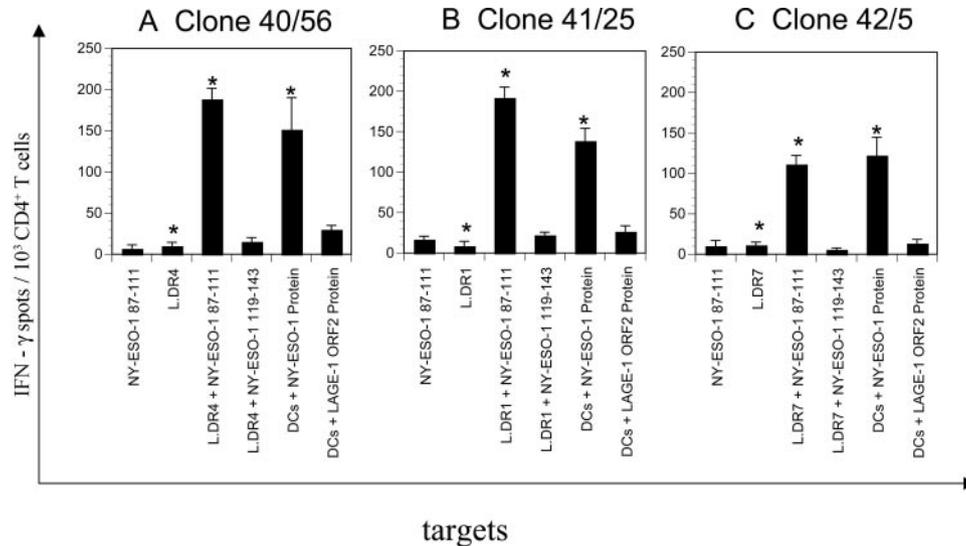
**FIGURE 1.** Recognition of peptide NY-ESO-1 87–111 and the autologous NY-ESO-1 protein-loaded DCs by Th1-type CD4<sup>+</sup> T cells from melanoma patients and a normal donor. CD4<sup>+</sup> T cells isolated from: *A* and *B*, an HLA-DRB1\*0401<sup>+</sup>/DRB1\*1401<sup>+</sup>/DRB4\*0101<sup>+</sup>/DPB1\*0402<sup>+</sup> melanoma patient (UPCI-MEL 527); *C* and *D*, an HLA-DRB1\*0101<sup>+</sup>/DRB1\*0401<sup>+</sup>/DPB1\*0401<sup>+</sup> melanoma patient (UPCI-MEL 285); or *E*, an HLA-DRB1\*0701<sup>+</sup>/DRB1\*1101<sup>+</sup>/DPB1\*0401<sup>+</sup> normal donor underwent three rounds of *in vitro* stimulation with autologous DC pulsed with peptide NY-ESO-1 87–111 as described in *Materials and Methods*. Ten thousand of the resulting responder CD4<sup>+</sup> T cells were incubated in a 48-h IFN- $\gamma$  assay in the presence of L.DR1, LDR4, L.DR7, L.DR11, or L.DP4 cells pulsed with peptide NY-ESO-1 87–111, NY-ESO-1 87–101, or NY-ESO-1 97–111 (10  $\mu$ g/ml). The CD4<sup>+</sup> T cells were also incubated in the presence of the autologous melanoma cell line UPCI-MEL 527.1 or UPCI-MEL 285.1 with or without anti-HLA-DR Abs (L243); UPCI-MEL 527.1 or UPCI-MEL 285.1 with or without anti-HLA-DP Abs (B7/21); or UPCI-MEL 527.1 or UPCI-MEL 285.1 cells with or without anti-HLA-A, -B, and -C Abs (W6/32). Alternatively, in *C–E*, CD4<sup>+</sup> T cells were incubated in the presence of autologous DCs loaded with either the NY-ESO-1 protein or the LAGE-1 ORF2 protein. The HLA-DRB1\*0401<sup>+</sup>, NY-ESO-1<sup>-</sup> melanoma cell line UPCI-MEL 136.1 was used as a negative control in *A*. IFN- $\gamma$  spots were developed and counted by computer-assisted video image analysis. Each bar represents the mean spot number of triplicate determinations  $\pm$  SD, with 10<sup>4</sup> CD4<sup>+</sup> T cells initially seeded per well (\*,  $p < 0.05$  was considered significant). Data from one representative experiment of three performed are depicted.

Interestingly, the CD4<sup>+</sup> T cells produced IFN- $\gamma$  not only in the presence of NY-ESO-1 87–111 peptide-pulsed DCs, but also in the presence of autologous DCs loaded with the NY-ESO-1 protein, as shown in Fig. 1*D*. No significant amount of IFN- $\gamma$  was produced by CD4<sup>+</sup> T cells in the presence of peptide NY-ESO-1 87–111 alone. The autologous DCs pulsed with an irrelevant peptide or protein were not recognized. L.DR cells expressing allogenic HLA-DR molecules (i.e., LDR7 and L.DR11) and pulsed with peptide NY-ESO-1 87–111 were not recognized (data not shown).

Bulk CD4<sup>+</sup> T cells isolated from donor 1 previously stimulated with the NY-ESO-1 87–111 peptide specifically recognized L.DR7 cells, L.DR11 cells, or L.DP4 cells pulsed with peptides NY-ESO-1 87–111 or NY-ESO-1 87–101 (Fig. 1*E*). CD4<sup>+</sup> T cells failed to produce a significant amount of IFN- $\gamma$  in the presence of APCs pulsed with either NY-ESO-1 97–111 or NY-ESO-1 119–

143, which was used as an irrelevant peptide. Importantly, these CD4<sup>+</sup> T cells recognized autologous DCs loaded with the NY-ESO-1 ORF1 protein, but not with an irrelevant protein, LAGE-1 ORF2 (11). No significant amount of IFN- $\gamma$  was produced by CD4<sup>+</sup> T cells in the presence of peptide NY-ESO-1 87–111 alone. L.DR cells expressing allogenic HLA-DR molecules (i.e., L.DR1 and L.DR4) and pulsed with peptide NY-ESO-1 87–111 were not recognized (data not shown).

From the NY-ESO-1 87–111-specific bulk CD4<sup>+</sup> T cells presented in Fig. 1, we have generated CD4<sup>+</sup> T cell clones by limiting dilution. Clone 40/56 was obtained from the NY-ESO-1 87–111-specific CD4<sup>+</sup> T cells from patient 1 and was capable of producing IFN- $\gamma$  in the presence of not only L.DR4 cells pulsed with peptide NY-ESO-1 87–111, but also autologous DCs previously loaded with the NY-ESO-1 protein (Fig. 2*A*). Of note, no significant amount of IFN- $\gamma$  production was observed in the presence of



**FIGURE 2.** Peptide NY-ESO-1 87–111 stimulates HLA-DR-restricted CD4<sup>+</sup> T cell clones. The CD4<sup>+</sup> T cell clones 40/56, 41/25, and 42/5 were obtained by limiting dilution from the NY-ESO-1 87–111-specific bulk CD4<sup>+</sup> T cells of patient 1, donor 1, and patient 2, respectively. One thousand CD4<sup>+</sup> T cells from clone 40/56 (A), clone 41/25 (B), or from clone 42/5 (C) were incubated in a 24-h IFN- $\gamma$  assay in the presence of peptide NY-ESO-1 87–111 alone (10  $\mu$ g/ml), L.DR cells pulsed with either peptide NY-ESO-1 87–111 (10  $\mu$ g/ml) or peptide NY-ESO-1 119–143 (10  $\mu$ g/ml), and autologous DCs previously loaded with protein NY-ESO-1 ORF1 (30  $\mu$ g/ml) or protein LAGE-1 ORF2 (30  $\mu$ g/ml) as previously described in *Materials and Methods*. IFN- $\gamma$  spots were developed and counted by computer-assisted video image analysis. Each bar represents the mean spot number of triplicate determinations  $\pm$  SD with 10<sup>3</sup> CD4<sup>+</sup> T cells initially seeded per well (\*,  $p < 0.05$  was considered significant).

peptide NY-ESO-1 87–111 alone, autologous DCs alone, DCs fed with an irrelevant protein (Fig. 2A), or other L.DR cells pulsed with peptides (data not shown).

Clone 41/25 was obtained by limiting dilution from the NY-ESO-1 87–111-specific CD4<sup>+</sup> T cells from donor 1 and produced IFN- $\gamma$  in the presence of not only L.DR1 cells pulsed with peptide NY-ESO-1 87–111, but also autologous DCs fed with the NY-ESO-1 protein (Fig. 2B). No significant amount of IFN- $\gamma$  production was observed in the presence of peptide NY-ESO-1 87–111 alone, DCs alone, autologous DCs fed with an irrelevant protein (Fig. 2B), or other L.DR cells pulsed with peptides (data not shown).

Clone 42/5 was obtained from the NY-ESO-1 87–111-specific CD4<sup>+</sup> T cells from patient 2 and produced IFN- $\gamma$  not only in the presence of L.DR7 cells pulsed with peptide NY-ESO-1 87–111, but also autologous DCs fed with the NY-ESO-1 protein (Fig. 2B). No significant amount of IFN- $\gamma$  production was observed in the presence of peptide NY-ESO-1 87–111 alone, DCs alone, autologous DCs fed with an irrelevant protein (Fig. 2C), or other L.DR cells pulsed with peptides (data not shown).

These data corroborate our binding data and demonstrate the ability of peptide NY-ESO-1 87–111 to stimulate CD4<sup>+</sup> T cells and CD4<sup>+</sup> T cell clones capable of recognizing peptide NY-ESO-1 87–111 in the context of the HLA-DRB1\*0101, -DRB1\*0701, and -DRB1\*1101 and HLA-DPB1\*0401 and -0402 molecules. These bulk CD4<sup>+</sup> T cells were also determined to be tumor reactive and capable of recognizing autologous DCs loaded with the relevant protein.

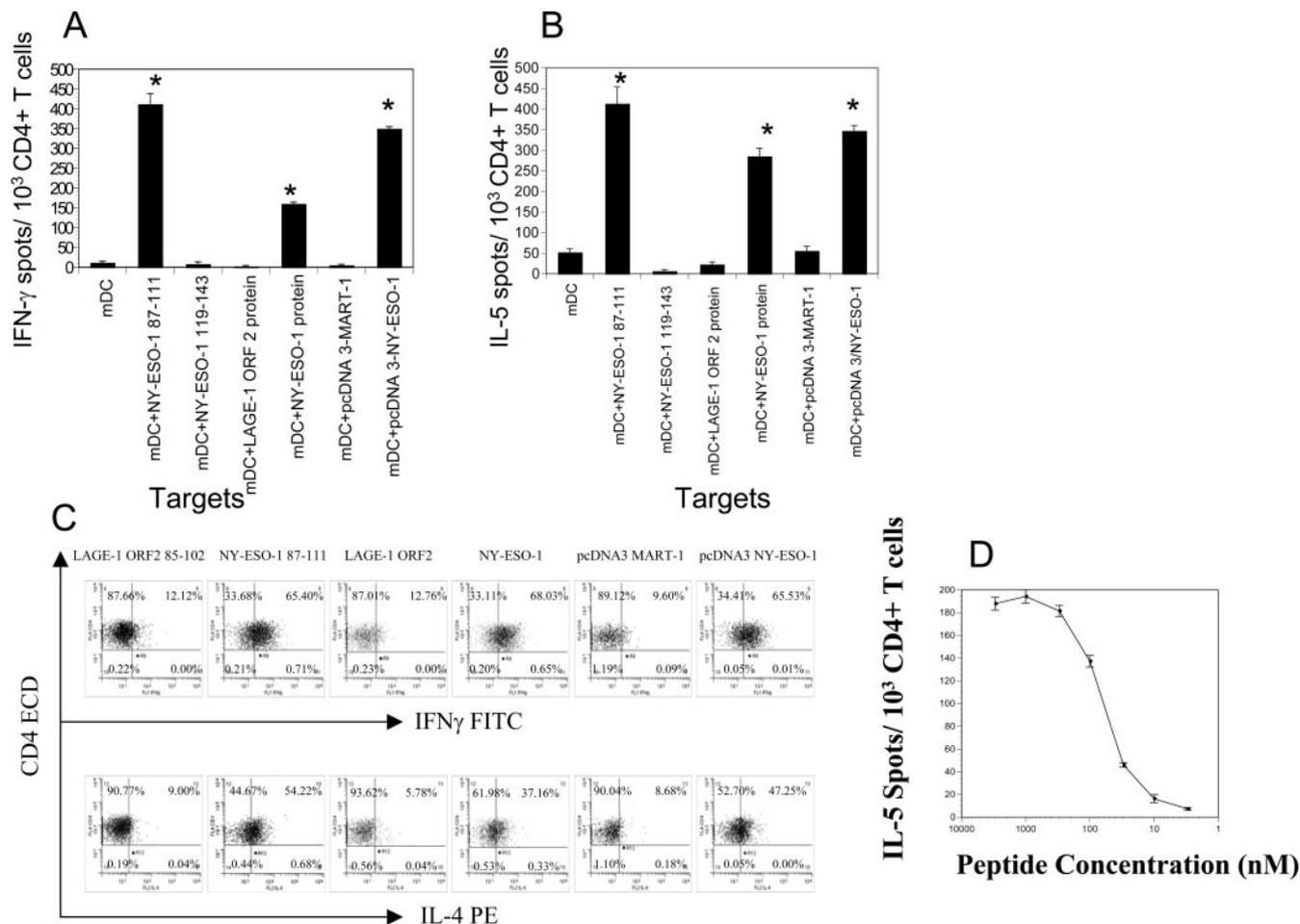
*Peptide NY-ESO-1 87–111 stimulates Th0 and Th2-type CD4<sup>+</sup> T cell clones isolated from PBL of patients with melanoma that are capable of recognizing autologous protein-loaded DCs*

Several CD4<sup>+</sup> T cell clones were obtained by limiting dilution from the blood of patient UPCI-MEL527 after in vitro stimulation with peptide NY-ESO-1 87–111. One representative clone, 40/1, specifically produced IL-4, IL-5, and IFN- $\gamma$  in the presence of autologous DCs pulsed with peptide NY-ESO-1 87–111, loaded

with the NY-ESO-1 ORF1 protein, or transfected with NY-ESO-1 cDNA in ELISPOT and cytokine release assays as described in *Materials and Methods* (Fig. 3, A–C). Unloaded DCs, DCs pulsed with an irrelevant peptide (i.e., NY-ESO-1 119–143 or LAGE-1 ORF2 85–102), and DCs fed with the LAGE-1 ORF2 protein served as baseline and controls. Clone 40/1 did not recognize the autologous tumor cell line, UPCI-MEL 527.1 (data not shown). Of note, the same CD4<sup>+</sup> T cell clone produced only IL-5 spots in the presence of L.DP4 cells pulsed with peptide NY-ESO-1 87–111 (Fig. 3D). No IL-5 spots were produced in the presence of L.DR cells pulsed with peptide NY-ESO-1 87–111 (data not shown). The ability of CD4<sup>+</sup> T cell clone 40/1 to produce IL-5 in the presence of L.DP4 cells, preincubated with the NY-ESO-1 87–111 peptide at various concentrations, was evaluated to determine the peptide-dose threshold for effector T cell recognition. The half-maximal stimulation of clone 40/1 required peptide loading concentrations of  $\sim$ 50–60 nM (Fig. 3D).

An additional CD4<sup>+</sup> T cell clone, 40/10, produced both IFN- $\gamma$  and IL-5 in ELISPOT assays in the presence of L.DP4 cells or autologous DCs pulsed with peptide NY-ESO-1 87–111. Clone 40/10 also produced IFN- $\gamma$  and IL-5 in the presence of autologous DCs loaded with the NY-ESO-1 ORF1 protein or transfected with NY-ESO-1 cDNA (Fig. 4, A and B). IFN- $\gamma$  and IL-5 production by clone 40/1 was inhibited by addition of anti-HLA-DP mAb (B7/21), but not anti-HLA-DR mAb (L243). The half-maximal stimulation of clone 40/10 required peptide loading concentrations of  $\sim$ 20 nM in IFN- $\gamma$  ELISPOT assays (Fig. 4C). IL-5 production by clone 40/10 in the presence of titrated doses of peptide NY-ESO-1 87–111 is represented as a dome-shaped curve with maximal production of IL-5 at peptide concentrations ranging from 20–200 nM (Fig. 4D).

Both CD4<sup>+</sup> T cell clones 40/1 and 40/10 recognized peptide NY-ESO-1 87–111 and peptide NY-ESO-1 87–101, but not peptide NY-ESO-1 92–105 or peptide NY-ESO-1 97–111 (data not shown). These data corroborate our binding data (Table II) and our ELISPOT data with NY-ESO-1 87–111-specific bulk CD4<sup>+</sup> T cells (Fig. 1).



**FIGURE 3.** Peptide NY-ESO-1 87–111 stimulates Th2/Th0-type CD4<sup>+</sup> T cell clone 40/1, the phenotype of which depends on the type of APCs used in the assay. CD4<sup>+</sup> T cell clone 40/1 was obtained by limiting dilution from the NY-ESO-1 87–111-specific bulk CD4<sup>+</sup> T cells of patient UPCI-MEL 527 as described in *Materials and Methods*. One thousand CD4<sup>+</sup> T cells from clone 40/1 were incubated in an IFN- $\gamma$  ELISPOT assay (A), an IL-5 ELISPOT assay (B), or an IFN- $\gamma$  and IL-4 cytokine release assay (C) in the presence of autologous DCs pulsed with peptide NY-ESO-1 87–111 (10  $\mu$ g/ml), peptide NY-ESO-1 119–143 (10  $\mu$ g/ml), protein NY-ESO-1 ORF1 (30  $\mu$ g/ml), or protein LAGE-1 ORF2 (30  $\mu$ g/ml) or were transfected with pcDNA3-NY-ESO-1 or pcDNA3-MART-1 as described in *Materials and Methods*. D, IL-5 ELISPOT assays in the presence of L.DP4 cells pulsed with titrated doses of peptide NY-ESO-1 87–111. Spots were developed and counted by computer-assisted video image analysis. Each bar represents the mean spot number of triplicate determinations  $\pm$  SD with  $10^3$  CD4<sup>+</sup> T cells initially seeded per well (\*,  $p < 0.05$  was considered significant). Data from one representative experiment of three performed are depicted.

Taken together, these data demonstrate the capability of peptide NY-ESO-1 87–111 to stimulate Ag-specific CD4<sup>+</sup> T cells whose phenotype (i.e., Th2 and Th0) may depend on the type of APCs used to stimulate the autologous responder T cells.

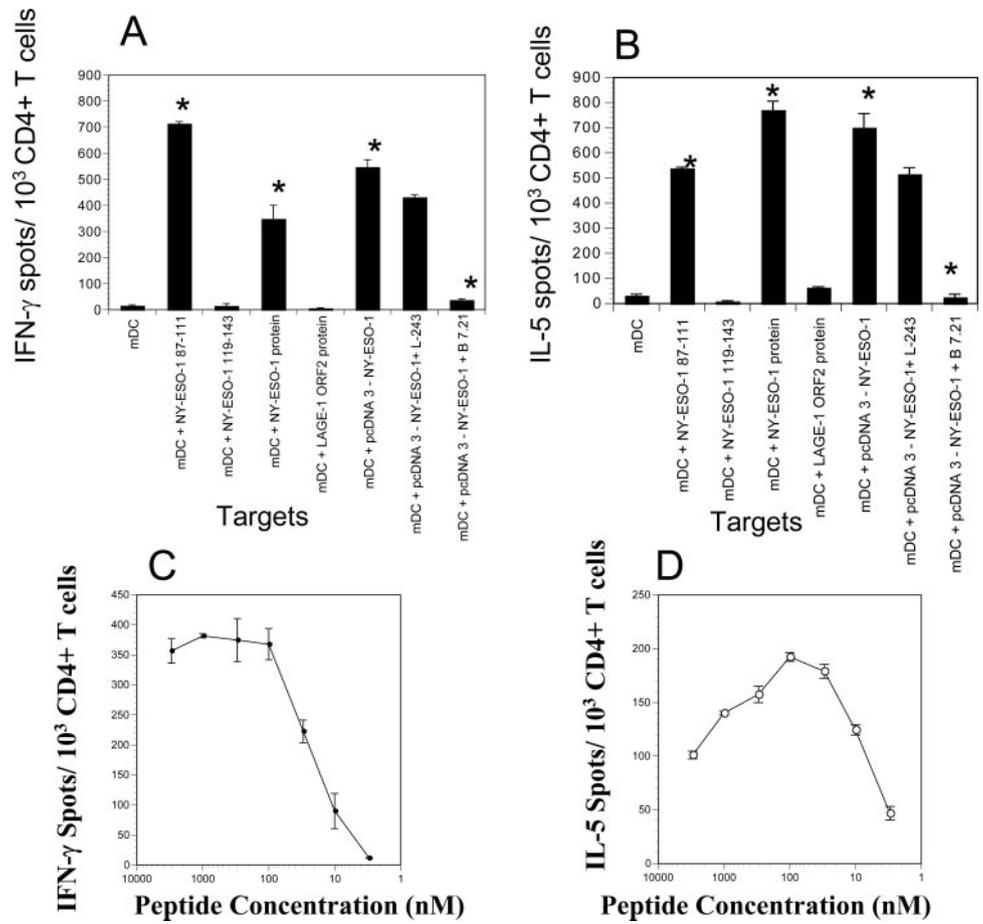
#### Detection of peptide NY-ESO-1 87–111-specific CD4<sup>+</sup> T cells in peripheral blood of melanoma patients

We next investigated the blood of 21 patients with melanoma, four long-term survivors, and six normal donors for the presence of CD4<sup>+</sup> T cells capable of recognizing peptide NY-ESO-1 87–111 (Table I). CD4<sup>+</sup> T cells were first isolated from PBLs and stimulated with peptide-pulsed APCs for 7 days before the performance of IFN- $\gamma$  and IL-5 ELISPOT assays, as previously described in *Materials and Methods*. We observed that the PBL of 5 of 12 HLA-DP4 patients with melanoma contained CD4<sup>+</sup> T cells were capable of recognizing L.DP4 cells pulsed with peptide NY-ESO-1 87–111 (Fig. 5A). Five patients developed Ag-specific Th1-type CD4<sup>+</sup> T cells, whereas one had Th0-type CD4<sup>+</sup> T cells (producing a low number of IL-5 spots). One of the three HLA-DPB1\*0401<sup>+</sup> long-term survivors with NY-ESO-1-expressing

tumors and none of the four HLA-DP4<sup>+</sup> normal donors had detectable levels of NY-ESO-1 87–111-specific HLA-DP4-restricted CD4<sup>+</sup> T cells. As a control and as shown in Fig. 5B, we also observed that 5 of 12 HLA-DP4 patients had NY-ESO-1 157–170-specific DP4-restricted CD4<sup>+</sup> T cells: four had Th1-type CD4<sup>+</sup> T cells, and one had Th0-type CD4<sup>+</sup> T cells (with a high number of IL-5 spots), respectively. Two of three long-term survivors with NY-ESO-1-expressing tumors developed Th1- and Th0-type HLA-DP4-restricted CD4<sup>+</sup> T cells recognizing peptide NY-ESO-1 157–170, respectively. No normal donor displayed NY-ESO-1 157–170-specific CD4<sup>+</sup> T cells. Altogether 8 of the 12 patients with NY-ESO-1-expressing tumors developed CD4<sup>+</sup> T cells responses against either NY-ESO-1 87–111 or NY-ESO-1 157–170. Of note, four patients developed HLA-DP4-restricted Th1-type CD4<sup>+</sup> T cells against both NY-ESO-1 87–111 and NY-ESO-1 157–170 epitopes (Fig. 5C). The two HLA-DP4<sup>+</sup> long-term survivors with NY-ESO-1-expressing tumors had only NY-ESO-1 157–170-specific CD4<sup>+</sup> T cells, not NY-ESO-1 87–111-specific CD4<sup>+</sup> T cells.

Finally, we also observed that one HLA-DRB1\*0401<sup>+</sup>/DPB1\*0401<sup>+</sup> patient with NY-ESO-1-expressing tumor and

**FIGURE 4.** Peptide NY-ESO-1 87–111 stimulates Th0-type CD4<sup>+</sup> T cell clone 40/10, the phenotype of which is stable and independent of the type of APCs used in the assay. CD4<sup>+</sup> T cell clone 40/10 was obtained by limiting dilution from the NY-ESO-1 87–111-specific bulk CD4<sup>+</sup> T cells of patient UPCI-MEL 527 as described in *Materials and Methods*. One thousand CD4<sup>+</sup> T cells from clone 40/10 were incubated for 48 h in: 1) IFN- $\gamma$  (A) and IL-5 (B) ELISPOT assays in the presence of autologous DCs pulsed with peptide NY-ESO-1 87–111 (10  $\mu$ g/ml), peptide NY-ESO-1 119–143 (10  $\mu$ g/ml), protein NY-ESO-1 ORF1 (30  $\mu$ g/ml), or protein LAGE-1 ORF2 (30  $\mu$ g/ml) or were transfected with pcDNA-3-NY-ESO-1 or pcDNA3-LAGE-1 ORF2 as described in *Materials and Methods*; or 2) IFN- $\gamma$  (C) and IL-5 (D) ELISPOT assays in the presence of L.DP4 cells pulsed with titrated doses of peptide NY-ESO-1 87–111. IFN- $\gamma$  spots were developed and counted by computer-assisted video image analysis. Each bar represents the mean spot number of triplicate determinations  $\pm$  SD with 10<sup>3</sup> CD4<sup>+</sup> T cells initially seeded per well (\*,  $p < 0.05$  was considered significant). Data from one representative experiment of three performed are depicted.



NY-ESO-1 87–111-specific CD4<sup>+</sup> T cells (patient 15) developed both HLA-DR4- and HLA-DP4-restricted CD4<sup>+</sup> T cell responses against peptide NY-ESO-1 87–111 (data not shown).

#### Analysis of NY-ESO-1-specific Ab responses in conjunction with NY-ESO-1-specific CD4<sup>+</sup> T cells

We then analyzed sera from melanoma patients for the presence of NY-ESO-1-specific Abs. ELISAs with the NY-ESO-1 protein and sera from patients were performed as previously reported (17). Among the six HLA-DP4<sup>+</sup> patients with NY-ESO-1-expressing tumors and NY-ESO-1 87–111-specific CD4<sup>+</sup> T cells, two had high titers of NY-ESO-1-specific Abs, whereas four had no detectable level of NY-ESO-1-specific Abs (Fig. 5D). Additionally, three patients had low to high titers of Abs, but no evidence of NY-ESO-1 87–111-specific CD4<sup>+</sup> T cells.

Among the five patients with NY-ESO-1-expressing tumors and NY-ESO-1 157–170-specific CD4<sup>+</sup> T cells, two had high titers of NY-ESO-1-specific Abs, and three had no NY-ESO-1-specific Abs (Fig. 5E). Additionally, three melanoma patients had low to high titers of Abs, but no evidence of NY-ESO-1 157–170-specific CD4<sup>+</sup> T cells.

We also observed that two of the three HLA-DP4<sup>+</sup> long-term survivors with NY-ESO-1-expressing tumors displayed high titers of NY-ESO-1-specific Abs.

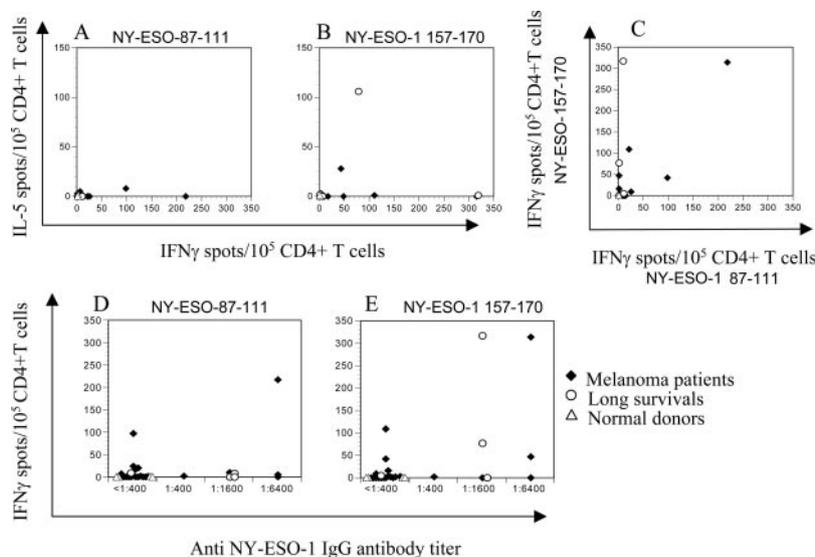
Of note, the presence of NY-ESO-1-specific Abs was not always correlated with the expression of HLA-DPB1\*0401/0402 molecules, as previously reported by others (4); one melanoma patient and one long-term survivor with NY-ESO-1-specific Abs did not express HLA-DP4.

## Discussion

In this study we have effectively used a strategy to identify pan-MHC class II tumor epitopes based on the combination of extensive binding studies to multiple HLA-DR and HLA-DP4 molecules with in vitro stimulation of CD4<sup>+</sup> T cells from the blood of normal individuals, long-term survivors, or patients with melanoma. We demonstrated the good binding capabilities of peptide NY-ESO-1 87–111 to five molecules of the most commonly expressed class II 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 87% of the American-Caucasian population. We have also shown that peptide NY-ESO-1 87–111 binds well to HLA-DRB5\*0101. Using binding assays to HLA-DPB1\*0401 and -0402 that we previously developed (14), we found that peptide NY-ESO-1 87–111 binds well to these molecules, which are expressed by a high frequency of Caucasians (Table II). We also demonstrated that the minimal peptide sequence encompassing the HLA-DP4 core epitope(s) is NY-ESO-1 87–101.

In a series of in vitro experiments using DCs and PBLs isolated from melanoma patients and normal donors, we have confirmed the implications of the binding data and demonstrated the immunogenicity of peptide NY-ESO-1 87–111 in the context of several HLA-DR and HLA-DP4 molecules. Moreover, our data demonstrate the ability of peptide NY-ESO-1 87–111 to stimulate both HLA-DR- and HLA-DP4-restricted CD4<sup>+</sup> T cells from the PBLs of the same patient.

We also demonstrated that the CD4<sup>+</sup> T cells and clones were capable of recognizing either autologous melanoma cell lines or



**FIGURE 5.** NY-ESO-1-specific Th1- vs Th2-type immunoreactivity of CD4<sup>+</sup> T cells and NY-ESO-1-specific Th1-type immunoreactivity vs anti-NY-ESO-1 Ab responses in HLA-DP4<sup>+</sup> melanoma patients. CD4<sup>+</sup> T cells from melanoma patients, long-term survivors, and normal donors were isolated and stimulated for 7 days in the absence of exogenous cytokines with APCs pulsed with either NY-ESO-1 87–111 or NY-ESO-1 157–170 as described in *Materials and Methods*. CD4<sup>+</sup> T cells were then analyzed in IFN- $\gamma$  and IL-5 ELISPOT assays for their reactivity to L.DP4 cells pulsed with peptide NY-ESO-1 87–111 (A) or L.DP4 cells pulsed with peptide NY-ESO-1 157–170 (B). Each symbol represents the combined IFN- $\gamma$  and IL-5 ELISPOT data for each individual melanoma patient, long-term survivor, or normal donor. C, Combined IFN- $\gamma$  ELISPOT assays with L.DP4 pulsed with either NY-ESO-1 87–111 or peptide NY-ESO-1 157–170 for each individual melanoma patient, long-term survivor, or normal donor. ELISAs for NY-ESO-1-specific Abs were performed as previously reported in *Materials and Methods*. The combined data of IFN- $\gamma$  ELISPOT assays and anti-NY-ESO-1 Ab responses from melanoma patients, long-term survivors, and normal donors are presented for HLA-DP4-restricted CD4<sup>+</sup> T cells against peptide NY-ESO-1 87–111 (D) and HLA-DP4-restricted CD4<sup>+</sup> T cells against peptide NY-ESO-1 87–111 (E). Each symbol represents the combined IFN- $\gamma$  ELISPOT and NY-ESO-1-specific Ab data for each individual melanoma patient, long-term survivor, or normal donor.

autologous DCs previously loaded with NY-ESO-1 protein or transfected with NY-ESO-1 cDNA. This indicates that peptides NY-ESO-1 87–111 and 87–101 contain epitopes that are processed and presented at the surface of multiple HLA-DR and HLA-DP4 molecules.

Gnjatic et al. (7) have recently reported the existence of circulating CD4<sup>+</sup> T cells against peptide 80–109 in HLA-DR7<sup>+</sup> patients with NY-ESO-1-expressing tumors. These authors have noted NY-ESO-1 80–109-specific CD4<sup>+</sup> T cells in three of five HLA-DP4<sup>+</sup> patients with NY-ESO-1-expressing tumors. Our data confirm and expand these observations, demonstrating precisely at the molecular level the capability of peptide NY-ESO-1 87–111 to bind to multiple HLA-DR and HLA-DP4 molecules and to stimulate HLA-DR- and HLA-DP4-restricted CD4<sup>+</sup> T cell lines and clones.

We have also demonstrated the ability of the NY-ESO-1 87–111 peptide to stimulate both Th1/Th0- and Th2-type CD4<sup>+</sup> T cells. We have observed that the phenotype of cloned CD4<sup>+</sup> T cells may vary with the type of APCs used in the assay, in particular when using mature DCs vs the L.DP4 cell line as APCs. One category of clones, such as clone 40/1, appears capable of producing both IFN- $\gamma$  and IL-5 only in the presence of autologous DCs, not L.DP4 cells. Another category, such as clone 40/10, appears to exhibit a well-defined and fixed Th phenotype that does not change based on the type of APCs added to stimulate these T cells. This may illustrate the flexibility of cytokine gene expression by certain tumor Ag-specific human CD4<sup>+</sup> T cell subsets (18). This observation represents one major issue for the phenotype study of Ag-specific CD4<sup>+</sup> T cells after in vitro stimulation, in particular any potential disease or therapy-associated Th1/Th2 bias from the blood of patients with cancer (19), because cytokine production may depend on the type of APCs used in the assay.

We also observed that the peptide concentration may modulate the level of IL-5 secretion by the CD4<sup>+</sup> T cells, such as clone 40/10. In particular, high doses of peptide (i.e., >500 nM/ml) were better at stimulating IFN- $\gamma$  production than IL-5 production by the Th0-type CD4<sup>+</sup> T cell clone. In contrast, low peptide doses (i.e., 5–10 nM) better stimulated IL-5 production. These results are in agreement with several studies suggesting that low doses of soluble Ag may optimally stimulate Th2-type CD4<sup>+</sup> T cells (20, 21).

We have shown that patients with active melanoma may develop naturally occurring HLA-DR4- and HLA-DP4-restricted CD4<sup>+</sup> T cell responses against NY-ESO-1 87–111. Nearly 67% of the HLA-DP4<sup>+</sup> patients (8 of 12) with NY-ESO-1-expressing melanomas displayed either NY-ESO-1 87–111- or NY-ESO-1 157–170-specific CD4<sup>+</sup> T cells, and nearly 25% displayed both (3 of 12). Patients with active melanoma had mainly Th1-type (seven of eight) and less often Th0-type (one of eight) NY-ESO-1-specific CD4<sup>+</sup> T cells. We did not measure any Th1/Th2-associated bias in CD4<sup>+</sup> T cell responses to tumor Ag, in contrast to reports by others (19).

No correlation of CD4<sup>+</sup> T cell responses against NY-ESO-1 87–111 or NY-ESO-1 157–170 with NY-ESO-1-specific Ab responses was observed; three of eight patients with NY-ESO-1-specific CD4<sup>+</sup> T cells did not exhibit NY-ESO-1-specific Abs.

Surprisingly, we detected NY-ESO-1-specific Abs in the sera of two of three long-term survivors. These patients have been disease free for >10 years and are clinically re-evaluated on a yearly basis. Although there was no obvious evidence of active disease in these patients, we cannot exclude that they may still be tumor-bearing, because the presence of NY-ESO-1-specific Abs has been previously correlated with the presence of NY-ESO-1-expressing tumors in the host (8).

Both the immunogenicity and the pan-MHC class II promiscuity of peptide NY-ESO-1 87–111 support its relevance in cancer vaccines designed to treat patients with NY-ESO-1<sup>+</sup> tumors. Because NY-ESO-1 is expressed by ~30% of metastatic melanoma, breast and prostate carcinomas, non-small cell lung cancer, and bladder, head, and neck tumors (22), peptide vaccines including NY-ESO-1 87–111 may be applicable to a large number of patients with tumors of different histological types.

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