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Cross-Reactive CD4⁺ T Cells against One Immunodominant Tumor-Derived Epitope in Melanoma Patients¹

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TCRs exhibit a high degree of specificity but may also recognize multiple and distinct peptide-MHC complexes, illustrating the so-called cross-reactivity of TCR-peptide-MHC recognition. In this study, we report the first evidence of CD4⁺ T cells recognizing the same tumor peptide-epitope from NY-ESO-1, in the context of multiple HLA-DR and HLA-DP molecules. These cross-reactive CD4⁺ T cells recognized not only autologous but also allogenic dendritic cells previously loaded with the relevant protein (i.e., the normally processed and presented epitope). Using clonotypic real-time RT-PCR, we have detected low frequencies of CD4⁺ T cells expressing one cross-reactive TCR from circulating CD4⁺ T cells of patients with stage IV melanoma either spontaneously or after immunization but not in normal donors. The maintenance of cross-reactive tumor Ag-specific CD4⁺ T cells in PBLs of cancer patients required the presence of tumor Ag/epitope in the context of the MHC molecule used to prime the Ag-specific CD4⁺ T cells. Our findings have significant implications for the optimization of TCR gene transfer immunotherapies widely applicable to cancer patients. *The Journal of Immunology*, 2007, 179: 7932–7940.

T cell receptor recognition of MHC-presented peptides is a central event in the development of Ag-specific immune responses. The high degree of specificity of Ag recognition by TCRs depends on a number of constraints imposed by the MHC molecules on the binding peptides and direct contacts between the TCRs and residues on both the MHC molecules and peptides (1). CD4⁺ T cells recognize short amino acid sequences presented in the context of an MHC class II molecule on the surface of APCs and/or less frequently on MHC class II⁺ tumor cells. On the one side, peptide-binding specificity to MHC class II molecules is due to polymorphic residues that preferentially lie within the peptide-binding site and are distributed into five pockets within the MHC molecule (2, 3). A series of peptide-binding motifs that accommodate these pockets has been previously defined for the main HLA-DR, DQ, and DP molecules (2–5). The repertoire of peptides binding to MHC class II molecules usually share common binding properties and can be regrouped into HLA II supertypes (6–8). This so-called degeneracy of peptide-binding specificity has been supported by the identification of a number of “promiscuous” peptide sequences capable of binding to multiple HLA-DR molecules and stimulating T cells in the context of these multiple HLA-DR molecules. In particular, we and others have identified a

series of tumor epitopes from tumor Ags capable of broadly binding to multiple MHC class II molecules (9–13).

On the other side, TCRs exhibit a high degree of specificity for peptide-MHC (pMHC)³ complexes. However, the number of pMHC ligands that can be encountered by T cells largely exceeds the limited diversity of the $\alpha\beta$ -TCR in one individual at a given time (14), suggesting the degeneracy or cross-reactivity of TCR recognition (15). There is now ample experimental evidence that a single TCR may recognize either multiple peptides in the context of a single MHC molecule (16), or one single peptide by multiple MHC molecules (17–19) or complexes made of both distinct peptides and MHC molecules (20, 21). The structural basis for the CD4⁺ T cell TCR cross-reactivity appears to be supported mainly by molecular mimicry: i.e., either the homology of the peptide sequence presented in the context of the MHC molecule or the minimal residue similarity involved in TCR contacts (22).

In this study, we report the first example of promiscuous CD4⁺ T cells capable of recognizing the same tumor epitope in the context of multiple HLA-DR and HLA-DP4 molecules. Our data illustrate the plasticity of TCR recognition of tumor pMHC class II complexes. They provide new tools for the monitoring of tumor Ag-specific promiscuous CD4⁺ T cells as well as for the optimization of TCR gene transfer immunotherapeutic approaches in cancer patients.

Materials and Methods

Cell lines, media

Blood samples used for all studies reported in this manuscript were obtained under the University of Pittsburgh Cancer Institute (UPCI) Institutional Review Board-approved protocols 96-099 and 00-079. The list of melanoma patients included in this study with HLA genotyping and disease stage is presented in Table I. HLA-DR and HLA-DP genotyping of melanoma patients and normal donors was performed using commercial typing

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³ Abbreviations used in this paper: pMHC, peptide-MHC; DC, dendritic cell; β -Gus, β -glucuronidase.

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Table I. HLA typing, stage of disease, frequency of specific TCR CDR3 β -expressing cells, and NY-ESO-1 Ab status of the melanoma patients and normal donors evaluated in this study^a

Patients	HLA-DR	Stage of Disease	CDR3 β (11/4)	CDR3 β (30/79)	RT-PCR NY-ESO-1	NY-ESO-1 Ab
			(/10 ⁶ CD4 ⁺ T cells)	(/10 ⁶ CD4 ⁺ T cells)		
Melanoma patients						
MP1	0401,1701	IV	6.8	0.0	+	+
MP1 NED	0401,1701	NED	0.0	0.0	NA	-
MP2	0101,0401	IV	9.5	0.0	+	+
MP2 NED	0101,0401	NED	0.0	0.0	NA	-
MP3	0401, -	IV	6.4	0.0	+	+
MP4	0401, -	IV	10.0	0.0	+	+
MP5	0401, -	IV	4.9	0.0	+	+
MP6	0401, -	IV	0.0	0.0	-	-
MP7	0401, -	IV	0.0	0.0	-	-
MP8-Day 1	0101,1301	IV	0.0	0.0	+	+
MP8-Day 112	0101,1301	IV	0.0	0.0	+	+
MP9-Day 1	0101,0401	IV	6.7	0.0	+	+
MP9-Day 112	0101,0401	IV	24.4	0.0	+	+
MP10	1301,1302	IV	0.0	0.0	+	+
MP11	0101,1301	IV	0.0	0.0	+	+
MP12	0301,1501	IV	0.0	0.0	+	+
MP13	1201,1501	IV	0.0	0.0	+	+
MP14-Day 1	0701,1301	IV	0.0	0.0	+	+
MP14-Day 112	0701,1301	IV	0.0	0.0	+	+
MP15-Day 1	0501, -	IV	0.0	0.0	+	+
MP15-Day 112	0501, -	IV	0.0	0.0	+	+
Normal donors						
ND1	0701,1101	-	0.0	0.0	-	-
ND2	0401,1401	-	0.0	0.0	-	-
ND3	0101,0301	-	0.0	0.0	-	-
ND4	0401,0701	-	0.0	0.0	-	-
ND5	0701,0804	-	0.0	0.0	-	-
ND6	1301,1501	-	0.0	0.0	-	-
ND7	0101,1201	-	0.0	0.0	-	-
ND8	0301,1301	-	0.0	0.0	-	-
ND9	0301,0401	-	0.0	0.0	-	-
ND10	0402,1501	-	0.0	0.0	-	-

^aMelanoma patients 8, 9, 14, and 15 have been evaluated pre- (day 1) and postvaccine (day 112). NED, No evidence of disease; NA, not available

panels of PCR primers according to the manufacturer's instructions (DynaL Biotech). HLA-DR-transfected mouse cells, i.e., LDR cells, were previously described (10). 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).

Peptide synthesis

The NY-ESO-1-derived peptides, synthesized using standard F-moc chemistry by the University of Pittsburgh Peptide Synthesis Facility (shared resource), were >90% pure as indicated by analytical HPLC, and were validated by mass spectrometry. Lyophilized peptides were dissolved in 100% DMSO at a concentration of 2 mg/ml and stored at -20°C until use.

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 full-length LAGE-1 ORF2 protein was produced in *E. coli* as previously described (13).

HLA-DP and HLA-DR peptide-binding assays

The binding to the multiple purified HLA-DR and HLA-DP4 molecules was performed as previously reported (8, 23, 24). Maximal binding was determined by incubating 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 the biotinylated peptides as described previously (8, 24). 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 dendritic cells (DCs) and peptide NY-ESO-1 119-143 was performed as previously reported (10,

25). The CD4⁺ T cells were cloned by limiting dilution using allogenic PBL and EBV-B cells as feeders in the presence of IL-2 and PHA, and were subsequently tested for specificity in IFN- γ ELISPOT and cytokine release assays.

IFN- γ and IL-5 ELISPOT assays

The recognition of APCs pulsed with peptides or proteins by the NY-ESO-1 119-143-specific CD4⁺ T cell clones expanded in vitro was assessed by ELISPOT assays specific for human IFN- γ and human IL-5 as previously reported (10, 25). Spot numbers and spot sizes were determined with computer-assisted video image analysis (Cellular Technologies).

IFN- γ and IL-4 cytokine secretion assays

The recognition of DCs pulsed with peptides (10 μ g/ml) or proteins (30 μ g/ml) was also assessed by MACS secretion assays for IFN- γ and IL-4 (Miltenyi Biotec) as previously described (13).

ELISA

Serum Abs against the NY-ESO-1 recombinant protein were measured with ELISA as previously reported. Sera were tested over a range of 4-fold dilutions as previously described (26).

TCR β gene usage

Total RNA was isolated from 1×10^6 CD4⁺ T cells using the RNeasy Mini kit (Qiagen) and reverse transcription was conducted as previously reported (27). The PCR product was cloned into pCR4-TOPO vector (Invitrogen Life Technologies) and sequenced using the ABI 3100 automated DNA sequencer.

Real-time quantitative RT-PCR

cDNAs from the CD4⁺ T cell clones were used as templates in TaqMan real-time PCR assays on an ABI 7700 Sequence Detection System (Applied Biosystems). Forward (F) and reverse (R) gene-specific PCR primers and fluorescent probes (FP) were designed using Primer Express Software (Applied Biosystems). The primers used in this study were: CDR3 β 11/4 F (5'-CAAATATCCGGTCCACAAAGC-3'), CDR3 β 11/4 R (5'-GCTCGTATCGTC TCCGCTA-3), CDR3 β 11/4 P (5'-AGGACTCAGCCATGTACTTCTGTGCCAGC-3'), CDR3 β 30/79 F (5'-CTGGAGCTTGGTGACTCTGCT-3'), CDR3 β 30/79 R (5'-TTGGCCCCAGAGAGGGAC-3'), CDR3 β 30/79 P (5'-TCTGTGCCAGCAGCCCGGG-3'), β -glucuronidase (β -Gus) S (5'-CTCATTTTGAATTTTCCGATT-3'), β -Gus F (5'-CCGAGTGAAGATCCCCTTTTA-3'), β -Gus P (5'-TGAACAGTCACACGAGAGTGGT-3'). Sample data were analyzed according to the comparative cycle threshold method and were normalized by β -Gus expression in each sample.

The sensitivity of the real-time RT-PCR to detect one specific TCR CDR3 β region in PBLs of melanoma patients with NY-ESO-1-expressing tumors was determined using serial dilutions from each NY-ESO-1-specific CD4⁺ T cell clone in PBLs from normal donors (1/10, 1/100, 1/1,000, 1/10,000, 1/100,000, and 1/1,000,000). The data are expressed as the percentage of fractions of the TCR CDR3 β region gene expression obtained from the cDNA of each clone. The relative expression of the TCR CDR3 β gene region obtained by real-time quantitative RT-PCR was correlated with the different dilution ratios of each NY-ESO-1 119-143-specific CD4⁺ T cell clone in PBLs by a power regression curve. The equations of power regression curves ($y = 4325.9 \times x^{1.1956}$, $R^2 = 0.997$ (clone 11/4) and $y = 13,431 \times x^{1.3004}$, $R^2 = 0.989$ (clone 30/79)) and relative expression values were used to estimate the number of T cell precursors expressing one specific clonotype in the PBLs of melanoma patients.

Results

Peptide NY-ESO-1 119-143 stimulates promiscuous CD4⁺ T cell clones recognizing the same epitope in the context of multiple MHC class II molecules

We have previously shown that peptide NY-ESO-1 119-143 is a promiscuous and immunodominant epitope capable of binding to multiple HLA-DR and HLA-DP4 molecules and also stimulates autologous CD4⁺ T cells in the context of these molecules (10). Using DCs and autologous CD4⁺ T cells from melanoma patients and normal donors, we have generated a number of NY-ESO-1-specific CD4⁺ T cell clones (10, 25). Th1-type CD4⁺ T cell clone 11/4 was derived from PBLs of an HLA-DR β 1*0401⁺/-DR β 1*1701⁺/-DP β 1*0402⁺ melanoma patient (MP1) with NY-ESO-1-expressing tumor (25), and recognized peptide NY-ESO-1 119-143 in the context of HLA-DR β 1*0401. Th0-type

CD4⁺ T cell clone 30/79 was obtained from an HLA-DR β 1*0701⁺/-DR β 1*1101⁺/-DP β 1*0401⁺ normal donor (ND1) and recognized peptide NY-ESO-1 119-143 in the context of DR β 1*1101. To investigate the cross-reactivity of these Ag-specific CD4⁺ T cell clones, we incubated them in IFN- γ and IL-5 ELISPOT assays in the presence of L.DR1, L.DR4, L.DR7, L.DR11, and L.DP4 cells pulsed with titrated doses of either peptide NY-ESO-1 119-143 or the shorter peptide sequence NY-ESO-1 123-137. As expected, clone 11/4 recognized well peptides NY-ESO-1 123-137 and NY-ESO-1 119-143 in the context of the autologous HLA-DR β 1*0401 molecule with a half-maximal stimulation of 200 and 100 nM, respectively (Fig. 1A). Strikingly, we observed that clone 11/4 produced IFN- γ in the presence of both peptides in the context of the allogenic molecules HLA-DR β 1*0101 and HLA-DR β 1*1101 (i.e., L.DR1 and L.DR11, respectively). The half-maximal stimulation of clone 11/4 required NY-ESO-1 123-137 peptide concentrations of ~20 and 200 nM in the presence of L.DR1 and L.DR11 cells, respectively. Accordingly, NY-ESO-1 119-143 peptide concentrations for half-maximal stimulation of clone 11/4 were measured at 20 and 10 nM in the presence of L.DR1 and L.DR11 cells, respectively (Fig. 1A). As control, clone 11/4 did not recognize L.DR cells pulsed with the irrelevant pan-MHC class II epitope, NY-ESO-1 87-111 (Ref. 10 and data not shown).

Clone 30/79 stimulated with peptide NY-ESO-1 123-137 produced IFN- γ (Fig. 1B) and IL-5 (Fig. 1C) at low peptide dose with a half maximal stimulation of 20–30 nM in the presence of L.DR11 cells (i.e., autologous molecules) and 400–500 nM in the presence of the other L.DR cells. NY-ESO-1 119-143 peptide concentrations for half-maximal stimulation of clone 30/79 were measured at 30–40 nM in the presence of L.DR11 cells and 400–500 nM in the presence of the other L.DR cells in IFN- γ and IL-5 ELISPOT assays (Fig. 1, B and C). As control, clone 30/79 did not recognize L.DR cells pulsed with the irrelevant pan-MHC class II epitope, NY-ESO-1 87-111 (data not shown).

Altogether, our data report the existence of tumor-Ag specific cross-reactive CD4⁺ T cell clones derived from PBLs of a melanoma patient and a normal donor, respectively, capable of recognizing promiscuously and specifically the same epitope/peptide in the context of multiple MHC class II molecules.

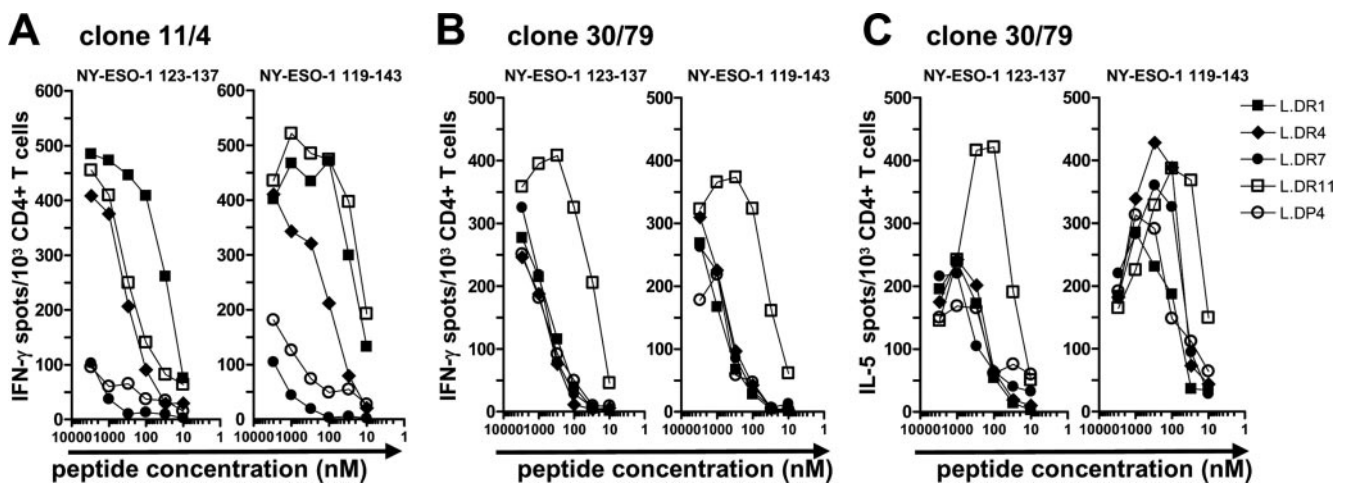


FIGURE 1. Reactivity of the two promiscuous CD4⁺ T cell clones, 11/4 and 30/79, to titrated doses of peptides NY-ESO-1 119-143 and NY-ESO-1 123-137. L.DR cells were pulsed with titrated doses of peptide NY-ESO-1 123-137 or peptide NY-ESO-1 119-143 and incubated either in the presence of CD4⁺ T cell clone 11/4 (A) or clone 30/79 (B and C) in 48 h IFN- γ and IL-5 ELISPOT assays as described in *Materials and Methods*. Spots were developed and counted by computer-assisted video image analysis. Data represent the mean spot number of triplicates with 10³ CD4⁺ T cells initially seeded per well. One representative experiment of three performed is depicted.

The promiscuous CD4⁺ T cell clones recognized naturally processed and presented NY-ESO-1-derived epitopes in the context of autologous and allogenic HLA-DR molecules

To further investigate whether the NY-ESO-1-specific promiscuous CD4⁺ T cell clones recognized naturally processed and presented epitopes from NY-ESO-1 in the context of allogenic MHC class II molecules, clones 11/4 and 30/79 were stimulated in IFN- γ and/or IL-4 secretion assays with autologous and allogenic DCs previously loaded with NY-ESO-1 119-143 peptide or NY-ESO-1 protein as previously reported (13). Unloaded DCs, DCs pulsed with an irrelevant promiscuous HLA-DR peptide, NY-ESO-1 87-111 (10), and DCs fed with the LAGE-1 ORF2 protein served as baseline and controls (13). As shown in Fig. 2A, clone 11/4 produced IFN- γ not only in the presence of autologous HLA-DR β 1*0401⁺/-DR β 1*1701⁺ DCs but also in the presence of allogenic HLA-DR β 1*0701⁺/-DR β 1*1101⁺ DCs previously loaded with the NY-ESO-1 protein. We also observed that the CD4⁺ T cell clone 30/79 produced IFN- γ and IL-4 not only in the

presence of autologous HLA-DR β 1*0701⁺/-DR β 1*1101⁺ DCs but also allogenic HLA-DR β 1*0401⁺/-DR β 1*1701⁺ DCs previously fed with the NY-ESO-1 protein but not with an irrelevant protein, LAGE-1 ORF2 (Fig. 2, B and C).

Altogether, our data demonstrate that the cross-reactive CD4⁺ T cell clones recognized normally presented and processed tumor-derived epitopes not only in the context of autologous but also allogenic MHC class II molecules.

Influence of single amino acid substitutions of peptide NY-ESO-1 123-137 on the peptide binding to MHC molecules and recognition by the promiscuous CD4⁺ T cell clones

To define the binding mode of the short peptide sequence NY-ESO-1 123-137, we tested the capability of single lysine-substituted peptides to bind to multiple purified MHC class II molecules, including HLA-DR β 1*0101, -DR β 1*0401, -DR β 1*0701, -DR β 1*1101, -DP β 1*0401, and -DP β 1*0402 (Table II). Peptide NY-ESO-1 123-137 was included as a control. We observed that

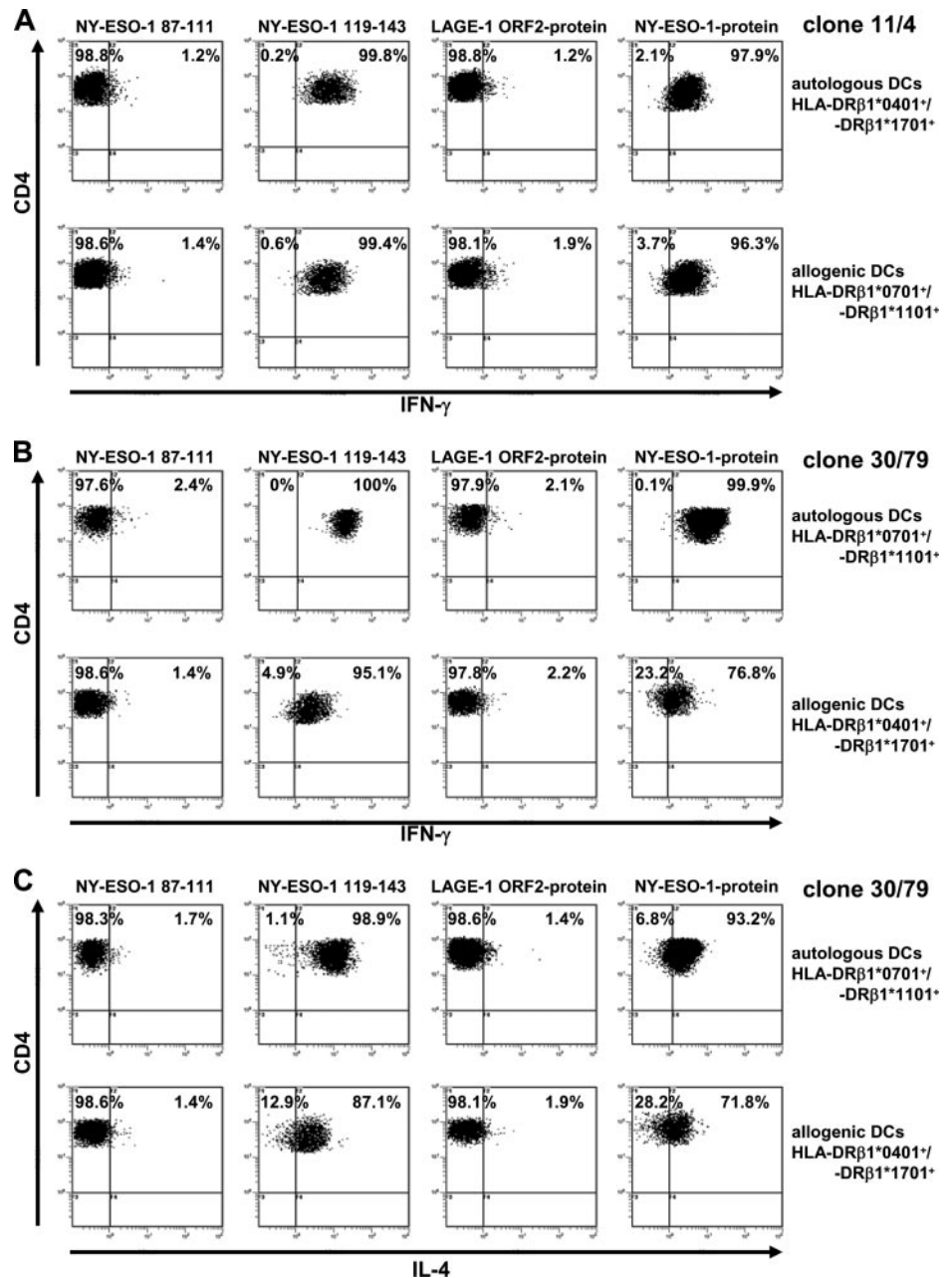


FIGURE 2. Two promiscuous CD4⁺ T cell clones recognized autologous and allogenic DCs previously loaded with the NY-ESO-1 protein. CD4⁺ T cell clones 11/4 (A) and 30/79 (B and C) were incubated in IFN- γ and/or IL-4 cytokine-release assays in the presence of autologous or allogenic DCs pulsed with either peptide NY-ESO-1 119-143 (3 μ M), peptide NY-ESO-1 87-111 (3 μ M), protein NY-ESO-1 (30 μ g/ml), or protein LAGE-1 ORF2 (30 μ g/ml). One representative of at least three independent experiments is shown.

Table II. Binding capacities to MHC class II molecules of lysine-substituted NY-ESO-1 123-137 peptides^a

Native Sequence	Peptides	IC ₅₀ (nM)					
		HLA-DR β1*0101	HLA-DR β1*0401	HLA-DR β1*0701	HLA-DR β1*1101	HLA-DP β1*0401	HLA-DP β1*0402
L	NY-ESO-1-123-137	3	60	19	20	398	256
	NY-ESO-1 123-137-K123	5	30	32	13	297	226
K							
E	NY-ESO-1 123-137-K125	4	38	14	13	531	464
F (P1)	NY-ESO-1 123-137-K126	>10,000	>10,000	>10,000	9,000	>10,000	10,000
T	NY-ESO-1 123-137-K127	1	5	4	5	321	189
V	NY-ESO-1 123-137-K128	3	36	6	26	255	232
S	NY-ESO-1 123-137-K129	63	3,162	237	43	766	565
G	NY-ESO-1 123-137-K130	2	31	8	10	169	104
N	NY-ESO-1 123-137-K131	217	>10,000	141	6	125	319
I	NY-ESO-1 123-137-K132	9	169	40	49	>10,000	10,000
L	NY-ESO-1 123-137-K133	6	189	45	26	>10,000	2,549
T	NY-ESO-1 123-137-K134	120	1,250	42	1,000	1170	609
I	NY-ESO-1 123-137-K135	4	45	34	9	>10,000	1,523
R	NY-ESO-1 123-137-K136	8	108	30	86	489	592
L	NY-ESO-1 123-137-K137	2	24	10	3	501	33

^aThe NY-ESO-1-123-137 peptide and the lysine-substituted peptides were submitted to MHC class II-binding assays as described in *Materials and Methods*. A reference peptide was used to validate each assay. Data are expressed as IC₅₀ and are the means of at least two independent experiments. Active peptides have an IC₅₀ inferior to 1000 nM. Bold: Significant loss of binding (at least 10-fold the values of the native peptide).

the lysine substitution at position 126 abolished the binding to all tested HLA molecules, strongly suggesting that this position served as a P1 anchor position for all the tested MHC class II molecules. Accordingly, lysine substitution at positions 129 (P4), 131 (P6), and 134 (P9) affected the peptide binding to HLA-DRβ1*0101, -DRβ1*0401, and -DRβ1*0701 in agreement with HLA-DR peptide-binding motifs (2, 3). For HLA-DRβ1*1101, the slight positive effect provoked by the lysine substitution at position 131 (P6) and the negative effects at positions 129 (P4) and 134 (P9) also correspond to the binding preferences of its pockets (2, 3). In contrast, the influence of the lysine substitutions on the binding to the HLA-DPβ1*0401 and -DPβ1*0402 molecules did not reflect their canonical motifs as previously described (8).

We then tested the capability of the lysine-substituted peptides to stimulate the two promiscuous CD4⁺ T cell clones, 11/4 and 30/79, in IFN-γ ELISPOT assays in the context of multiple L·DR and L·DP4 cells. As shown in Fig. 3, lysine substitutions of peptide 123–137 at P1, P3, P5, P6, and P7 abolished recognition by clone 11/4 on HLA-DRβ1*0101, -DRβ1*0401, -DRβ1*1101, and HLA-DPβ1*0401 molecules. Lysine substitution at P-1 and P8 decreased recognition on the allogenic HLA-DRβ1*0101 and DRβ1*1101 molecules and abolished recognition on the autologous HLA-DRβ1*0401 molecule. Lysine substitution at P2 decreased recognition on HLA-DRβ1*1101 and abolished recognition on the HLA-DRβ1*0101, -DRβ1*0401, and HLA-DPβ1*0401 molecules. Although peptide NY-ESO-1 123-137 appears to bind with the same mode to all HLA-DR and HLA-DP molecules, our findings suggest that the pMHC complex exhibits distinct TCR contacts with clone 11/4 depending on the MHC-presenting molecule.

Lysine substitutions at positions P-1, P1, P2, P3, P5, P7, and P8 abolished the recognition by clone 30/79 on all HLA-DR and HLA-DP4 molecules (data not shown). In correlation with our binding data, we may conclude that aa residues in position P-1, P2, P3, P5, P7, and P8 are likely involved in contacts with TCR. Here, our findings suggest that peptide NY-ESO-1 123-137 within the multiple pMHC complexes exhibit the same TCR contacts with clone 30/79.

Collectively, our findings demonstrate that peptide NY-ESO-1 123-137 binds to HLA-DR and HLA-DP4 molecules according to

two slightly different peptide-binding modes with the same anchor residue P1. They also defined at least two distinct modes of interactions between peptide NY-ESO-1 123-137-MHC complexes and the two cross-reactive Ag-specific CD4⁺ T cells.

Ex vivo detection of cross-reactive TCR from PBLs of stage IV melanoma patients with NY-ESO-1-expressing tumors

We have cloned and sequenced the TCR CDR3β regions of each cross-reactive clone as previously reported (28, 29) and described in *Materials and Methods*. Each of the two NY-ESO-1-specific CD4⁺ T cell clones exhibited a distinct CDR3β region involving distinct Vβ and Jβ chains, named CDR3β 11/4 (SFYICSAQ-GLAYEQYFGPGTR:Vβ2.1-NDN-Jβ2.7) and CDR3β 30/79 (VYFCASSPGRVSLSGANVLTFG:Vβ9-NDN-Jβ2.6), respectively. The sequences that are underlined correspond to the NOW regions.

We next investigated whether we could detect one of the two previously identified cross-reactive TCR from PBLs of 10 normal donors, 13 patients with stage IV NY-ESO-1-expressing melanoma, 2 patients with NY-ESO-1-negative melanoma, and 2 patients with a history of NY-ESO-1-expressing tumors who became disease free (Table I). Total RNA was extracted from PBLs and based on the sequences of the TCR CDR3β regions of clone 11/4 and 30/79, we have engineered specific primers to perform quantitative clonotypic real-time PCR. To correlate the expression level of CDR3β gene expression with the number of Ag-specific cells present in PBLs from patients, we have made serial dilutions of each clone from 10⁻¹ to 10⁻⁶ in PBLs from normal donors and performed clonotypic real-time RT-PCR. Assuming that the signal observed from each pure clone represents 100% of CDR3β gene expression, we expressed the results obtained from the serial dilutions and PBLs from patients as a fraction of the total CDR3β gene expression obtained from each clone (Fig. 4). As shown in Fig. 4A, 6 of 15 patients with stage IV melanoma had detectable levels of clone CDR3β 11/4 gene expression with a precursor frequency of CD4⁺ T cells from 4.9 to 10 × 10⁻⁶ CD4⁺ T cells. These six patients were typed HLA-DRβ1*0401⁺ and had NY-ESO-1-expressing tumors. Interestingly, these patients had spontaneous Ab responses against NY-ESO-1 (Table I). The two other HLA-DRβ1*0401⁺ patients (MP6 and MP7) in our study had

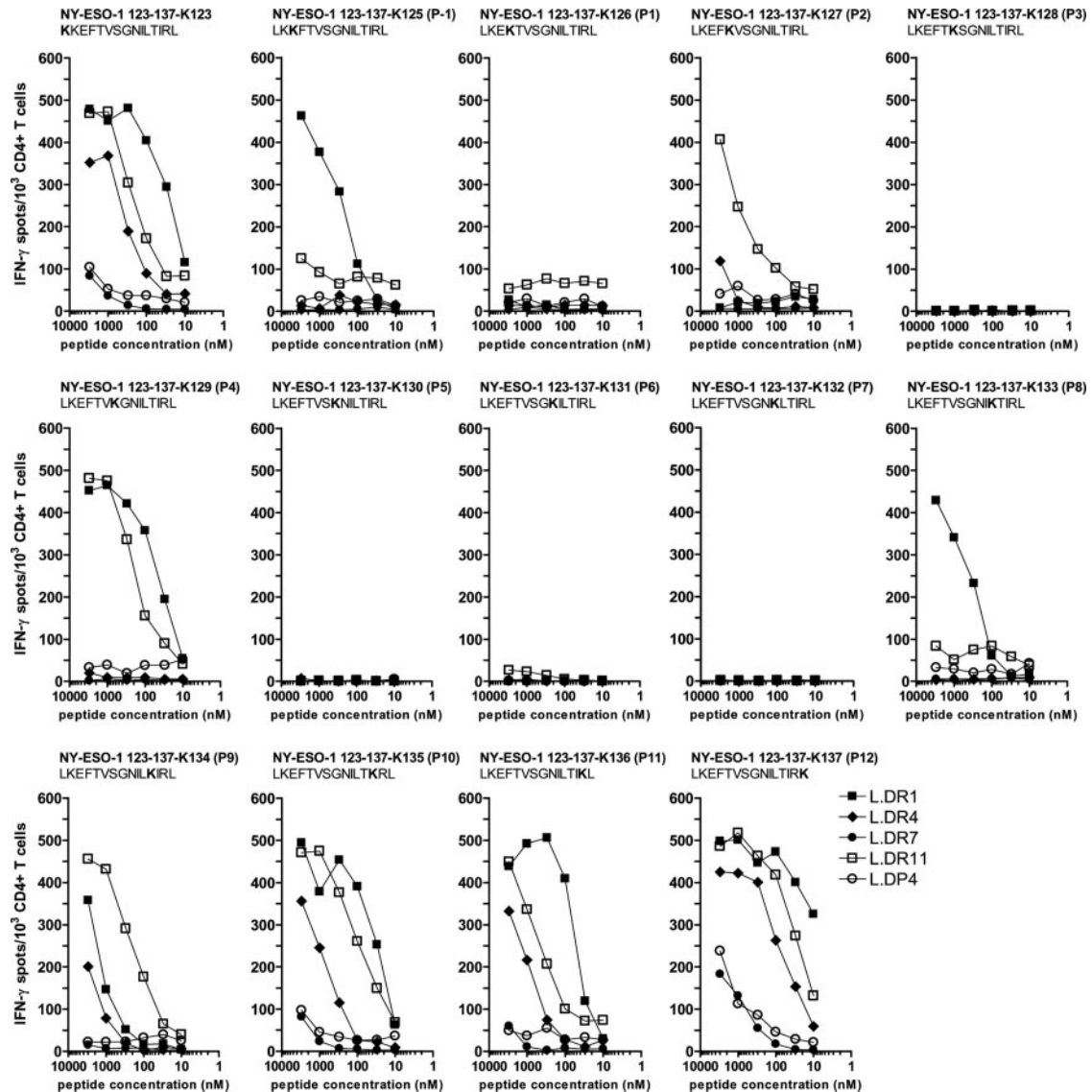


FIGURE 3. Recognition of titrated doses of single lysine-substituted peptides by CD4⁺ T cell clone 11/4. Clone 11/4 was incubated in a 48-h IFN- γ ELISPOT assay in the presence of either L.DR1, L.DR4, L.DR7, and L.DR11 or L.DP4 cells pulsed with titrated doses of single-lysine-substituted NY-ESO-1 123-137 peptides. IFN- γ spots were developed and counted by computer-assisted video image analysis. Data represent the mean spot number of triplicates with 10^5 CD4⁺ T cells initially seeded per well. One representative experiment of three performed is depicted.

NY-ESO-1-negative tumors and no detectable level of circulating cross-reactive T cells. As negative controls, none of the normal donors had NY-ESO-1-specific CD4⁺ T cell frequency $\geq 1 \times 10^{-6}$ CD4⁺ T cells. As positive controls, we observed that CDR3 β 11/4 gene expression increased significantly after three rounds of in vitro stimulation of CD4⁺ T cells from patient 1 (MP1) with peptide-pulsed APCs. Two of the 6 HLA-DR β 1*0401⁺ melanoma patients became long-term disease free (MP1 NED and MP2 NED) following surgery and/or chemotherapy with no detectable level of CDR3 β 11/4 gene expression (Fig. 4A) and no detectable anti-NY-ESO-1 serum Abs (Table I).

We further investigated whether patients with stage IV melanoma undergoing vaccine therapy with CpG 7909/PF3512676 and NY-ESO-1-derived peptides (UPCI trial 00-079) had increased CDR3 β 11/4 gene expression from CD4⁺ T cells isolated from circulating PBLs after eight biweekly s.c. immunizations (day 112). Four patients have been included in this study (Fig. 4B). These patients received either the HLA-A2-restricted peptide NY-ESO-1 157-165 V (patient 8), or the pan-

MHC class II peptide NY-ESO-1 119-143 (patients 9 and 15), or both (patient 14). As expected, patient 8 who did not have a detectable CDR3 β 11/4 gene expression before immunization remained negative for CDR3 β 11/4 gene expression. Only 1 of the 3 patients (patient 9) who was immunized with peptide NY-ESO-1 119-143 alone or in combination with peptide NY-ESO-1 157-165V, had a 3.6-fold increase of CDR3 β 11/4 gene expression postimmunization from 6.7 to 24.4×10^{-6} CD4⁺ T cells. Of note, patient 9 was the only HLA-DR β 1*0401⁺ patient among the 4 immunized patients.

As shown in Fig. 4C, none of the 15 melanoma patients had significant detectable levels of CDR3 β 30/79 gene expression (i.e., NY-ESO-1-specific CD4⁺ T cell frequency $\geq 1 \times 10^6$ CD4⁺ T cells). However, CDR3 β 30/79 gene expression increased significantly after three rounds of in vitro stimulation of CD4⁺ T cells from the HLA-DR β 1*0701^{+/−}DR β 1*1101^{+/−}DP β 1*0401⁺ normal donor (ND1) from whom clone 30/79 was originally derived. Furthermore, none of the three melanoma patients immunized with peptide NY-ESO-1 119-143 had

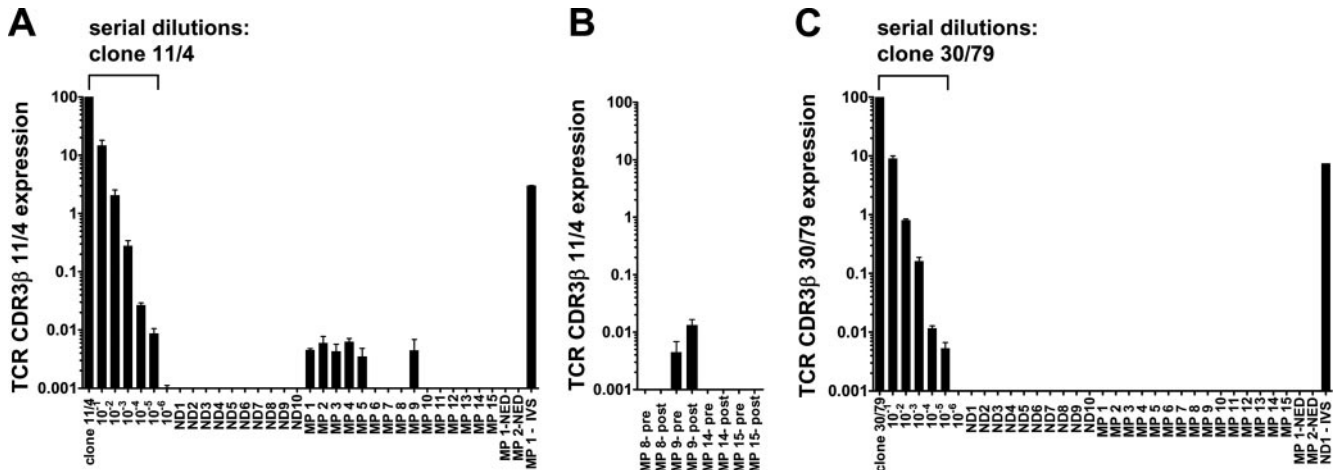


FIGURE 4. Ex vivo detection of cross-reactive CD4⁺ T cells from PBLs of melanoma patients. CD4⁺ T cells were isolated from PBLs of 15 melanoma patients with active disease (MP), 2 melanoma patients with no evidence of disease (MP NED), and 10 normal donors (ND) as shown in Table I. The relative expressions of the TCR CDR3 β regions of each cross-reactive clone were evaluated with quantitative real-time RT-PCR as described in *Materials and Methods*: clone 11/4 for each MP and ND (A) and for four MP pre-(day 1)- and post-(day 112) immunizations with s.c. injections of CpG 7909/PF3512676 and peptide NY-ESO-1 119-143 (B); clone 30/79 for each MP and ND (C). The relative expression of the TCR CDR3 β gene region was correlated to different dilution ratios of each of the two NY-ESO-1 119-143 specific CD4⁺ T cell clones in PBLs from ND as described in *Materials and Methods*. The mean and SDs of three independent experiments are presented.

detectable levels of CDR3 β 30/79 gene expression from circulating CD4⁺ T cells (data not shown).

Collectively, our data indicate that the promiscuous TCR recognition of peptide NY-ESO-1 119-143 is supported by multiple V β chains usage and TCR CDR3 β sequences. They further demonstrate the persistence of one cross-reactive TCR detectable from PBLs of the DR β 1*0401⁺ melanoma patients with active NY-ESO-1-expressing tumors and circulating anti-NY-ESO-1 Abs but not in normal donors and in HLA-DR β 1*0401⁺ melanoma patients with NED or NY-ESO-1-negative melanoma.

Discussion

The capability of a single TCR to recognize disparate peptide-MHC complexes illustrates the so-called degeneracy or cross-reactivity of TCR-pMHC recognition. T cell degeneracy allows the recognition of any potential pMHC complex by the limited number of T cells present in one individual at a time (15). CD8⁺ T cell cross-reactivity appears to play an important role for intrathymic T cell positive selection, homeostatic survival in the periphery, T cell immunodominance, and alloreactivity (30–34). Here, our findings report the first evidence of tumor-Ag-specific CD4⁺ T cells capable of recognizing the same promiscuous tumor epitope-peptide, NY-ESO-1 119-143 in the context of multiple HLA-DR and HLA-DP molecules. Although pre-existing affinity of TCR for MHC molecules has been reported and appeared sufficient for peptide-independent T cell activation (35), our data clearly demonstrate that the NY-ESO-1-specific CD4⁺ T cells act in a peptide-specific interaction, and MHC-dependent but unrestricted fashion. Human CD4⁺ T cells capable of recognizing the same tetanus toxoid peptide in the context of multiple MHC class II molecules have been previously reported by Panina-Bordignon et al. (19) and named “promiscuous” T cells. Additional reports of human promiscuous CD4⁺ T cell clones recognizing pathogen-derived epitopes (like hemagglutinin, herpes simplex type 2, and mycobacterium-derived epitopes) (17, 18, 22) have been made. To the best of our knowledge, all previously identified epitopes recognized by human promiscuous CD4⁺ T cells were derived from infectious pathogens. It is thus tempting to speculate that in the context of an infection and in a short period of time, cross-reactivity

may allow the limited repertoire of T cells present in one individual to recognize a large number of peptide MHC complexes, enhancing immune responses to infectious agents. Furthermore, in animal models, the capability of T cells to cross-react with unrelated viral epitopes appeared to play a central role in promoting the immunodominance and maintenance of memory T cells (34).

Several lines of evidence support the biological relevance of the cross-reactive tumor Ag-specific CD4⁺ T cells. First, among the NY-ESO-1-specific CD4⁺ T cell clones that we generated and cultured according to the same experimental conditions, only a fraction was promiscuous. Therefore, cross-reactivity cannot be only explained by an increase in sensitivity of the TCR to its ligands observed only in well differentiated/hyperactivated T cells in vitro. Furthermore, in our study, the NY-ESO-1-specific promiscuous CD4⁺ T cell clones were not only capable of producing IFN- γ at low-peptide dose but also of recognizing the normally processed and presented Ag, supporting the biological relevance of cross-reactivity.

Our findings contribute to define the conditions required for the generation and maintenance of the promiscuous tumor Ag-specific CD4⁺ T cells in vivo. First, the peptide must bind and be presented in the context of multiple MHC class II molecules. Second and in agreement with previous experimental models, the persistence and proliferation of CD4⁺ T cells in vivo required the continued presence of Ag (36). Third, the expansion and persistence of a given promiscuous clonotype appeared to require Ag presentation in the context of the same MHC class II molecule used to prime CD4⁺ T cells. A number of observations supported this last statement. The promiscuous CD4⁺ T cell clone 11/4 was previously derived from an HLA-DR β 1*0401⁺ melanoma patient and we have found detectable CDR3 β 11/4 gene expression only from PBLs of HLA-DR β 1*0401⁺ melanoma patients. In contrast, no detectable level of CDR3 β 30/79 gene expression was found from PBLs of the melanoma patients with NY-ESO-1-expressing tumors. Interestingly, clone 30/79 was derived from the PBLs of an HLA-DR β 1*1101⁺ normal donor after several rounds of in vitro stimulation and none of the melanoma patients included in our

study were HLA-DR β 1*1101⁺. Collectively, our data suggest that the cross-reactive TCR of clone 11/4 identified in this study exhibits a “public” clonotype present only in HLA-DR β 1*0401⁺ patients chronically exposed to their selecting Ag (37). Strikingly, we observed low levels of specific CDR3 β gene expression from circulating CD4⁺ T cells of melanoma patients, reflecting a low precursor frequency, unlikely to promote tumor rejection in patients with large tumor burden. The reason why we could not detect major spontaneous expansion of cross-reactive CD4⁺ T cells in patients with NY-ESO-1-expressing tumors still needs to be investigated. One likely explanation may be the suboptimal MHC class II presentation by tumor cells in the absence of a significant number of tumor Ag-loaded DCs (38). Alternatively, multiple mechanisms of tumor-induced T cell immunosuppression have been recently reported and may contribute to limit the expansion of the tumor Ag-specific CD4⁺ T cells in patients with active disease (39).

Our findings raise the question of sequence homology between the natural peptide sequence and potential cross-reactive sequences. NY-ESO-1 is a tumor-specific Ag expressed by tumor of different histological types but not by normal tissue, except testis, and exhibiting no sequence homology with known foreign Ags. This is in sharp contrast with another melanoma-associated Ag, Melan-A/MART-1 that shows sequence similarities with viral and bacterial proteins (40), thus supporting the high Melan-A/MART-1 27–35-specific precursor frequency in normal donors. Whether the NY-ESO-1-specific CD4⁺ T cells may recognize unknown foreign or self-Ags remains to be defined.

Cross-reactive cerebrospinal fluid-infiltrating T cell clones from a relapsing-remitting multiple sclerosis patient have been shown to recognize one epitope presented in a single binding mode by multiple MHC class II molecules, suggesting the role of the peptide-binding mode to MHC class II molecules in promoting promiscuous CD4⁺ T cells (41). Our findings further support this hypothesis. The molecular basis of the CD4⁺ T cell cross-reactivity for MHC class II-presented epitopes is now better understood because of crystallographic studies of the HA1.7 TCR bound to the self MHC class molecule, HLA-DR β 1*0101 or an allogenic MHC class II molecule HLA-DR β 1*0401 (22). In this model, the allelic sequences that are different between DR β 1*0101 and DR β 1*0401 were not exposed to TCR contacts and the two pMHC complexes recognized by the same TCR were very similar from a TCR perspective, supporting the concept of molecular mimicry. An additional model for TCR cross-reactivity has shown that the flexible CDR3 region could adapt to structurally different pMHC complexes (42). Whether this new model of cross-reactivity shown for mouse CD8⁺ T cells is relevant to human TCR recognition of CD4 epitopes in the context of MHC class II molecules remains to be demonstrated.

In summary, our findings demonstrate the existence of tumor Ag-specific cross-reactive CD4⁺ T cells in melanoma patients with active disease, illustrating the considerable plasticity of TCR recognition of tumor pMHC class II complexes. From a clinical standpoint, the identification of promiscuous TCR motifs targeting complexes of tumor epitopes with MHC molecules, would prove particularly useful for the design of clonotypic probes for the molecular follow-up of memory T cell responses of defined specificity, in patients with cancers. Our findings further hold promises for the optimization of adoptive transfer of promiscuous T cells and the design of TCR gene transfer immunotherapies applicable to the majority of cancer patients independently of their MHC class II phenotype.

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Disclosures

The authors have no financial conflict of interest.

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