# Identification of CD4$^+$ T Cell Epitopes from NY-ESO-1 Presented by HLA-DR Molecules

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**In previous studies, the shared cancer-testis Ag, NY-ESO-1, was demonstrated to be recognized by both Abs and CD8$^+$ T cells. Gene expression of NY-ESO-1 was detected in many tumor types, including melanoma, breast, and lung cancers, but was not found in normal tissues, with the exception of testis. In this study, we describe the identification of MHC class II-restricted T cell epitopes from NY-ESO-1. Candidate CD4$^+$ T cell peptides were first identified using HLA-DR4 transgenic mice immunized with the NY-ESO-1 protein. NY-ESO-1-specific CD4$^+$ T cells were then generated from PBMC of a patient with melanoma stimulated with the candidate peptides in vitro. These CD4$^+$ T cells recognized NY-ESO-1 peptides or protein pulsed on HLA-DR4$^+$ EBV B cells, and also recognized tumor cells expressing HLA-DR4 and NY-ESO-1. A 10-mer peptide (VLLKEFTVSG) was recognized by CD4$^+$ T cells. These studies provide new opportunities for developing more effective vaccine strategies by using tumor-specific CD4$^+$ T cells. This approach may be applicable to the identification of CD4$^+$ T cell epitopes from many known tumor Ags recognized by CD8$^+$ T cells.** *The Journal of Immunology,* **2000, 165: 1153–1159.**

T cells play an important role in controlling tumor growth and mediating tumor regression. To understand the molecular basis of T cell-mediated antitumor immunity, a number of tumor Ags recognized by CD8$^+$ T cells have been identified in melanoma as well as in other types of cancers (1, 2). These studies have led to several clinical trials using peptides derived from the molecularly defined tumor Ags (3–6). Although the clinical trial using a modified peptide derived from gp100 provided some evidence of therapeutic efficacy for the treatment of patients with metastatic melanoma (3), these studies mainly focused on the use of CD8$^+$ T cells. Increasing evidence from both human and animal studies has indicated that optimal cancer vaccines require the participation of both CD4$^+$ and CD8$^+$ T cells (7, 8). Moreover, tumor-specific CD4$^+$ T cells are required for generating protective immunity against MHC class II-negative tumor cells (9, 10). Identification of such Ags is thus important for the development of cancer vaccines as well as for our understanding of the mechanism by which CD4$^+$ T cells regulate host immune responses.

To date, only a limited number of MHC class II-restricted tumor Ags have been identified. Several known MHC class I-restricted tumor Ags, such as tyrosinase, gp100,[3] and MAGE-3, were demonstrated to contain MHC class II-restricted epitopes recognized by CD4$^+$ T cells (11–15). Recently, a genetic approach was developed to identify unknown MHC class II-restricted tumor Ags by using tumor-specific CD4$^+$ T cells (16). This has led to the identification of several mutated tumor Ags, including CDC27, triosephosphate isomerase, and LDLR-FUT (16, 17). Among them, triosephosphate isomerase is a mutated Ag that was independently identified by a biochemical approach (18).

The NY-ESO-1 gene was previously identified by Ab screening (19), and was recently identified as an MHC class I-restricted tumor Ag as well (20, 21). High titers of Abs against NY-ESO-1 were also detected from patients with cancer (22). The NY-ESO-1 cDNA encoded two gene products from two overlapping open reading frames (20). Because of its strict tumor-specific expression pattern, with the exception of expression in normal testis, as well as its high frequency of expression in many tumors, including melanoma, breast, prostate, lung, and other cancers (19, 20, 23), NY-ESO-1 is potentially an important immune target for the development of immunotherapies for a variety of cancer types (24).

Because both CTL and Ab immune responses against NY-ESO-1 were demonstrated in patients with cancer, identification of MHC class II-restricted T cell epitopes in the NY-ESO-1 protein could be important for the development of effective cancer vaccines. In this study, we report the identification of an HLA-DR4-restricted T cell epitope from the NY-ESO-1 Ag by using HLA-DR4 transgenic mice immunized with the purified NY-ESO-1 protein and in vitro stimulation of human PBMC with synthetic candidate peptides. We show that CD4$^+$ T cells generated from human PBMC are NY-ESO-1 specific, and recognize NY-ESO-1 peptides pulsed on HLA-DR4$^+$ EBV B cells as well as naturally processed peptides on melanoma cells.

## Materials and Methods

### *Purification and analysis of recombinant NY-ESO-1 protein*

To construct a bacterial expression vector encoding the full-length NY-ESO-1 gene, we generated a PCR fragment by using a pair of primers, ESO-5p (5′-GCTCCGGACATATGCAGGCCGAAGGCCGGGG) containing an *Nde*I site and ESO-3p (5′-AAGGGGCTCGAGGCTGGGCT TAGCGCCTCT) containing an *Xho*I site. After digestion with restriction enzymes and gel purification of the PCR product, a DNA fragment encoding NY-ESO-1 was fused to DNA encoding a polyhistidine peptide in frame in pET-28$^{(+)}$ (Novagen, Madison, WI). A similar strategy was also used to construct an expression vector for a truncated NY-ESO-1, ESO1–74, which contained only the first 74 aa residues. *Escherichia coli* strain BL21(DE3) bearing the correct plasmid construct was grown at 37°C to log

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Received for publication February 4, 2000. Accepted for publication May 8, 2000.

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[3] Abbreviations used in this paper: gp, glycoprotein; TRP, tyrosinase-related protein; LDLR-FUT, low density lipid receptor (LDLR) and GDP-L-fucose $\beta$-D-galactoside 2-$\alpha$-L-fucosyltransferase (FUT) fusion protein; CU, cetus unit.



0022-1767/00/$02.00

phase, then induced for protein production by adding isopropyl $\beta$-*d*-thiogalactoside to a final concentration of 0.5 mM and shaking for 3 h. Soluble fractions of bacterial extract were obtained; and NY-ESO-1 was purified by $Ni^{2+}$ affinity chromatography. SDS-PAGE analysis of the purified protein was performed as previously reported (25). The N-terminal sequence of the purified protein was determined by automatic Edman degradation.

### *Serum and PBMC*

Sera from patients with metastatic melanoma were stored at −80°C. Sera of normal donors were obtained from the Blood Bank at the Clinical Center of National Institutes of Health. The MHC class II genotype of patient TE with metastatic melanoma was HLA-DR$\beta$1*0401, $\beta$1*1501. The patient was treated with the gp100:209–217(210 M) peptide plus high dose of IL-2, and experienced an objective tumor regression.

### *Detection of Abs against NY-ESO-1 protein*

About 50 ng of purified NY-ESO-1 protein diluted in 50 $\mu$l PBST (PBS with 0.1% Tween-20) was adsorbed to each well of a 96-well MaxiSorp plate (Nunc, Roskilde, Denmark) overnight at room temperature. Control plates were coated with 150 ng BSA/well. Plates were blocked with 5% dry milk in PBST for at least 2 h, washed, and were loaded with 100 $\mu$l of diluted serum samples. All serum samples were diluted at 1/25, 1/250, and 1/2500 with 3% dry milk in PBST. Each sample at the three different dilutions was loaded onto NY-ESO-1-coated plates as well as BSA-coated plates. After 1-h incubation at room temperature, plates were washed and loaded with secondary Ab (goat anti-human IgG conjugated with HRP; Sigma, St. Louis, MO) diluted with 1% dry milk in PBST. Plates were developed after a 0.5-h incubation, and absorbance at 450 nm was read by using an ELISA reader (Dynatech, Chantilly, VA). A positive reaction was defined as an OD value against NY-ESO-1 that exceeded the mean OD value plus three times SDs of normal donors at serum dilutions of both 1/25 and 1/250. Western blot was performed as described (25) to confirm the specificity of the Ab in a few representative sera samples.

### *Cell lines and Abs*

Melanoma lines F049 and F050 were early cultures of fine needle asparate samples, provided by Adam Riker at the Surgery Branch of National Cancer Institute. All other melanoma lines and EBV B lines were generated and maintained in RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 10% FCS (Biofluids, Gaithersburg, MD). 293IMDR1 and 293IMDR4 were genetically engineered to express human invariant chain, DMA, DMB, and DR molecules, and were cultured in RPMI 1640 supplemented with 10% FCS (16). Culture medium for murine lymphocytes was RPMI 1640 with 0.05 mM 2-ME, 5 CU/ml IL-2, plus 10% FCS, provided by HyClone (Logan, UT). Medium used for human T cell culture was RPMI 1640 with 0.05 mM 2-ME, 50 CU/ml IL-2, plus 10% human AB serum, provided by Sigma. Ab-blocking experiments were performed as previously described (15). Hybridoma HB55 and HB95 were obtained from American Type Culture Collection (Manassas, VA). Control Ab was purchased from PharMingen (San Diego, CA).

### *Transgenic animals and immunization procedures*

HLA-DR4 transgenic (DR4-Tg) mice were murine class II deficient, and expressed HLA-DR$\alpha$-IE-$\alpha$ and HLA-DR $\beta$1*0401-IE-$\beta$ chimeric molecules (26). Founder mice were obtained through Paul Lehmann at Case Western Reserve University (Cleveland, OH). Mice were inbred and maintained at Biocon (Rockville, MD). Female mice aged between 6 and 10 wk were immunized with the full-length recombinant NY-ESO-1 protein. About 50 $\mu$g of purified protein were emulsified in CFA, divided evenly, and given to each mouse via s.c. injection into rear footpads and the base of tail. Eleven days after the injection, mice were sacrificed, and the bilateral hind limb popliteal and the inguinal lymph nodes were harvested. Single cell suspensions were obtained from the lymph nodes of two immunized animals, and followed by in vitro stimulation.

### *Peptide synthesis*

Synthetic peptides used in this study were made using a solid-phase method on a peptide synthesizer (Gilson, Worthington, OH) at the Surgery Branch of National Cancer Institute. The purity of each peptide was evaluated by mass spectrometry (Bio-synthesis, Lewisville, TX).

### *In vitro sensitization procedure and cytokine release assays*

Peptides at a final concentration of 10 $\mu$M were mixed with $2.5 \times 10^5$ mouse lymphocytes for 1 wk before cytokine release assays were conducted. For in vitro sensitization of human PBMC, $2.5 \times 10^5$ cells were pulsed with peptides at 10 $\mu$M concentration and incubated in each well of a flat-bottom 96-well plate. After two in vitro stimulations, cells were tested against various targets and supernatants were harvested for cytokine release assays. Rapid expansion and cloning of human T cells were performed as described (20).

Peptide at a final concentration of 10 $\mu$M or protein at a final concentration of 5 $\mu$g/ml was pulsed onto target cells. After 4-h incubation, cells were washed in serum-free RPMI medium, and $\sim 3 \times 10^4$ target cells were incubated with the same number of TE4-1 cells overnight, and cytokine release was measured using GM-CSF ELISA kits (R&D Systems, Minneapolis, MN) for human or IFN-$\gamma$ kits (Endogen, Woburn, MA) for mouse. Other cytokines, such as human IFN-$\gamma$, IL-10, TNF-$\alpha$, and IL-4, were measured using ELISA kits from Endogen or R&D Systems, according to the manufacturer's instructions.

## Results

### *Recombinant NY-ESO-1 protein and detection of NY-ESO-1-reactive Ab*

NY-ESO-1-reactive Abs and CTL have been reported in patients with cancer (19, 22). It thus appeared that NY-ESO-1-specific CD4$^+$ T cells might play a role in orchestrating the development of Abs as well as CTLs against the NY-ESO-1 Ag. To identify MHC class II-restricted CD4$^+$ T cell epitopes, we began by purifying NY-ESO-1 protein from a bacterial expression system as the starting material. To facilitate NY-ESO-1 expression and protein purification, a cDNA fragment encoding NY-ESO-1 was fused to a polyhistidine tag in frame located at the N terminus in the pET28 expression vector, and a high level production of recombinant protein was obtained. Several milligrams of the NY-ESO-1 protein were purified by using a $Ni^{2+}$-charged affinity chromatography column. The purified protein showed an apparent molecular mass of ~26 kDa on an SDS polyacrylamide gel (Fig. 1*A*). To confirm the identity of the purified protein, N-terminal microsequencing of protein was performed by automatic Edman degradation. All 25 aa residues obtained by Edman degradation matched the predicted amino acid sequences (data not shown). A short version of NY-ESO-1 containing the first 74 aa residues, ESO1–74, was also purified by the same approach (Fig. 1*A*).

To determine whether melanoma patients developed Abs against the NY-ESO-1 protein, sera from 88 metastatic melanoma patients enrolled in cancer vaccine treatment protocols in the Surgery Branch, National Cancer Institute, were screened. Sera from eight normal donors were used as controls for screening. Eleven of eighty-eight patients (13%) were found to have high titers of Abs against NY-ESO-1 (Fig. 1*C*). These data were consistent with results obtained by other groups (22). To exclude the possibility that patients' sera reacted with a minor contaminant present in the purified NY-ESO-1 protein, Western blot was performed using representative sera samples. Fig. 1*B* showed that the NY-ESO-1-reactive sera from a patient reacted only with cell lysates from NY-ESO-1-expressing bacteria and the purified NY-ESO-1 protein, but not with extracts from bacteria containing the control vector. A nonreactive serum sample was also tested (Fig. 1*B*, *lanes 4*, *5*, and *6*).

### *Identification of putative MHC class II-restricted epitopes from HLA-DR4 transgenic mice*

To identify CD4$^+$ T cell epitopes, DR4 transgenic mice were immunized in the tail base and rear footpads with ~50 $\mu$g of full-length NY-ESO-1 protein in CFA. Eleven days after the injection, single cell suspensions obtained from bilateral hind limb popliteal and inguinal lymph nodes of two immunized mice were prepared and used for in vitro sensitization with synthetic peptides derived from the NY-ESO-1 protein based on the predicted peptide-binding properties of the HLA-DR4 molecules (27).

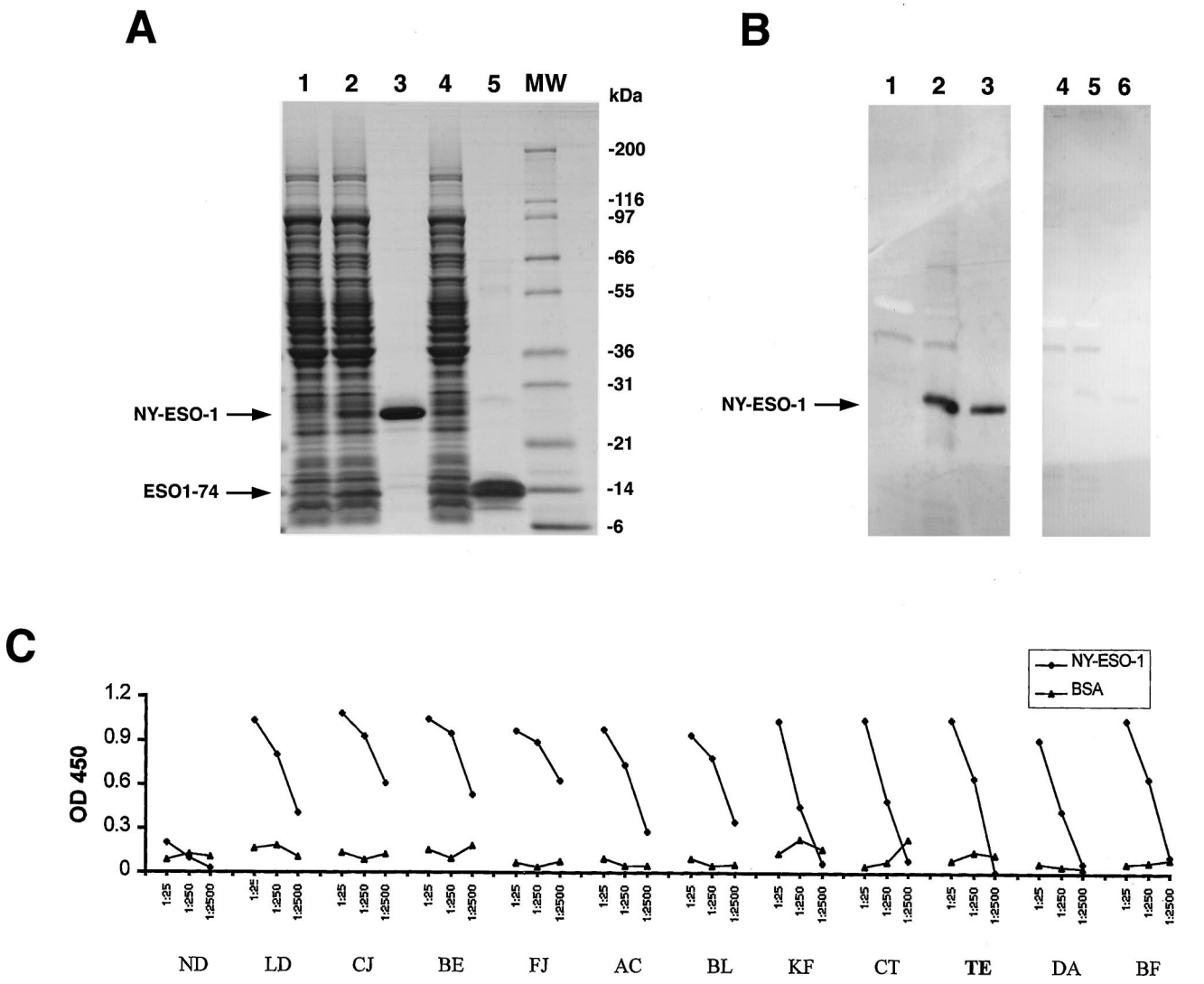


**FIGURE 1.** *A*, Purification of full-length NY-ESO-1 protein using a $Ni^{2+}$ chromatography column. SDS polyacrylamide gel showed the crude extract from *E. coli* strain BL21(DE3) bearing pET28 vector (*lane 1*), pNY-ESO-1 (*lane 2*), the purified NY-ESO-1 protein (*lane 3*), bacterial extract encoding the truncated NY-ESO-1 (*lane 4*), and the purified truncated NY-ESO-1 protein, ESO1–74 (*lane 5*). *B*, Western blot to confirm the specificity of Abs against NY-ESO-1. Sera at 1/2000 dilution from two representative patients, one with (*lanes 1–3*) and one without (*lanes 4–6*) detectable NY-ESO-1 Abs by ELISA, were used against bacterial extract encoding the vector only (*lanes 1* and *4*), encoding NY-ESO-1 (*lanes 2* and *5*), and the purified NY-ESO-1 protein (*lanes 3* and *6*). *C*, Patient TE was among the melanoma patients who had Abs against NY-ESO-1 protein. ELISA was performed using sera from 88 patients for the presence of Abs against NY-ESO-1 (BSA as control protein). Values of OD 450 at 1/25, 1/250, and 1/2500 of sera dilutions were plotted. Sera from normal donors were used as controls, and their mean OD value was also plotted (ND).

Eight high-binding peptides containing amino acid sequence segments predicted to bind to HLA-DR4 were used for the in vitro sensitization experiments. Six days after the initial in vitro sensitization, murine lymphocytes were tested for cytokine release against human HLA-DR4-positive 1359EBV B cells alone and 1359EBV B pulsed with the corresponding peptide used for stimulation. Three peptides were recognized by murine T cells based on cytokine secretion from T cells, while other five peptides showed no recognition (Fig. 2). The ESO p116–135 showed the strongest activity among the positive peptides, suggesting that this peptide might contain an epitope presented by the HLA-DR4 molecule for T cell recognition. This peptide was thus chosen for further analysis.

### *Generation of human $CD4^+$ T cells specific for NY-ESO-1*

PBMCs from patient TE, who had high titered Abs against NY-ESO-1 (Fig. 1*C*), were used for in vitro stimulation with the ESO p116–135 peptide. After 1 wk of in vitro stimulation, PBMC from patient TE showed marked expansion. IL-2 was added in the second week of stimulation. The cell line thus established was named TE4-1, which continued growth for more than 2 wk in the presence of 20 CU/ml IL-2. The TE4-1 T cells were 90% $CD4^+$ T cells based on FACS analysis. TE4-1 contained Th1-type $CD4^+$ T cells as they secreted GM-CSF, IFN-$\gamma$, and TNF-$\alpha$, but not IL-10 or IL-4 (data not shown). After depletion of a few percent of $CD8^+$ T cells, the purified population of $CD4^+$ T cells still retained its reactivity. Some T cell clones derived from TE4-1 cell line were also shown to recognize the ESO p116–135 peptide (data not shown).

TE4-1 recognized EBV B cells pulsed with the full-length NY-ESO-1 protein as well as the ESO p116–135 peptide in the context of HLA-DR4, but not with the truncated NY-ESO-1 protein containing the first 74 aa (Fig. 3*A*). The TE4-1 cell line was also reactive specifically with DR4-positive dendritic cells infected with adenovirus encoding NY-ESO-1, but not adenovirus encoding the green fluorescence protein (data not shown).

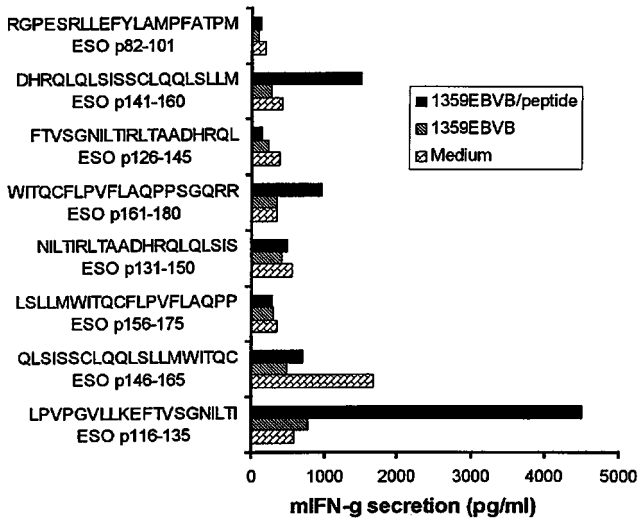


**FIGURE 2.** Testing of putative NY-ESO-1 epitopes using HLA-DR4-Tg mice immunized with NY-ESO-1 protein. Eight peptides based on the predicted binding affinity to HLA-DR4 were used for in vitro sensitization of lymphocytes from immunized mice. Murine lymphocytes were tested for IFN-$\gamma$ production against either medium alone, 1359EBV B (HLA-DR4$^+$) cells alone, or 1359EBV B cells pulsed with the peptide used for in vitro stimulation.

To test whether T cell recognition by TE4-1 was restricted by HLA-DR4, two overlapping peptides (ESO p116–135 and ESO p111–130) and a control peptide (ESO p91–110) were pulsed onto 293IMDR1 and 293IMDR4 cells in serum-free medium. Cells were washed and subsequently incubated with TE4-1 cells overnight. As shown in Fig. 3*B*, both peptide 116–135 and peptide 111–130 were recognized by TE4-1 in the context of HLA-DR4. Interestingly, peptide 116–135 was also capable of stimulating cytokine secretion from T cells when pulsed onto 293IMDR1 cells. No activity was detected with 293IMDR4 pulsed with the control ESO p91–110 peptide (Fig. 3*B*). The recognition of ESO-p116–135-pulsed 293IMDR4 was completely inhibited by an anti-HLA-DR Ab (HB55), but not by the control and anti-HLA class I Abs (HB95) (Fig. 3*C*). A gp100-specific CD8$^+$ T cell line (CTL-C3G1) and an HLA-DR1-restricted CD4$^+$ T cell line (T3–80) were used as specificity controls for the Ab blocking.

*Recognition of tumor cells by TE4-1*

Although peptide-specific CD4$^+$ and CD8$^+$ T cell activities can often be generated against a putative tumor Ag, in many cases tumor reactivity could not be demonstrated due to either the low affinity of the T cells or the failure of presentation of naturally processed peptides on the tumor cell surface (2). To test whether TE4-1 could recognize NY-ESO-1 epitopes naturally processed and presented by tumor cells, several melanoma lines were used as targets. The expression of NY-ESO-1 in each line was determined by RT-PCR, while the expression of HLA-DR alleles was determined by FACS analysis (data not shown). As shown in Fig. 4, TE4-1 was capable of recognizing NY-ESO-1/HLA-DR4-positive tumors (1359 mel and F049 mel), but failed to recognize tumor cell lines 397 mel and 624.38 mel (NY-ESO-1$^+$/HLA-DR$^-$), nor 526 mel (NY-ESO-1$^-$/HLA-DR4$^+$). T cell reactivity was also detected on F050 mel (DR1$^+$/NY-ESO-1$^+$), but not on 1300 mel expressing DR1 and a low level of NY-ESO-1. One possible explanation is that CD4$^+$ T cells may recognize the same peptide presented by DR1 molecule when the peptide concentration is high. Alternatively, ESO p116–135 may bind to DR1 and subsequently released and picked up by TE4-1 and presented to each other as TE4-1 cells express DR4 molecules. The recognition of F049 mel could be specifically blocked in the presence of anti-HLA-DR Ab, but not the anti-MHC class I Ab (data not shown). These studies suggested that the TE4-1 cell line recognized a naturally processed peptide on the tumor cell surface.

*Characterization of the NY-ESO-1 epitope recognized by TE4-1*

Because the two reactive peptides shared 15 aa (LPVPGVLLKEFTVSG), the minimal length of peptide was determined by testing a series of N- and C-terminal truncated peptides. Peptides were pulsed onto DR4$^+$ 1088 EBV B cells and tested for their ability to stimulate TE4-1 cells. The valine residue at position 128 was found to be critical for T cell recognition (Fig. 5*A*). The peptides with the N-terminal deletions up to leucine residue at position 123 did not affect T cell recognition, but the peptide with further deletions partially lost its ability to stimulate T cells. The leucine residue at position 123 may be a P1 anchor residue because the P1, P4, P6, and P7 residues contributed to the peptide binding to MHC class II molecules. Further deletions are required to determine the critical residues for binding to MHC class II molecules.

Although CD4$^+$ TE4-1 cells reacted with positive peptides at a relatively high peptide concentration, we found that the ESO p119–130 peptide exhibited a higher activity and required a lower peptide concentration to achieve a similar level of T cell recognition than other two short peptides, ESO p120–130 and ESO p121–130, in a titration experiment (data not shown). Thus, we used the ESO p119–130 peptide to determine the binding affinity of the peptide recognized by TE4-1. Peptides were pulsed onto 1088EBV B cells (HLA-DR4$^+$) as targets at different peptide concentrations. As shown in Fig. 5*B*, no or little T cell activity was observed at 33 nM or lower concentrations of the ESO p119–130 peptide; high activities were detected at 0.33 $\mu$M peptide concentration, and the T cell activity did not reach a plateau at a 33 $\mu$M peptide concentration. The control peptide was not recognized by TE4-1 even at a 33 $\mu$M peptide concentration.

## Discussion

NY-ESO-1 is an important immune target because it gives rise to both humoral and cellular immune responses (19–21). Although its expression pattern is similar to Ags in the MAGE gene family, NY-ESO-1 is more frequently expressed in breast, prostate, and lung cancers than any member of the MAGE family (19, 20, 23). More interestingly, high titered NY-ESO-1-reactive Abs were frequently detected in patients with cancer (Fig. 1, *B* and *C*), while a very low percentage of patients developed high titers of Abs against the MAGE Ags or differentiation Ags such as tyrosinase, gp100, TRP-1, and TRP-2 (data not shown) (22). It is possible that Abs reacting with glycosylated proteins, such as gp100, TRP-1, and TRP-2, may not react with bacteria-derived unglycosylated proteins. However, these studies strongly suggest that NY-ESO-1-reactive CD4$^+$ T cells may be involved in Ab production and CTL proliferation. In this study, we identified the HLA-DR4-restricted T cell epitope derived from NY-ESO-1 by the use of HLA-DR4 transgenic mice and in vitro stimulation of human PBMC with candidate peptides. To our knowledge, this is the first demonstration that T cell epitopes from NY-ESO-1 were shown to be presented by HLA-DR4 (DR$\beta$1*0401) molecule to CD4$^+$ T cells. Because NY-ESO-1-specific Abs and CTL were detected in patients with different HLA genotypes, other CD4$^+$ T cell epitopes presented by HLA class II molecules other than HLA-DR4 probably exist. Indeed, after this work was submitted, a study describing the identification of NY-ESO-1 epitopes presented by DR$\beta$4*0101–0103 was reported (28).

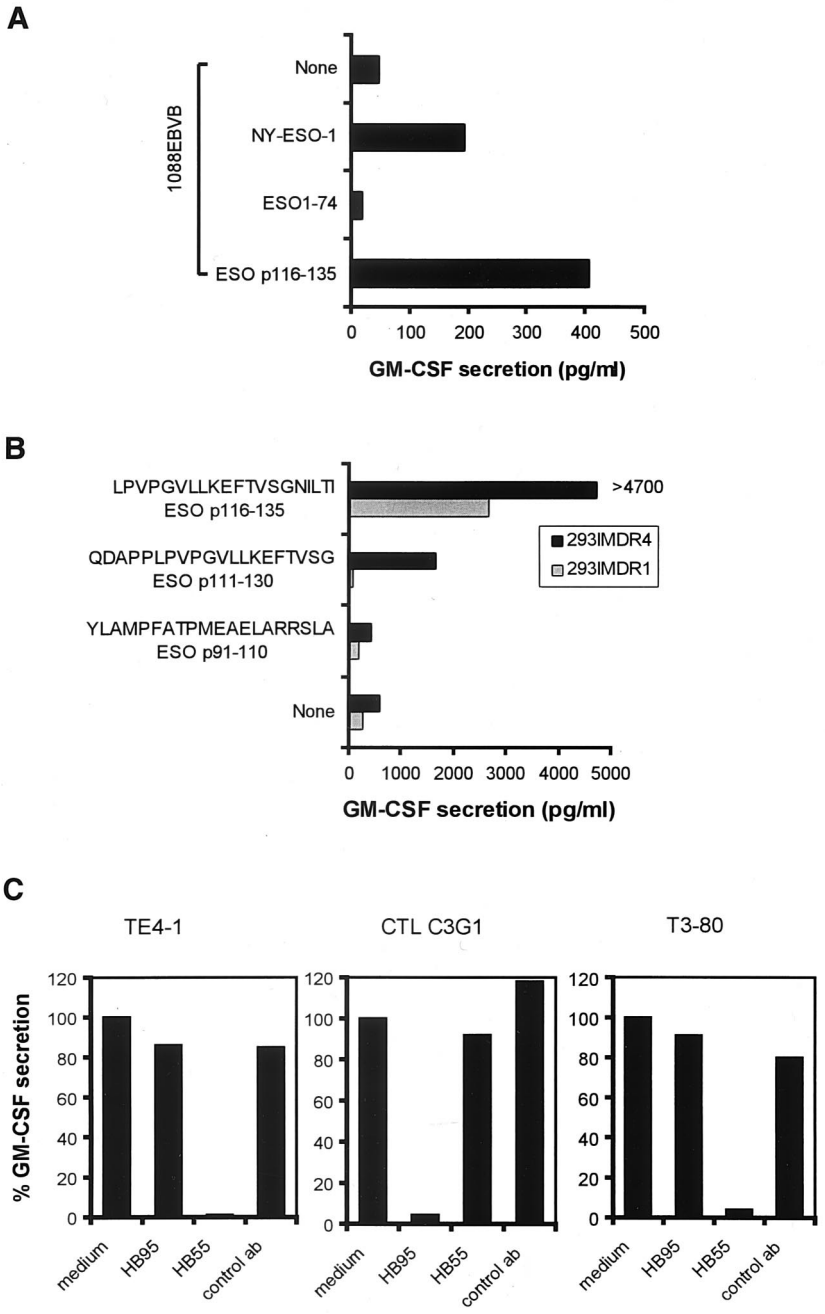


**FIGURE 3.** Characterization of the TE4-1 $CD4^+$ T cell line. *A*, TE4-1 specifically recognized 1088 EBV B cells (HLA-$DR4^+$) pulsed with ESO p116–135 peptide or purified NY-ESO-1 protein, but not ESO1–74 protein, which lacked the putative epitope. *B*, HLA-DR4 restriction was required for the recognition of NY-ESO-1 by TE4-1. Two overlapping peptides, ESO p111–130 and p116–135, were recognized when pulsed onto 293IMDR4 cells. A total of $1 \times 10^5$ target cells was cocultured with $4 \times 10^4$ TE4-1 cells overnight before GM-CSF secretion was measured. *C*, Recognition of 293IMDR4 pulsed with ESO p116–135 peptide was specifically inhibited by the anti-HLA-DR Ab (HB55), but not by the anti-class I Ab (HB95). The amount of GM-CSF secreted by TE4-1 in the absence of Abs was used as the reference, against which the percentage of GM-CSF release in the presence of Abs was calculated. Inhibition by the control (mouse IgG2a) and the anti-MHC class I Abs (HB95) had little effect. CTL C3G1 (courtesy of C. Macalli, Surgery Branch, National Cancer Institute) was a gp100-specific $CD8^+$ T cell line that recognized 624.38 mel, and was used as a control for the activity of HB95. T3-80 was a $CD4^+$ T cell line that recognized 1362 mel and was used as the control for the activity of HB55.

Recently, two groups reported the identification of MHC class II-restricted T cell epitopes from the known MHC class I-restricted tumor Ag, MAGE-3. $CD4^+$ T cell clones generated from PBMC stimulated with DC pulsed with purified MAGE-3 protein recognized peptide or protein pulsed on HLA-DR13-matched EBV B cells, but not MAGE-$3^+$/$DR13^+$ tumor cells (13). However, in another study, $CD4^+$ T cells generated from PMBC stimulated with peptides predicted by a computer-assisted algorithm were capable of recognizing both peptide pulsed on EBV B cells and MAGE-$3^+$/$DR11^+$ tumor cells (15). In the case of NY-ESO-1, we show in this study that $CD4^+$ T cells can recognize the NY-ESO-1 protein or peptide pulsed on DR4-matched EBV B cells as well as tumor cells expressing NY-ESO-1 (Figs. 3 and 4). Utilization of HLA-DR transgenic mice may have advantages in identifying putative peptides because immunized transgenic mice presumably have a high precursor frequency of specifically reactive T cells. Once candidate peptides were identified, $CD4^+$ T cells could be generated from PBMC stimulated with synthetic candidate peptides. Therefore, the combined use of transgenic mice immunized with the whole protein and stimulated with the peptides predicted by a computer-assisted algorithm may avoid the need to stimulate human PBMC with a large number of peptides and several rounds of in vitro stimulation. Furthermore, candidate peptides identified by using the immunized transgenic mice are likely to be peptides that are naturally processed and presented on the cell surface. This may increase the likelihood that peptide-specific $CD4^+$ T cells can recognize tumor cells as well. Finally, the use of PBMC from a patient (TE), who developed a high titer of Ab and a high precursor frequency of CTL against NY-ESO-1, may make it easier to generate tumor-specific $CD4^+$ T cells because both Ab production and CTL require the help of $CD4^+$ T cells. This approach has been used to identify a number of MHC class II-restricted T cell epitopes from known autoantigens involved in autoimmune disease (29). Therefore, the strategy used in this study may be applicable to many other known MHC class I-restricted tumor Ags, while other strategies such as a

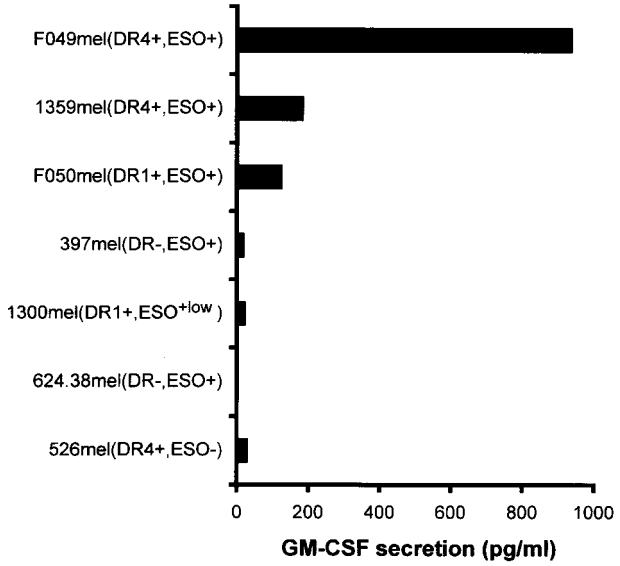


**FIGURE 4.** Recognition of tumor cells by the CD4+ T cell line TE4-1. All melanoma lines used as targets for TE4-1 were analyzed for the expression of HLA-DR4 and NY-ESO-1 by FACS and RT-PCR, respectively. TE4-1 was able to recognize NY-ESO-1+ tumor lines constitutively expressing the HLA-DR4 molecule (1359 mel and F049 mel). F050 mel expressing DR1 and NY-ESO-1 was also recognized by T cells. There was no reactivity against control targets 526 mel (DR4 positive and NY-ESO-1 negative), 397 mel, 624.38 mel (DR negative and NY-ESO-1 positive), or 1300 mel (DR1 positive and NY-ESO-1 weakly positive).

direct gene-cloning approach may facilitate the identification of unknown MHC class II-restricted tumor Ags.

In this study, we observed that ESO p116–135 was recognized by TE4-1 when pulsed on 293IMDR1 cells at a relatively high peptide concentration (Fig. 3*B*). T cell recognition was significantly reduced when peptide concentrations decreased from 33 to 3.3 $\mu$M for the peptide titration experiment (data not shown). In a separate experiment, the peptide ESO p116–135 as well as the whole NY-ESO-1 protein were weakly recognized when pulsed on DR1+ 586 EBV B cells. The recognition was specifically blocked by anti-DR Ab, but not anti-class I Ab and control Ab (data not shown). TE4-1 cells also only recognized DR1-positive tumor cell line F050 mel expressing a high level of NY-ESO-1, but not DR1-positive1300 mel expressing a low level of NY-ESO-1 (Fig. 4). Thus, this T cell recognition may be explained by two possibilities. The peptide ESO p116–135 may be promiscuous and can be recognized by TE4-1 in the context of either DR1 or DR4, but with different affinities. It has been shown that T cells can degenerately recognize peptides presented by multiple HLA class II alleles (30, 31). Alternatively, ESO p116–135 might bind to DR1 molecule. The carried-over peptide is subsequently released and presented to each other by DR4-positive TE4-1 T cells. T cell-T cell presentation has been reported in CD4+ TIL1363 (17).

Clinical trials using peptides derived from tissue-specific differentiation Ags such as gp100 showed some evidence of therapeutic efficacy in the treatment of patients with melanoma (3). Although no significant toxic side effects were observed in the patients treated with the modified gp100 peptides, vitiligo or depigmentation was often found in patients who responded to therapy (32), suggesting that antitumor immunity induced by immunization with self Ags may cause autoimmunity. In animal studies using TRP-1 as an immune target, similar results (antitumor immunity and coat depigmentation) were also obtained (33–35). Interestingly, antitumor immunity and autoimmunity mediated by gp75/TRP-1 appeared to involve CD4+ T cells and Abs (36). Immunization of mice with human TRP-2 (37), but not murine TRP-2 (38), broke tolerance to the self Ag, and the antitumor immunity required the participation of both CD4+ and CD8+ T cells (36). These studies suggested that antitumor immunity could be mediated by either Abs or CD8+ T cells, but both require the critical help of CD4+ T cells (24, 36).

The MHC class II-restricted NY-ESO-1 peptides identified in this study may be useful in clinical applications because CTL and Abs against NY-ESO-1 were detected in patients with cancer. Immunization with both MHC class I- and II-restricted peptides or

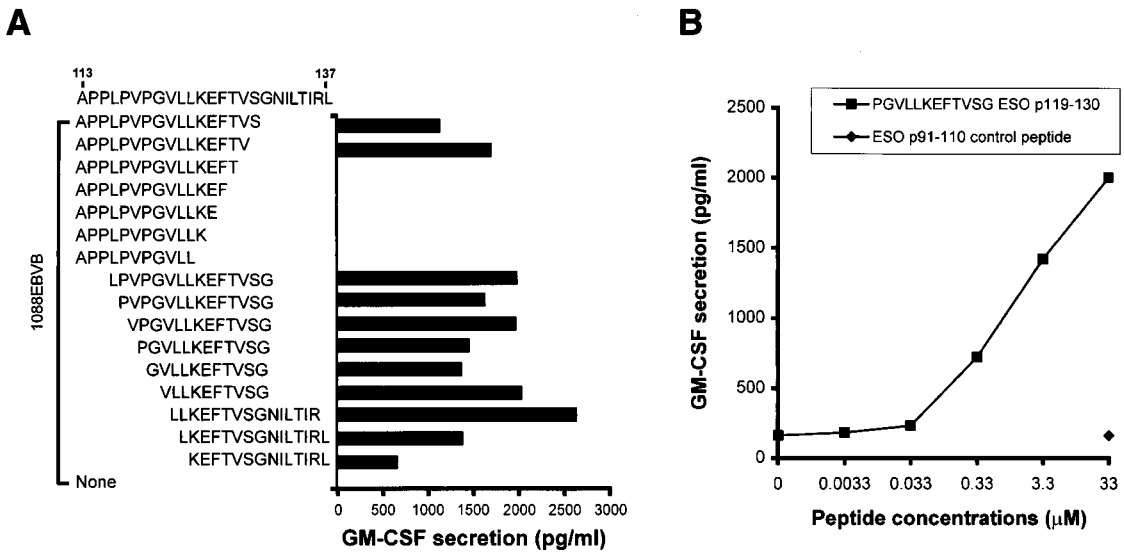


**FIGURE 5.** Characterization of the NY-ESO-1 peptide epitope recognized by TE4-1. *A*, Determination of the anchor positions for the HLA-DR4-restricted NY-ESO-1 epitope. The 1088 EBV B cells were pulsed with 20 $\mu$M of the indicated peptides. TE4-1 cells were cocultured with the target cells overnight before GM-CSF was measured. *B*, Peptide titration experiment using ESO p119–130. ESO p119–130 was chosen based on its recognition shown in *A*. ESO p119–130 diluted at the indicated concentrations were pulsed onto 1088 EBV B cells, which were used as targets for recognition by TE4-1. Recognition of a control peptide, ESO p91–110, was measured only at the highest concentration of 33 $\mu$M.

with a purified NY-ESO-1 protein may induce NY-ESO-1-specific $CD4^+$, $CD8^+$ T cells as well as Abs. Alternatively, patients could be immunized with dendritic cells loaded with both class I and II peptides or infected with recombinant viruses encoding the NY-ESO-1 gene. Because testicular germ cells do not express MHC class I and II molecules (39), immune responses against NY-ESO-1 should be specific for tumor cells, and thus generate little or no autoimmune responses. Similar studies using MHC class I-restricted peptides of MAGE-3 or peptides pulsed on dendritic cells indicated that while antitumor immunity (CTL responses) and slow tumor regression were demonstrated, no depigmentation/vitiligo or other significant side effects were observed (5, 6). Antitumor immunity may be enhanced by providing tumor-specific $CD4^+$ T cell help.

## Acknowledgments

We thank Dr. John Wunderlich and members of Tumor Infiltrating Lymphocyte Laboratory in the Surgery Branch for providing clinical samples for this study, Alicia Atwood for technical assistance, Arnold Mixon for FACS, Maria Parkhurst and John Riley for synthetic peptides, and Pat Spinella (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health) for protein N-terminal sequencing. We thank Dr. Paul Robbins for helpful discussions of the manuscript.

## References

1. Houghton, A. N. 1994. Commentary: cancer antigens: immune recognition of self and alterted self. *J. Exp. Med. 180:1.*
2. Wang, R.-F., and S. A. Rosenberg. 1999. Human tumor antigens for cancer vaccine development. *Immunol. Rev. 170:85.*
3. Rosenberg, S. A., J. C. Yang, D. J. Schwartzentruber, P. Hwu, F. M. Marincola, S. L. Topalian, N. P. Restifo, M. E. Dudley, S. L. Schwarz, P. J. Spiess, et al. 1998. Immunologic and therapeutic evaluation of a synthetic tumor-associated peptide vaccine for the treatment of patients with metastatic melanoma. *Nat. Med. 4:321.*
4. Nestle, F. O., S. Alijagic, M. Gilliet, Y. Sun, S. Grabbe, R. Dummer, G. Burg, and D. Schadendorf. 1998. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat. Med. 4:328.*
5. Marchand, M., N. van Baren, P. Weynants, V. Brichard, B. Dreno, M. H. Tessier, E. Rankin, G. Parmiani, F. Arienti, Y. Humblet, et al. 1999. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. *Int. J. Cancer 80:219.*
6. Thurner, B., I. Haendle, R. d. C. D. Dieckmann, P. Keikavoussi, H. Jonuleit, A. Bender, C. Maczek, D. Schreiner, P. von Den Driesch, E. B. Brocker, et al. 1999. Vaccination with Mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. *J. Exp. Med. 190:1669.*
7. Hung, K., R. Hayashi, A. Lafond-Walker, C. Lowenstein, D. Pardoll, and H. Levitsky. 1998. The central role of $CD4^+$ T cells in the antitumor immune response. *J. Exp. Med. 188:2357.*
8. Toes, R. E., F. Ossendorp, R. Offringa, and C. J. Melief. 1999. CD4 T cells and their role in antitumor immune responses. *J. Exp. Med. 189:753.*
9. Ossendorp, F., E. Mengede, M. Camps, R. Filius, and C. J. Melief. 1998. Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. *J. Exp. Med. 187:693.*
10. Mumberg, D., P. A. Monach, S. Wanderling, M. Philip, A. Y. Toledano, R. D. Schreiber, and H. Schreiber. 1999. $CD4^+$ T cells eliminate MHC class II-negative cancer cells in vivo by indirect effects of IFN-γ. *Proc. Natl. Acad. Sci. USA 96:8633.*
11. Topalian, S. L., M. I. Gonzales, M. Parkhurst, Y. F. Li, S. Southwood, A. Sette, S. A. Rosenberg, and P. F. Robbins. 1996. Melanoma-specific $CD4^+$ T cells recognize nonmutated HLA-DR-restricted tyrosinase epitopes. *J. Exp. Med. 183: 1965.*
12. Li, K., M. Adibzadeh, T. Halder, H. Kalbacher, S. Heinzel, C. Muller, J. Zeuthen, and G. Pawelec. 1998. Tumor-specific MHC-class-II-restricted responses after in vitro sensitization to synthetic peptides corresponding to gp100 and annexin II eluted from melanoma cells. *Cancer Immunol. Immunother. 47:32.*
13. Chaux, P., V. Vantomme, V. Stroobant, K. Thielemans, J. Corthals, R. Luiten, A. M. Eggermont, T. Boon, and P. van der Bruggen. 1999. Identification of MAGE-3 epitopes presented by HLA-DR molecules to $CD4^+$ T lymphocytes. *J. Exp. Med. 189:767.*
14. Touloukian, C. E., W. W. Leitner, S. L. Topalian, Y. F. Li, P. F. Robbins, S. A. Rosenberg, and N. P. Restifo. 2000. Identification of an MHC class II-restricted human gp100 epitope using DR4-IE transgenic mice. *J. Immunol. 164: 3535.*
15. Manici, S., T. Sturniolo, M. A. Imro, J. Hammer, F. Sinigaglia, C. Noppen, G. Spagnoli, B. Mazzi, M. Bellone, P. Dellabona, and M. P. Protti. 1999. Melanoma cells present a MAGE-3 epitope to $CD4^+$ cytotoxic T cells in association with histocompatibility leukocyte antigen DR11. *J. Exp. Med. 189:871.*
16. Wang, R.-F., X. Wang, A. C. Atwood, S. L. Topalian, and S. A. Rosenberg. 1999. Cloning genes encoding MHC class II-restricted antigens: mutated CDC27 as a tumor antigen. *Science 284:1351.*
17. Wang, R.-F., X. Wang, and S. A. Rosenberg. 1999. Identification of a novel MHC class II-restricted tumor antigen resulting from a chromosomal rearrangement recognized by $CD4^+$ T cells. *J. Exp. Med. 189:1659.*
18. Pieper, R., R. E. Christian, M. I. Gonzales, M. I. Nishimura, G. Gupta, R. E. Settlage, J. Shabanowitz, S. A. Rosenberg, D. F. Hunt, and S. L. Topalian. 1999. Biochemical identification of a mutated human melanoma antigen recognized by $CD4^+$ T cells. *J. Exp. Med. 189:757.*
19. Chen, Y. T., M. J. Scanlan, U. Sahin, O. Tureci, A. O. Gure, S. Tsang, B. Williamson, E. Stockert, M. Pfreundschuh, and L. J. Old. 1997. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc. Natl. Acad. Sci. USA 94:1914.*
20. Wang, R.-F., S. L. Johnston, G. Zeng, D. J. Schwartzentruber, and S. A. Rosenberg. 1998. A breast and melanoma-shared tumor antigen: T cell responses to antigenic peptides translated from different open reading frames. *J. Immunol. 161:3596.*
21. Jager, E., Y. T. Chen, J. W. Drijfhout, J. Karbach, M. Ringhoffer, D. Jager, M. Arand, H. Wada, Y. Noguchi, E. Stockert, et al. 1998. Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. *J. Exp. Med. 187:265.*
22. Stockert, E., E. Jager, Y. T. Chen, M. J. Scanlan, I. Gout, J. Karbach, M. Arand, A. Knuth, and L. J. Old. 1998. A survey of the humoral immune response of cancer patients to a panel of human tumor antigens. *J. Exp. Med. 187:1349.*
23. Lee, L., R.-F. Wang, X. Wang, A. Mixon, B. E. Johnson, S. A. Rosenberg, and D. S. Schrump. 1998. NY-ESO-1 may be a potential target for lung cancer immunotherapy. *Can. J. Sci. Am. 5:20.*
24. Old, L. J., and Y. T. Chen. 1998. New paths in human cancer serology. *J. Exp. Med. 187:1163.*
25. Wang, R. F., and J. I. Mullins. 1995. Mammalian cell/vaccinia virus expression vectors with increased stability of retroviral sequences in *Escherichia coli*: production of feline immunodeficiency virus envelope protein. *Gene 153:197.*
26. Ito, K., H. J. Bian, M. Molina, J. Han, J. Magram, E. Saar, C. Belunis, D. R. Bolin, R. Arceo, R. Campbell, et al. 1996. HLA-DR4-IE chimeric class II transgenic, murine class II-deficient mice are susceptible to experimental allergic encephalomyelitis. *J. Exp. Med. 183:2635.*
27. Southwood, S., J. Sidney, A. Kondo, M. F. del Guercio, E. Appella, S. Hoffman, R. T. Kubo, R. W. Chesnut, H. M. Grey, and A. Sette. 1998. Several common HLA-DR types share largely overlapping peptide binding repertoires. *J. Immunol. 160:3363.*
28. Jager, E., D. Jager, J. Karbach, Y.-T. Chen, G. Ritter, Y. Nagata, S. Gnjatic, E. Stockert, M. Arand, L. J. Old, and A. Knuth. 2000. Identification of NY-ESO-1 epitopes presented by human histocompatibility antigen (HLA)-DRB4*0101–0103 and recognized by $CD4^+$ T lymphocytes of patients with NY-ESO-1-expressing melanoma. *J. Exp. Med. 191:625.*
29. Sonderstrup, G., and H. McDevitt. 1998. Identification of autoantigen epitopes in MHC class II transgenic mice. *Immunol. Rev. 164:129.*
30. Valli, A., A. Sette, L. Kappos, C. Oseroff, J. Sidney, G. Miescher, M. Hochberger, E. D. Albert, and L. Adorini. 1993. Binding of myelin basic protein peptides to human histocompatibility leukocyte antigen class II molecules and their recognition by T cells from multiple sclerosis patients. *J. Clin. Invest. 91:616.*
31. Zeliszewski, D., P. Gaudebout, J. J. Golvano, I. Dorval, A. Prevost, F. Borras-Cuesta, and G. Sterkers. 1994. Molecular basis for degenerate T-cell recognition of one peptide in the context of several DR molecules. *Hum. Immunol. 41:28.*
32. Rosenberg, S. A., and D. E. White. 1996. Vitiligo in patients with melanoma: normal tissue antigens can be targeted for cancer immunotherapy. *J. Immunother. 19:81.*
33. Hara, I., Y. Takechi, and A. N. Houghton. 1995. Implicating a role for immune recognition of self in tumor rejection: passive immunization against the *Brown* locus protein. *J. Exp. Med. 182:1609.*
34. Weber, L. W., W. B. Bowne, J. D. Wolchok, R. Srinivasan, J. Qin, Y. Moroi, R. Clynes, P. Song, J. J. Lewis, and A. N. Houghton. 1998. Tumor immunity and autoimmunity induced by immunization with homologous DNA. *J. Clin. Invest. 102:1258.*
35. Overwijk, W. W., D. S. Lee, D. R. Surman, K. R. Irvine, C. E. Touloukian, C. C. Chan, M. W. Carroll, B. Moss, S. A. Rosenberg, and N. P. Restifo. 1999. Vaccination with a recombinant vaccinia virus encoding a "self" antigen induces autoimmune vitiligo and tumor cell destruction in mice: requirement for $CD4^+$ T lymphocytes. *Proc. Natl. Acad. Sci. USA 96:2982.*
36. Bowne, W. B., R. Srinivasan, J. D. Wolchok, W. G. Hawkins, N. E. Blachere, R. Dyall, J. J. Lewis, and A. N. Houghton. 1999. Coupling and uncoupling of tumor immunity and autoimmunity. *J. Exp. Med. 190:1717.*
37. Wang, R.-F., E. Appella, Y. Kawakami, X. Kang, and S. A. Rosenberg. 1996. Identification of TRP-2 as a human tumor antigen recognized by cytotoxic T lymphocytes. *J. Exp. Med. 184:2207.*
38. Bloom, M. B., D. Perry-Lalley, P. F. Robbins, Y. Li, M. El-Gamil, S. A. Rosenberg, and J. C. Yang. 1997. Identification of tyrosinase-related protein 2 as a tumor rejection antigen for the B16 melanoma. *J. Exp. Med. 185:453.*
39. Haas, G. G. J., O. J. D'Cruz, and L. E. D. Bault. 1988. Distribution of human leukocyte antigen-ABC and -D/DR antigens in the unfixed human testis. *Am. J. Reprod. Immunuol. Microbiol. 18:47.*