Spontaneous CD4$^+$ T Cell Responses against TRAG-3 in Patients with Melanoma and Breast Cancers[1]

Bratislav Janjic,* Pedro Andrade,* Xiao-Fei Wang,[§] Julien Fourcade,* Christine Almunia,[§] Pavol Kudela,*[‡] Adam Brufsky,* Samuel Jacobs,* David Friedland,* Ronald Stoller,* Daniel Gillet,[§] Ronald B. Herberman,* John M. Kirkwood,* Bernard Maillere,[§] and Hassane M. Zarour[2*†]

The taxol resistance gene *TRAG-3* was initially isolated from cancer cell lines that became resistant to taxol in vitro. TRAG-3 is a cancer germline Ag expressed by tumors of different histological types including the majority of melanoma, breast, and lung cancers. In the present study, we report that patients with stage IV melanoma and breast cancers developed spontaneous IFN-γ-producing CD4$^+$ T cell responses against a single immunodominant and promiscuous peptide epitope from TRAG-3 presented in the context of multiple HLA-DR molecules. The TRAG-3-specific CD4$^+$ T cells and clones were expanded in vitro and recognized not only peptide pulsed APCs but also autologous dendritic cells (DCs) loaded with the TRAG-3 protein. All stage IV melanoma patients with TRAG-3-expressing tumors developed spontaneous CD4$^+$ T cell responses against TRAG-3, demonstrating its strong immunogenicity. None of these patients had detectable IgG Ab responses against TRAG-3. *TCRβ* gene usage studies of TRAG-3-specific CD4$^+$ T cell clones from a melanoma patient and a normal donor suggested a restricted TCR repertoire in patients with TRAG-3-expressing tumors. Altogether, our data define a novel profile of spontaneous immune responses to cancer germline Ag-expressing tumors, showing that spontaneous TRAG-3-specific CD4$^+$ T cells are directed against a single immunodominant epitope and exist independently of Ab responses. Because of its immunodominance, peptide TRAG-3$_{34-48}$ is of particular interest for the monitoring of spontaneous immune responses in patients with TRAG-3-expressing tumors and for the development of cancer vaccines. *The Journal of Immunology,* 2006, 177: 2717–2727.

The CD4$^+$ Th cells play a crucial role in B cell activation for Ab production and in the generation and the maintenance of Ag-specific effector CD8$^+$ T cells (1–4). Therefore, the identification of Th epitopes is of first importance not only for the study of spontaneous Ag-specific immune responses in patients with cancer but also for the development of cancer vaccines with Th epitopes that may optimally stimulate antitumor CD8$^+$ memory T cells. We and others (5–15) have developed strategies to identify novel MHC class II epitopes capable of stimulating Th1-type tumor Ag-specific CD4$^+$ T cells. These efforts have led to the identification of a number of epitopes that are currently used in phase I/II trials peptide-based vaccines.

We have focused our research efforts on the discovery of epitopes from cancer germline Ags (CGAs),[3] expressed by tumors of different histological types but not by normal tissues, except testis. Because the germ cells in testis do not express MHC class I and class II molecules (16), the CGA-derived epitopes are specifically expressed by tumor cells. CGAs appear to give rise to multiple MHC class II-restricted epitopes recognized by CD4$^+$ T cells (8–10, 12, 17, 18). In patients with active cancers, CGAs may spontaneously stimulate Abs and circulating CD4$^+$ T cells that are most often detectable only after one round of in vitro stimulation with peptide-pulsed APCs (18, 19). Whenever detectable, these CGA-specific CD4$^+$ T cells appeared to be associated with the presence of circulating Ag-specific Abs (18). However, few correlative analyses of Ab and T cell responses have been performed, and more studies are needed to better understand the immunological basis of spontaneous immune responses to tumor Ags in cancer patients and to further optimize cancer vaccines strategies.

Here, we have investigated the spontaneous CD4$^+$ T cell and Ab responses in patients with active solid tumors that commonly expressed the taxol resistance-associated gene-3 (*TRAG-3*), including melanoma, breast, and lung cancers. TRAG-3 is expressed by a wide range of solid tumors including 78% of melanoma cell lines and all tested melanoma tumor samples (20), 60% of breast cancers (21), and 54% of non-small cell lung carcinoma (NSCLC) (22). The *TRAG-3* gene was originally isolated from tumor cell lines that became resistant to taxol in vitro (23) and is induced by demethylation of tumor cell lines (24). Multiple HLA-A2-presented peptides capable of stimulating CD8$^+$ T cells from PBL of normal donors or patients with tumors have been reported (25, 26). However, only the epitope TRAG-3$_{56-68}$ successfully stimulated CTLs that recognized and lysed TRAG-3-expressing tumor cells. To date, no MHC class II epitope from TRAG-3 has yet been reported.

*Department of Medicine and Division of Hematology/Oncology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213; [†]Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213; [‡]Cancer Research Institute, Slovak Academy of Sciences, Bratislava, Slovakia; and [§]Protein Engineering and Research Department, CEA-Saclay, Gif-sur-Yvette, France

Received for publication March 29, 2006. Accepted for publication June 2, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Institutes of Health/National Cancer Institute Grants CA90360 and CA112198 (to H.M.Z.), a Clinical Trial Grant/Melanoma Initiative from the Cancer Research Institute (to H.M.Z.), and a grant from the Association pour la Recherche sur le Cancer (to B.M.).

[2] Address correspondence and reprint requests to Dr. Hassane M. Zarour, Hillman Cancer Center, Research Pavilion, University of Pittsburgh Cancer Institute, Suite 1.32, 5117 Centre Avenue, Pittsburgh, PA 15213. E-mail address: zarourhm@upmc.edu

[3] Abbreviations used in this paper: CGA, cancer germline Ag; *TRAG,* taxol resistance gene; UPCI, University of Pittsburgh Cancer Institute; DC, dendritic cells; NSCLC, non-small cell lung carcinoma; IC$_{50}$, concentration of peptide that prevented binding of 50% of the labeled peptide.

0022-1767/06/$02.00

In this study, we observed that patients with stage IV melanoma and breast cancer developed IFN-γ-producing CD4+ T cell responses against a single immunodominant and promiscuous epitope from TRAG-3. All patients with stage IV melanoma and *TRAG-3*-expressing tumors had detectable TRAG-3-specific CD4+ T cells, but no circulating *TRAG-3*-specific Ab. Our findings add to the diversity of the spontaneous immune responses to CGA-expressing tumors observed in patients with advanced cancers. They also provide a good candidate for the monitoring of spontaneous immune responses in patients with TRAG-3-expressing tumors and for the development of *TRAG-3*-based cancer vaccines.

Materials and Methods

Cell lines, media, and Abs

Tissues and blood samples utilized for all studies reported in this araticle were obtained under the University of Pittsburgh Cancer Institute (UPCI) Institutional Review Board-approved protocols 96-099 and 99-088. 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 (Dylan). *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 (8, 27). All cell lines were cultured in RPMI 1640 (Invitrogen) supplemented with 10% FCS, L-arginine (116 mg/L), L-asparagine (36 mg/L), and L-glutamine (216 mg/L). The HB55 and HB95 hybridomas, secreting the L243 anti-HLA-DR (class II) mAb and the W6/32 anti-HLA-A, B, 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 (28).

Peptide synthesis

The *TRAG-3*-derived peptides were synthesized and stored as previously reported (8, 9). Synthesis of TRAG-3 peptides was based on the protein sequence encoded by the TRAG-3 cDNA obtained from three melanoma cell lines as detailed below.

Recombinant proteins

The TRAG-3 recombinant protein was expressed in *Escherichia coli* strain Bl21 (DE3) from a TRAG-3 cDNA cloned in vector pTriEx (Novagen) as described under *TRAG-3 cDNA sequencing*. The protein was extracted from inclusion bodies in the presence of 8 M urea and purified by ion metal affinity chromatography using the 6-his tag at the N terminus of the protein. After elution, the protein sample was dialyzed against a buffer containing 2 M urea, 50 mM NaCl, and 20 mM Tris-HCl, pH 8. The TRAG-3 protein was concentrated up to 5 mg/ml. The full length NY-ESO-1 recombinant protein was produced in *E. coli* and was kindly provided by Drs. Lloyd J. Old and Gerd Ritter (Ludwig Institute for Cancer Research, New York Branch, New York, NY).

TRAG-3 cDNA sequencing

Total RNA was extracted from the three melanoma tumor cell lines UPCI-Mel 285, UPCI-Mel 558, and UPCI-Mel 527.1 using the RNeasy Mini Kit (Qiagen) and following the manufacturer's instructions. Reverse transcription was conducted as previously reported (29). The PCRs were performed in the presence of AmpliTaq DNA polymerase (Applied Biosystems), using the TRAG-3-specific forward primer, HZ30 (5′-TGTGGATGGGCCT CATCCAATTAGTT-3′) and reverse primer, HZ27 (5′-AGCGGCG GTCTTTTATGGAGAGC-3′). Both primers were designed according to the published TRAG-3 cDNA sequence (23). The PCR amplification was conducted in 50 μl as follows: 5 min at 94°C for 5 min; then 32 cycles (1 min at 94°C, 1 min at 60°C, 1 min at 72°C); and finally 10 min at 72°C. The PCR products were run on 1.5% agarose gel, purified using the QIAquick gel extraction kit (Qiagen), and sequenced using the ABI 3100 automated DNA sequencer.

Construction of pTriEx4-neo-TRAG-3 plasmid

TRAG-3 cDNA was amplified with RT-PCR from the melanoma cell line UPCI-MEL 285 using pfu DNA polymerase (Stratagene Cloning System), and the previously described TRAG-3-specific primers HZ30 and HZ27. The PCR amplification was conducted in 50 μl as follows: 5 min at 94°C for 5 min; then 32 cycles (1 min at 94°C, 1 min at 60°C, 1 min at 72°C); and finally 10 min at 72°C. The PCR product was phosphorylated and then run on a 1% agarose gel before purification using QIAquick (Qiagen). pTriEx4-neo eucaryotic expression vector (Novagen) was digested with *Sma*I restriction enzyme (New England Biolabs), dephosphorylated, and used for ligation with the *TRAG-3* cDNA before transformation of NovaBlue Singles Competent Cells using a Clonables Ligation/Transformation Kit (Novagen) as recommended by the manufacturer.

HLA-DR peptide-binding assays

HLA-DR and HLA-DP were purified from homozygous EBV cell lines by affinity chromatography using the monomorphic mAb L243 and B7/21, respectively, which were coupled to protein A-Sepharose CL4B gel (Amersham Pharmacia Biotech) as previously described (30, 31). The binding to the multiple HLA-DR and HLA-DP4 molecules was performed as previously reported (30–32). 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 (31, 32). Data were expressed as the concentration of peptide that prevented binding of 50% of the labeled peptide (IC_{50}).

Induction of CD4+ T cells with peptides

The induction of CD4+ T cells in vitro with dendritic cells (DCs) and the *TRAG-3*-derived peptides was performed as previously reported (7, 9). The CD4+ 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+ T cell cultures was analyzed in IFN-γ and IL-5 ELISPOT assays. The APCs used in ELISPOT assays were either autologous T blasts or 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). The CD4+ T cells were cloned by limiting dilution using allogeneic PBL and EBV-B cells as feeders in the presence of IL-2 and PHA and subsequently tested for specificity in IFN-γ ELISPOT and cytokine release assays. The CD4+ T cell clones were maintained by restimulation every 2 wk, by alternating irradiated allogeneic PBL and EBV-B cells or autologous peptide-pulsed DCs as stimulators.

In vitro sensitization with peptides

In vitro sensitization of CD4+ T cells purified from PBLs of patients and normal donors was performed as previously described (33). Briefly, CD4+ T cells were purified with immunomagnetic beads (Miltenyi Biotec). Two million PBLs previously depleted of CD8+ and CD4+ T cells, were pulsed with 10 μg/ml of peptide TRAG-3_{34-48} (4 h, 37°C), irradiated (3000 rads), washed, and used to stimulate 2×10^6 autologous CD4+ T cells in Iscove's medium (Mediatech) supplemented with 10% human AB serum (Sigma-Aldrich). After 8 h of incubation, 10 U/ml IL-2 and 5 ng/ml IL-7 were added to the culture wells, and this was repeated every 3–4 days thereafter. The remaining fraction of CD4+ and CD8+ T cells was used to prepare T-APCs. The T-APC were harvested and used as target cells in IFN-γ and IL-5 ELISPOT assays.

IFN-γ and IL-5 ELISPOT assays

The recognition of APCs pulsed with peptides or proteins by the *TRAG-3*-specific CD4+ T cells and clones expanded in vitro was assessed by ELISPOT assays specific for human IFN-γ and IL-5 as previously reported (8, 9). The protein-loaded DCs were prepared as previously reported in the presence of the recombinant TRAG-3 or NY-ESO-1 proteins (30 μg/ml). In the blocking experiments, 3 μg each of purified anti-HLA mAb were added in each ELISPOT well.

The recognition of T-APCs pulsed with peptides by CD4+ cells isolated from cancer patients and in vitro sensitized as previously in this article was assessed by IFN-γ and IL-5 ELISPOT assays. Briefly, 10^5 presensitized CD4+ T effector cells and 10^5 targets (T-APCs) pulsed with peptides (10 μg/ml) were added to each well and incubated for 48 h in AIM V medium (Invitrogen). Spot numbers and spot sizes were determined with computer-assisted video image analysis (Cellular Technologies). For statistical evaluation, a Student t test for unpaired samples was used. $p < 0.05$ was considered significant.

IFN-γ and IL-4 cytokine secretion assays

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

ELISA

Recombinant proteins were diluted in coating buffer (15 mM Na_2CO_3, 30 mM $NaHCO_3$ (pH 9.6), with 0.02% NaN_3) to the concentration of 1 μg/ml and adsorbed to polypropylene flat-bottom 96-well plates (Corning-Costar) overnight at 4°C. Plates were washed with PBS and saturated overnight at 4°C by addition of 200 μl/well PBS containing 2% BSA. After the plates were washed, serum was diluted in PBS-2% BSA buffer and added to the plates (50 μl/well). After 2 h of incubation at room temperature, plates were washed, the secondary Ab (goat anti-human IgG-AP; Caltag Laboratories) was added (50 μl/well), and the plates were further incubated for 1 h at room temperature. Following additional washes, 50 μl/well substrate solution (phosphatase substrate system; Kirkegaard & Perry Laboratories) were added, and plates were incubated for 25 min at room temperature and read immediately (MRX Microplate Reader; Dynatech). Sera were tested over a range of 4-fold dilutions as previously described (34).

TCRβ *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. A panel of 24 specific primers complementary to TCR β-chain variable and a primer for the constant region were used to determine the diversity of the TCR β-chain variable repertoire (35). The PCR amplification was conducted in 50 μl as follows: 5 min at 94°C for 5 min, then 40 cycles (1 min at 94°C, 1 min at 60°C, 1 min at 72°C) and finally 10 min at 72°C. The PCR products were run on 1.5% agarose gel and purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA). The PCR product was cloned into pCR4-TOPO vector (Invitrogen, Carlsbad, CA) and sequenced using the ABI 3100 automated DNA sequencer.

Results

Peptide TRAG-3$_{34-48}$ is a novel promiscuous epitope that binds to multiple HLA-DR molecules

Based on the published TRAG-3 cDNA sequence, we have designed specific oligonucleotide primers and used proof-reading polymerase to clone the *TRAG-3* cDNA from three different melanoma cell lines as previously described in *Materials and Methods*. We have found that all three cDNA sequences were identical and slightly different from the previously reported TRAG-3 cDNA: G instead of A at position 251; T instead of A at position 263; A instead of T at position 264; and A instead of G at position 340 (23). These nucleotide differences result in a 3-aa difference at the protein level: R instead of H at position 33; L instead of H at position 37; and T instead of A at position 63. On the basis of the new TRAG-3 sequence, we have chosen 15 overlapping 15-mer peptides. All the peptides include an aliphatic or aromatic residue in positions 1–5 because their presence at these positions is a requisite for the binding to HLA-DR and HLA-DP4 molecules. Indeed, aliphatic and aromatic residues are accommodated by the P1 pocket of these molecules and constitute the main anchor residue (32, 36). We have then synthesized the 15 overlapping peptides and evaluated their binding capacities to 10 different HLA-DR and 2 HLA-DP4 molecules including the 7 molecules encoded by the *HLA-DRB1* genes (i.e., *HLA-DRB1*0101, HLA-DRB1*0301, HLA-DRB1*0401, HLA-DRB1*0701, HLA-DRB1*1101, HLA-DRB1*1301,* and *HLA-DRB1*1501*), three molecules encoded by the *HLA-DRB3, HLA-DRB4*, and *HLA-DRB5* genes and the HLADPB1*0401 and -0402 molecules. The data are presented in Table I.

As compared with the IC_{50} values obtained with reference peptides that are defined as good binders, high and moderate binding was found for three distinct amino acid regions that exhibit significant binding to at least one HLA-DR molecule. In the N-terminal portion of TRAG-3, peptide TRAG-3$_{1-15}$ bound only to HLA-DRB1*0101. In the 38–66 region, each of the four overlapping peptides bound to two different MHC class II molecules, namely HLA-DRB1*0101, -DRB1*1101, -DRB5*0101, -DPB1*0401, and -DPB1*0402. Only one region (28–48) was composed of peptides that exhibit binding activities for a substantial number of MHC class II molecules. More precisely, peptide TRAG-3$_{34-48}$ was capable of binding very efficiently to multiple HLA-DR alleles, namely, *HLA-DRB1*0101, HLA-DRB1*0401, HLA-DRB1*0701, HLA-DRB1*1101, HLA-DRB1*1501,* and *HLA-DRB5*0101* (*HLA-DR51*).

We next performed a series of additional experiments to investigate the immunogenicity of these peptide sequences.

Peptide TRAG-3$_{34-48}$ was presented by multiple HLA-DR molecules to stimulate $CD4^+$ T cells from two melanoma patients and two normal donors

In an independent series of in vitro experiments, we primed $CD4^+$ T cells from 2 melanoma patients (patients 1 and 2) and 2 normal donors (normal donors 1 and 2) against each of the 15 overlapping

Table I. *Binding capacities to MHC class II molecules of overlapping peptides from TRAG-3*[a]

	HLA II Alleles											
Peptides	*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 B4*0101*	*HLA-DR B5*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	2	467	29	10	11	837	52	10	30	7	8	7
P_{1-15}	**794**	—	—	—	2939	—	—	—	—	—	—	—
P_{7-21}	—	—	—	—	—	—	—	—	—	—	—	—
P_{17-31}	—	—	—	—	—	—	—	—	—	—	—	—
P_{22-36}	2117	—	—	—	—	—	—	—	—	7000	—	—
P_{28-42}	**36**	**540**	**511**	1480	1068	**671**	—	—	4472	**160**	1249	—
P_{34-48}	**21**	—	**209**	**319**	**805**	—	**62**	—	—	**295**	—	1800
P_{38-52}	**775**	—	**7483**	6693	1691	—	—	—	—	**92**	6050	1876
P_{43-57}	7697	—	—	—	—	—	—	—	—	3146	**414**	**760**
P_{48-62}	—	—	—	—	1400	5848	—	—	—	**185**	**780**	1500
P_{52-66}	2191	—	—	—	**40**	—	—	1500	—	—	**442**	1949
P_{57-71}	—	—	—	—	—	—	—	—	—	—	—	—
P_{63-77}	—	—	—	—	—	—	—	—	—	—	—	—
P_{76-90}	—	—	—	—	—	—	—	—	—	—	—	—
P_{87-101}	—	—	—	—	—	—	—	—	—	—	—	—
P_{96-110}	—	—	—	—	—	—	—	—	—	—	—	—

[a]Peptides (P) encompassing the whole sequence of the TRAG-3 Ag have been submitted to MHC class II binding assays. Data are expressed as IC_{50} (nanomolar concentration) and are the means of three experiments. A reference peptide was used to validate each assay. This peptide is the nonbiotinylated form of the biotinylated peptide used in the assay. Active peptides have a IC_{50} inferior to 1000 nM and are bold. — indicates no binding observed at the maximum concentration of 10,000 nM.

peptide sequences from TRAG-3 as previously reported and described in *Materials and Methods* (7, 9). Patients 1 and 2 have been genotyped as *HLA-DRB1*0101$^+$/DRB1*0401$^+$/DRB4*0101$^+$/DPB1*0401$^+$*, and *-DRB1*0401$^+$/DRB1*1101$^+$/DRB4*0101$^+$/DPB1*0401$^+$*, respectively. Normal donor 1 and normal donor 2 have been genotyped as *HLA-DRB1*0701$^+$/DRB1*1101$^+$/DRB4*0101$^+$/DPB1*0401$^+$* (donor 1) and *HLA-DRB1*0401$^+$/DRB1*1401$^+$/DRB4*0101$^+$/DPB1*0402$^+$* (donor 2), respectively.

Only peptide TRAG-3_{34-48} was capable of stimulating IFN-γ-producing CD4$^+$ T cells derived from PBLs of the two melanoma patients and the two normal donors (Fig. 1).

Bulk CD4$^+$ T cells isolated from patient 1 (UPCI-MEL 285) and stimulated with peptide TRAG-3_{34-48} produced both IFN-γ and IL-5 spots in the presence of L.DR1 cells and L.DR4 cells pulsed with peptide TRAG-3_{34-48} but not with an irrelevant peptide (NY-ESO-$1_{119-143}$) (Fig. 2, *A* and *B*). These CD4$^+$ T cells also displayed reactivity against autologous DCs previously loaded with the TRAG-3 protein but not with an irrelevant protein (NY-ESO-1). This reactivity was partially inhibited by the addition of the anti-HLA-DR mAb (L243) but not the anti-HLA-A, B, C mAb (w6/32).

Bulk CD4$^+$ T cells isolated from normal donor 1 and stimulated with peptide TRAG-3_{34-48} peptide specifically recognized L.DR7 cells but not L.DR11 cells or L.DP4 cells pulsed with peptide TRAG-3_{34-48} in IFN-γ and IL-5 ELISPOT assays (Fig. 2, *C* and *D*). The CD4$^+$ T cells failed to produce significant amount of IFN-γ or IL-5 in the presence of APCs pulsed with peptide NY-ESO-$1_{119-143}$, used as an irrelevant peptide. These CD4$^+$ T cells displayed reactivity against autologous DCs previously loaded with the TRAG-3 protein that was inhibited by addition of the anti-HLA-DR mAb (L243) but not the anti-HLA-A, B, C mAb (w6/32). These CD4$^+$ T cells did not recognize autologous DCs loaded with the irrelevant protein NY-ESO-1 protein.

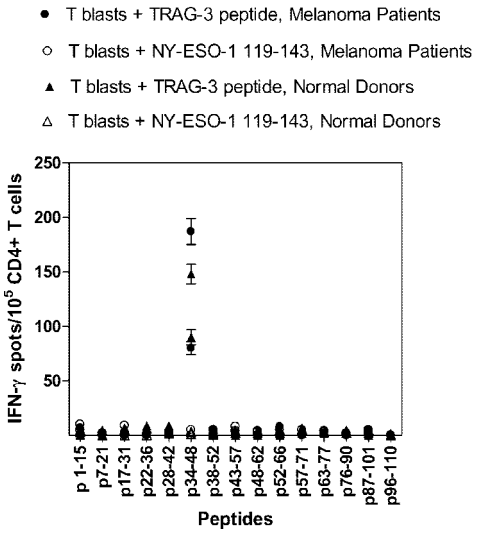

FIGURE 1. TRAG-3_{34-48} is the only TRAG-3-derived MHC class II-binding epitope capable of stimulating CD4$^+$ T cells from two melanoma patients and two normal donors. CD4$^+$ T cells isolated from 2 melanoma patients and 2 normal donors underwent 3 rounds of in vitro stimulation with autologous DCs pulsed with each of the 15 TRAG-3 overlapping peptides reported in Table I. Ten thousand of the responder CD4$^+$ T cells were incubated in 48-h IFN-γ ELISPOT assays in the presence of T blasts pulsed with either the relevant TRAG-3 peptide (p) or peptide NY-ESO-$1_{119-143}$ as control (10 μg/ml). Spots were developed and counted by computer-assisted video image analysis. Each symbol represents the mean spot number of triplicates with 10^4 CD4$^+$ T cells initially seeded per well.

Bulk CD4$^+$ T cells isolated from normal donor 2 and stimulated with peptide TRAG-3_{34-48} peptide produced IFN-$\gamma$and IL-5 in the presence of L.DR4 cells but not L.DP4 cells pulsed with peptide TRAG-3_{34-48} (Fig. 2, *D* and *E*). The CD4$^+$ T cells failed to produce significant amount of IFN-γ or IL-5 in the presence of APCs pulsed with peptide NY-ESO-$1_{119-143}$, used as an irrelevant peptide. These CD4$^+$ T cells displayed reactivity against autologous DCs previously loaded with the TRAG-3 protein but not with an irrelevant protein (NY-ESO-1). This reactivity was inhibited by addition of the anti-HLA-DR mAb (L243) but not the anti-HLA-A, B, C mAb (w6/32).

Altogether, our in vitro data demonstrate the ability of peptide TRAG-3_{34-48} to stimulate CD4$^+$ T cells capable of recognizing peptide TRAG-3_{34-48} in the context of the HLA-DRB1*0101, -DRB1*0401, and -DRB1*0701 molecules. Importantly, these TRAG-3_{34-48} CD4$^+$ T cells recognized autologous DCs loaded with the relevant protein.

*Peptide TRAG-3_{34-48} stimulated HLA-DRB1*0101 and -DRB1*0701-restricted CD4$^+$ T cell clones from a melanoma patient and a normal donor that recognized autologous protein-pulsed DCs*

Several clones were obtained by limiting dilution from the CD4$^+$ bulk T cells of patient 1 and normal donor 1 that recognized the TRAG-3_{34-48} peptide.

Clone 61/58 derived from PBLs of patient 1 specifically produced IFN-γ but not IL-5 in the presence of L.DR1 cells pulsed with peptide TRAG-3_{34-48} and autologous DCs loaded with the TRAG-3 protein in IFN-γ ELISPOT assays (Fig. 3*A*). The reactivity of this Th1-type CD4$^+$ T cell clone against autologous TRAG-3-loaded DCs was partially inhibited by the addition of the anti-HLA-DR mAb (L243) but not the anti-HLA-A, B, C mAb (w6/32). No significant amount of IFN-γ production was observed in the presence of peptide TRAG-3_{34-48} alone, other L.DR cells pulsed with peptides, or autologous DCs fed with an irrelevant protein (NY-ESO-1).

CD4$^+$ T cell clone 61/49 was derived from patient 1 and produced IFN-γ but not Th2-type cytokines (i.e., IL4, IL-5, and IL-10) in the presence of autologous DCs pulsed with peptide TRAG-3_{34-48} or loaded with the TRAG-3 protein but not with an irrelevant protein (NY-ESO-1) in IFN-γ-secretion assays (Fig. 3*B*). Autologous DCs pulsed with an irrelevant peptide (i.e., NY-ESO-$1_{119-143}$), and DCs loaded with the NY-ESO-1 protein served as baseline and controls.

Clone 62/4 derived from PBLs of normal donor 1 produced not only IFN-γ but also IL-5 in ELISPOT assays in the presence of L.DR7 pulsed with peptide TRAG-3_{34-48} and autologous DCs fed with the TRAG-3 protein (Fig. 3, *C* and *D*). No significant amount of IFN-γ production was observed in the presence of peptide TRAG-3_{34-48} alone, other L.DR cells pulsed with peptides, or autologous DCs fed with an irrelevant protein (NY-ESO-1). The ability of this Th0-type CD4 T cell clone to produce IFN-γ in the presence L.DR7 cells, preincubated with various concentrations of peptide TRAG-3_{34-48}, was evaluated. Half-maximal stimulation of clone 62/4 required peptide loading concentrations of ~600 nM (Fig. 3*E*).

We also observed that the peptide sequence TRAG-3_{34-48} 37H (with H instead of L at position 37) encoded by the previous reported TRAG-3 cDNA sequence (23) was not recognized by our CD4$^+$ T cell clones (Fig. 3, *A, C,* and *D*).

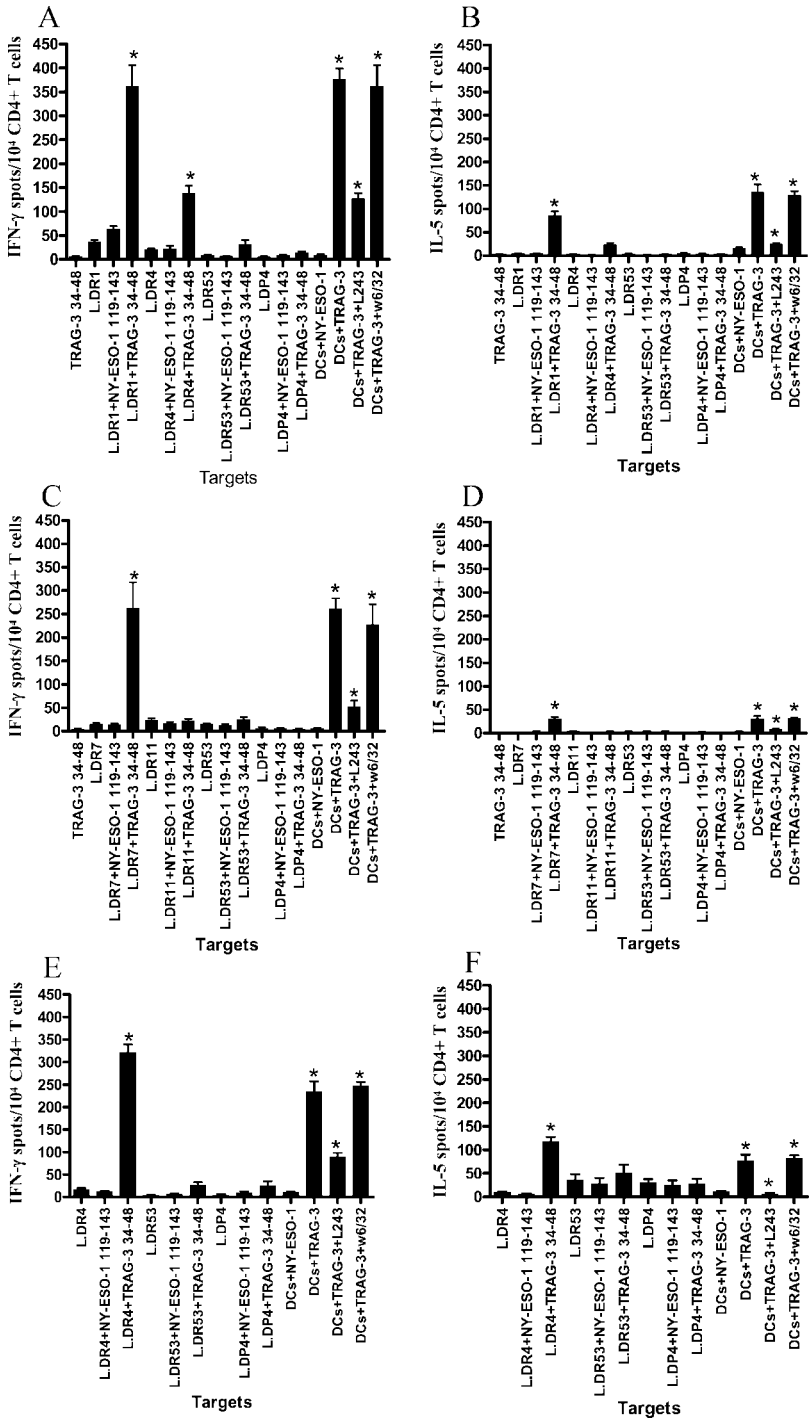

FIGURE 2. Peptide TRAG-3_{34-48} stimulates $CD4^+$ T cells from one melanoma patient and two normal donors that recognized peptide-pulsed APCs and autologous DCs loaded with the TRAG-3 protein. $CD4^+$ T cells isolated from: and melanoma patient 1 (*A* and *B*), normal donor 1 (*C* and *D*), and normal donor 2 (*E* and *F*) underwent four rounds of in vitro stimulation with autologous DC pulsed with peptide TRAG-3_{34-48}. Ten thousand of the resulting responder $CD4^+$ T cells were incubated in 48-h IFN-γ and IL-5 ELISPOT assays in the presence of L.DR1, LDR4, L.DR7, L.DR11, L.DR53, or L.DP4 cells pulsed with either peptide TRAG-3_{34-48} or peptide NY-ESO-$1_{119-143}$ (10 μg/ml). The $CD4^+$ T cells were also incubated in the presence of the autologous DCs loaded with either the TRAG-3 protein or the NY-ESO-1 protein. Spots were developed and counted by computer-assisted video image analysis. Each bar represents the mean spot number of triplicates ± SD with 10^4 $CD4^+$ T cells initially seeded per well. *, $p < 0.05$ were considered significant. Data from one representative experiment of three performed is depicted.

Detection of TRAG-3-specific IFN-γ producing $CD4^+$ T cells in the peripheral blood of melanoma and breast cancer but not lung cancer patients

We next wanted to address whether TRAG-3 could stimulate spontaneous $CD4^+$ T cell and Ab responses from the blood of patients with TRAG-3-expressing tumors. Therefore, we investigated the blood of 12 patients with melanoma, 13 patients with breast cancers, 14 patients with lung cancers, and 9 normal donors for the presence of $CD4^+$ T cells capable of recognizing peptide TRAG-3_{34-48} and peptide HA_{60-73} as control (Table II). $CD4^+$ T cells were first isolated from PBLs and stimulated with peptide-pulsed APCs for one round of in vitro stimulation before IFN-γ, IL-5, and IL-10 ELISPOT assays, as described in *Materials and Methods*.

We observed TRAG-3-specific $CD4^+$ T cells from 9 of 12 patients with stage IV melanoma (Fig. 4*A*). All of the nine patients with TRAG-3-specific $CD4^+$ T cells had TRAG-3-expressing melanoma as assessed by RT-PCR and shown in Table II. These TRAG-3-specific $CD4^+$ T cells were Th1-type $CD4^+$ T cells for six of nine patients and Th0-type $CD4^+$ T cells for the remaining three patients. We also detected HA-specific $CD4^+$ T cells in 11 of the 12 melanoma patients (Fig. 4*D*). Four of thirteen patients with breast cancers had circulating IFN-γ-producing (i.e., Th-1 type) TRAG-3-specific $CD4^+$ T cells (Fig. 4*B*). Two of the patients with TRAG-3-specific $CD4^+$ T cells had stage IV disease, and two had stage III disease. HA-specific $CD4^+$ T cell responses were evaluated in 9 of the 13 breast cancer patients and were detectable in

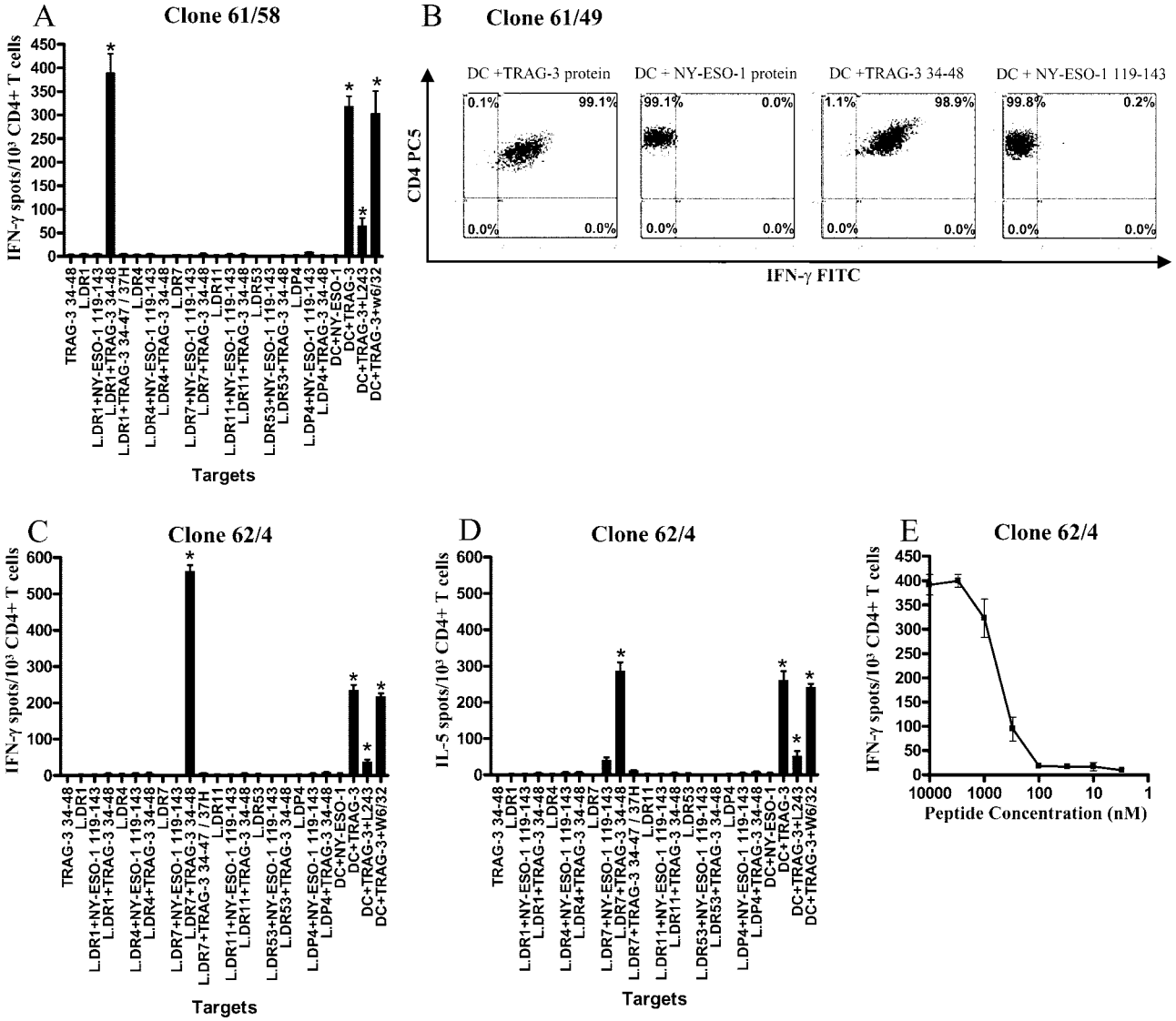

FIGURE 3. Peptide TRAG-3_{34-48} stimulates HLA-DR-restricted $CD4^+$ T cell clones. The $CD4^+$ T cell clones 61/58, 61/49, and 62/4 were obtained by limiting dilution from the TRAG-3_{34-48}-specific bulk $CD4^+$ T cells of patient 1 and donor 1, respectively. *A,* 1000 $CD4^+$ T cells from clone 61/58 were incubated in 48-h IFN-γ ELISPOT assays in the presence of peptide NY-ESO-1_{34-48} alone (10 μg/ml), L.DR cells or autologous DCs pulsed with either TRAG-3_{34-48} (10 μg/ml) or peptide NY-ESO-$1_{119-143}$ (10 μg/ml), and autologous DCs previously loaded with protein TRAG-3 (30 μg/ml) or protein NY-ESO-1 (30 μg/ml). *B,* Clone 61/49 was incubated in IFN-γ and IL-4 cytokine release assays in the presence of autologous DCs pulsed with either peptide TRAG-3_{34-48} (10 μg/ml), peptide NY-ESO-$1_{119-143}$ (10 μg/ml), protein TRAG-3 (30 μg/ml), or protein NY-ESO-1 (30 μg/ml). One thousand $CD4^+$ T cells from clone 62/4 were incubated in 48-h IFN-γ (*C*) and IL-5 (*D*) assays in the presence of peptide TRAG-3_{34-48} alone (10 μg/ml), L.DR cells or autologous DCs pulsed with either peptide TRAG-3_{34-48} (10 μg/ml) or peptide NY-ESO-$1_{119-143}$ (10 μg/ml), and autologous DCs previously loaded with protein TRAG-3 (30 μg/ml) or protein NY-ESO-1 (30 μg/ml). *E,* Clone 62/4 was also incubated in the presence of L.DR7 cells pulsed with titered doses of peptide TRAG-3_{34-48}. IFN-γ and IL-5 spots were developed and counted by computer-assisted video image analysis. Each bar represents the mean spot number of triplicates $\pm$ SD with 10^3 $CD4^+$ T cells initially seeded per well. $*$, $p < 0.05$ were considered significant.

3 patients (Fig. 4*E*). None of the 14 patients with stage IV NSLCC had detectable TRAG-3-specific $CD4^+$ T cells (Fig. 4*C*). HA-specific $CD4^+$ T cell responses were analyzed from nine patients and were detectable in only two patients (Fig. 4*F*). Of note and as controls, none of the nine normal donors had detectable TRAG-3-specific $CD4^+$ T cells (Fig. 4, *A–C*). HA-specific $CD4^+$ T cells were detectable in seven of nine normal donors (Fig. 4, *D–F*).

To assess the capability of the patients with advanced cancer in this study to develop Abs against CGA-expressing tumors, we have also evaluated the spontaneous NY-ESO-1-specific $CD4^+$ T cell and Ab responses in the 12 patients with melanoma. As shown in Fig. 4, *G* and *H,* we observed that 4 and 2 of the 12 melanoma patients had detectable $CD4^+$ T cells against peptides NY-ESO-1_{87-111} and NY-ESO-$1_{157-170}$, respectively. All patients with spontaneous NY-ESO-1-specific $CD4^+$ T cells in this study had circulating anti-NY-ESO-1 Abs detectable with ELISA (Fig. 4, *G* and *H*). In sharp contrast to what we observed for NY-ESO-1, none of the patients in this study, including the patients with evidence of TRAG-3-specific CD4+T cells, had circulating TRAG-3-specific Abs detectable by ELISA (Table II). As a positive control, we have generated anti-TRAG-3 and anti-NY-ESO-1 rabbit polyclonal Abs that showed similar levels of reactivity in ELISAs against the recombinant TRAG-3 protein and the recombinant NY-ESO-1 protein, respectively. The anti-TRAG-3 polyclonal Ab also

Table II. *HLA typing, stage of disease, taxane treatment, TRAG-3 expression in tumors, and TRAG-Ab status of the patients evaluated in the study*

Patient	HLA-DR	Stage of Disease	Taxane Treatment	RT-PCR TRAG-3	TRAG-3 Ab
Melanoma patients					
1	0401	IV	No	+	−
2	0401	IV	No	+	−
3	0401	IV	No	−	−
4	0401	IV	No	+	−
5	0401	IV	No	−	−
6	0401	IV	No	+	−
7	0401	IV	No	+	−
8	0401	IV	No	+	−
9	0401	IV	No	+	−
10	0401	IV	No	−	−
11	0401	IV	No	−	−
12	0401	IV	No	+	−
Breast cancer patients					
13	NA[a]	$T_2N_1M_0$	Yes	NA	−
14	0701, 1302	IV	Yes	NA	−
15	1502, 1501	IV	Yes	NA	−
16	0101, 1401	IV	Yes	NA	−
17	0403, 0701	$T_1N_0M_0$	No	NA	−
18	1302, 1301	$T_2N_1M_0$	Yes	NA	−
19	NA	$T_1N_0M_0$	No	NA	−
20	NA	IV	Yes	NA	−
21	0401	IV	Yes	NA	−
22	1104, 1501	$T_2N_1M_0$	No	NA	−
23	0103	$T_1N_0M_0$	No	NA	−
24	NA	$T_1cN_0M_0$	No	NA	−
25	0103, 1501	$T_1N_0M_0$	No	NA	−
Lung cancer patients					
26	0301, 1301	IV	Yes	NA	−
27	0701, 1501	IV	Yes	NA	−
28	0404, 0701	IV	No	NA	−
29	NA	III	Yes	NA	−
30	0405, 1301	IV	Yes	NA	−
31	0301, 0701	IV	Yes	NA	−
32	0701, 1501	IV	Yes	NA	−
33	0401, 0901	IV	Yes	NA	−
34	NA	IV	Yes	NA	−
35	0301, 1502	IV	Yes	NA	−
36	NA	IV	Yes	NA	−
37	0101	IV	Yes	NA	−
38	0404, 1301	IV	No	NA	−
39	0802, 1201	IV	Yes	NA	−
Normal donors					
40	0401, 1401				−
41	0701, 0701				−
42	0701, 1101				−
43	0401, 1302				−
44	0301, 0701				−
45	1302, 1501				−
46	0401, 1102				−
47	0701, 1301				−
48	1101, 1301				−

[a]NA, Not available; TNM, tumor-nodes-metastasis classification.

labeled TRAG-3-expressing paraffin-embedded tumor samples as well as TRAG-3-expressing tumor cell lines (as assessed by RT-PCR) and *TRAG-3*-transfected COS cells (data not shown).

We next investigated whether our immunological findings were correlated with taxane therapy (Table II). Eight of the thirteen breast cancer patients received taxanes, but only two patients previously treated with taxanes developed TRAG-3-specific $CD4^+$ T cells. Twelve of fourteen lung cancer patients were previously treated with taxanes; none of them developed TRAG-3-specific $CD4^+$ T cells. Most importantly, none of the nine melanoma patients with TRAG-3-expressing tumors and TRAG-3-specific $CD4^+$ T cells had received taxanes.

Altogether, our data demonstrate the existence of spontaneous TRAG-3-specific $CD4^+$ T cells in all stage IV melanoma patients with TRAG-3-expressing melanoma and in a limited number of patients with stage III/IV breast cancers but not in any of the patients with stage IV lung cancers. Furthermore, no circulating TRAG-3-specific Abs have been detected from the blood of these patients, although some of these patients had NY-ESO-1-specific $CD4^+$ T cell responses that coexist with the presence of circulating anti-NY-ESO-1 Abs.

TCRβ CDR3 studies of TRAG-3-specific $CD4^+$ T cell clones

To study the diversity of the T cell repertoire of the TRAG-3-specific $CD4^+$ T cells, we have cloned and sequenced the TCRβ CDR3 gene region of 10 different $CD4^+$ T cell clones isolated either from patient 1 or normal donor 1 as previously described in *Materials and Methods*. The data are presented in Table III. We

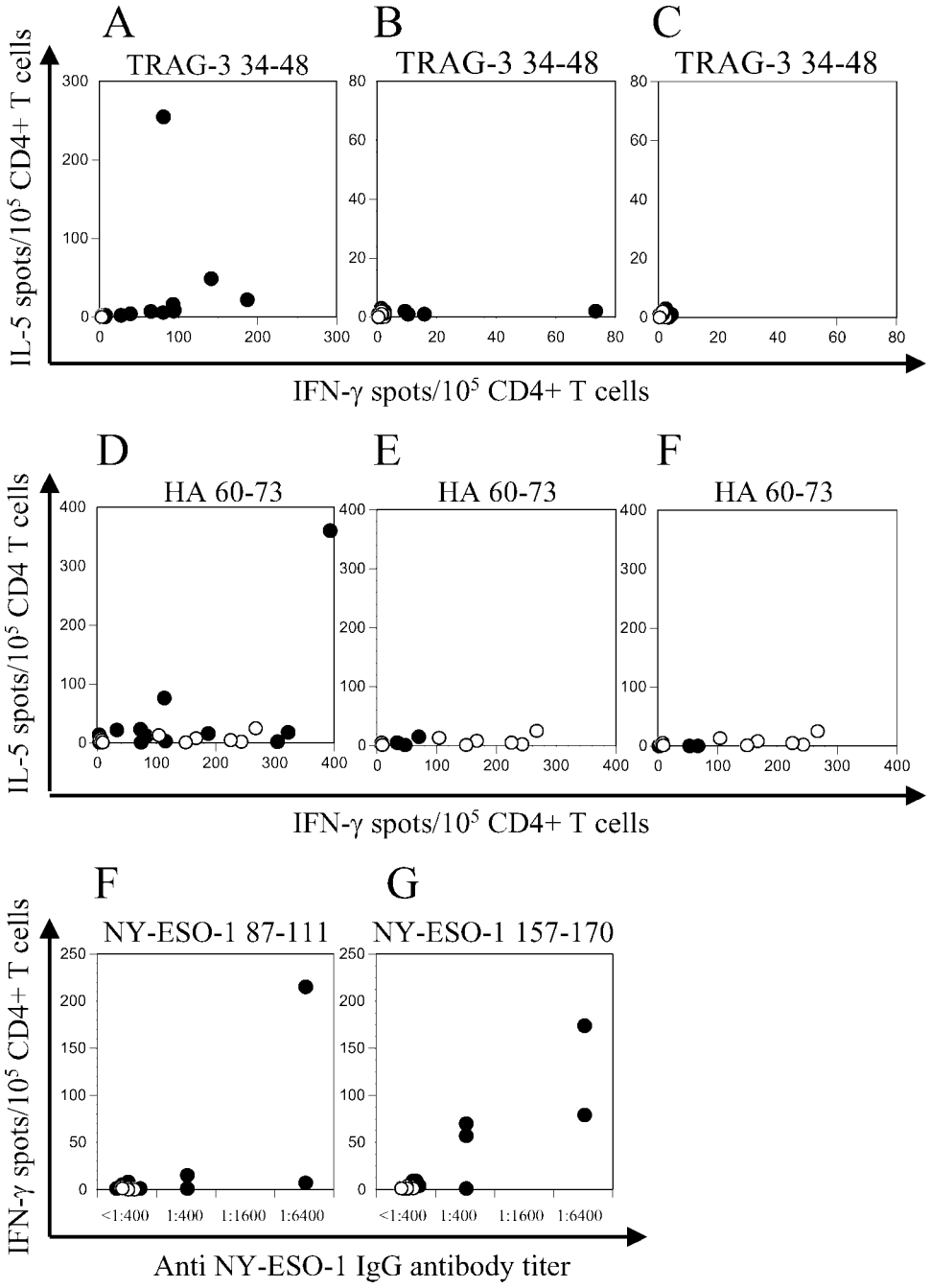

FIGURE 4. TRAG-3-specific Th1-type vs Th2-type immunoreactivity of $CD4^+$ T cells in patients with melanoma, breast, and lung cancers. $CD4^+$ T cells were isolated from the peripheral blood of patients with melanoma, breast, and lung cancers and normal donors and then stimulated with autologous APCs pulsed with peptide TRAG-3_{34-48}, peptide HA_{60-73}, peptide NY-ESO-1_{87-111}, or peptide NY-ESO-$1_{157-170}$. $CD4^+$ T cells were then analyzed in IFN-γ and IL-5 ELISPOT assays for their reactivity to autologous T-APCs pulsed with: peptide TRAG-3_{34-48} (*A–C*); peptide HA_{60-73} (*D–F*), for melanoma, breast cancer, and lung cancer patients, respectively. Each symbol represents the combined IFN-γand IL-5 ELISPOT data for each individual patient with melanoma, breast cancer, or lung cancer (●) and each normal donor (○). ELISA for NY-ESO-1-specific Abs were performed as previously reported in the section *Materials and Methods* (all data <1/400 are considered negative). We have also presented the combined data of anti-NY-ESO-1 Ab and IFN-γ ELISPOT assays from melanoma patients and normal donors for: peptide NY-ESO-1_{87-111} (*F*) and peptide NY-ESO-$1_{157-170}$ (*G*). Each symbol represents the combined IFN-γELISPOT and NY-ESO-1-specific Abs data for each individual melanoma patient (●) or normal donor (○).

observed that the 10 clones isolated from patient 1 exhibited 1 unique TCRβ CDR3 region, whereas the TRAG-3-specific $CD4^+$ T cell clones derived from normal donor 1 expressed 3 distinct TCRβ CDR3 regions.

Altogether, our data suggest a limited TCR repertoire of TRAG-3-specific $CD4^+$ T cells in patients with TRAG-3-expressing tumors.

Discussion

In the present study, we investigated the $CD4^+$ T cell responses against the CGA TRAG-3, commonly expressed by the majority of melanomas, breast cancers, and NSCLC. We observed that a single immunodominant epitope, TRAG-3_{34-48}, bound to multiple HLA-DR molecules including *HLA-DRB1*0101, HLA-DRB1*0401, HLA-DRB1*0701, HLA-DRB1*1101, HLA-DRB1*1501,* and *HLA-DRB5*0101* and stimulated autologous $CD4^+$ T cells from normal donors and melanoma patients when presented in the context of these HLA molecules. Because peptide TRAG-3_{28-42} binds to *HLA-DRB1*0301* and *HLA-DRB1*1301* and peptide TRAG-3_{34-48} binds to *HLA-DRB1*1501,* it is expected that the 21-mer TRAG-3_{28-48} may represent a more promiscuous HLA-DR-binding sequence that binds to the three HLA-DR alleles.

Importantly, TRAG-3-specific $CD4^+$ T cells recognized autologous DCs previously loaded with the TRAG-3 protein but not with an irrelevant protein, thus indicating that peptide TRAG-3_{34-48} encompasses one naturally processed and presented epitope. Our findings contrast with previous studies showing that CGAs usually give rise to multiple MHC class II epitopes recognized by $CD4^+$ T cells (8–10, 12, 17, 18), reporting the first example of tumor Ag-specific $CD4^+$ T cell responses directed against a single immunodominant epitope. The selectivity of the T

Table III. *TCRβ CDR3 sequences from TRAG-3-specific CD4$^+$ T cell clones*

Donor	Clones		Vβ	NDN	Jβ	
Patient 1	61/6, 61/7, 61/11, 61/18, 61/28 61/32, 61/35, 61/41, 61/57, 61/58	**T-CRBV20-1**	CSAW	GGLN	TDTQYF	**T-CRBJ2-3**
Normal donor 1	62/4, 62/52	**T-CRBV24-1**	CATSD	PGTG	TEKLF	**T-CRBJ1-4**
	62/3, 62/5, 62/18, 62/39, 62/43	**T-CRBV19**	CASS	MGQGYFKRP	YNEQF	**T-CRBJ2-1**
	62/8, 62/19, 62/25	**T-CRBV4-1**	CASSQ	ETT	YGYTF	**T-CRBJ1-2**

cells to respond to a very limited number of peptides within complex protein Ags is called immunodominance (37). A number of mechanisms appear to contribute to immunodominance including events related to Ag processing and presentation as well as T cell recognition (38). Our binding data on TRAG-3, showing that a single amino acid region is capable of binding to multiple HLA-DR molecules, strongly support the pivotal role of HLA-binding properties for the determinant selection of the immunodominant peptide, TRAG-3_{34-48} (37, 39). Furthermore, our data support the hypothesis that immunodominance is correlated with binding to multiple MHC class II molecules. The correlation between highly degenerate HLA-DR binding and immunodominance has been previously established for a number of epitopes derived from foreign and self-Ags (40–45). Such a correlation is also suggested by a recent study in cancer patients showing that the spontaneous NY-ESO-1-specific CD4$^+$ T cell responses are directed toward two immunodominant and promiscuous HLA-DR-restricted peptides, NY-ESO-1_{81-100} and NY-ESO-$1_{119-143}$, previously identified by us and others (8, 9, 18, 46).

There is now ample evidence that some CGAs may spontaneously give rise to T cell and Ab responses in patients with CGA-expressing tumors (18, 19, 47–49). Whenever detectable, spontaneous immune responses to CGAs were found only in a fraction of the patients with CGA-expressing tumor. In contrast, our findings show that all patients with TRAG-3-expressing stage IV melanoma had spontaneous TRAG-3-specific CD4$^+$ T cells, supporting the strong immunogenicity of TRAG-3. This observation may not apply to all types of CGA-expressing tumors as spontaneous CD4$^+$ T cell responses against TRAG-3 were found in only four tested stage III/IV breast cancer patients and in none of the tested patients with stage IV NSCLC. We have not investigated TRAG-3 expression in the primary tumor or metastases of the breast cancer and NSCLC patients and cannot rule out that few breast tumors and none of the 14 lung cancer patients' tumors expressed TRAG-3. However, this seems very unlikely given that previous works have demonstrated that ~60 and 54% of breast cancers and NSLCC express TRAG-3, respectively (21, 22). Furthermore, as only three of nine and two of nine patients with breast cancers and stage IV NSCLC, respectively, developed HA-specific CD4$^+$ T cells, we also cannot exclude that the capability of the Ag-specific CD4$^+$ T cells to produce cytokines may have been impaired because of their clinical status or ongoing chemotherapy.

To investigate a correlation between the presence of TRAG-3-specific CD4$^+$ T cells and TRAG-3-specific IgG Ab, we studied the occurrence of spontaneous humoral responses to TRAG-3 and NY-ESO-1 (as a control) with ELISA. Whereas the stage IV melanoma patients in this study with naturally occurring NY-ESO-1-specific CD4$^+$ T cells had circulating Abs against NY-ESO-1, none of the patients with spontaneous TRAG-3-specific CD4$^+$ T cells had detectable Ab responses against TRAG-3. Our observation of IFN-γ-producing CD4$^+$ T cells in the absence of circulating TRAG-3-specific Abs clearly indicates that spontaneous CGA-specific CD4$^+$ T cells may exist independently of the presence of CGA-specific circulating Abs, revealing a novel profile of spontaneously acquired immune response against CGAs. Interestingly, four of the melanoma patients with TRAG-3-specific CD4$^+$ T cells also had circulating NY-ESO-1-specific CD4$^+$ T cells and NY-ESO-1-specific Abs, suggesting that CGA-specific CD4$^+$ T cells within the same individual may differ in their capability to activate B cells and promote CGA-specific IgG Abs.

Two previous experimental studies in animals have suggested that the diversity of the CD4$^+$ T cell repertoire plays a critical role in the generation of isotype-switched IgG autoantibodies (50, 51). Another study in humans has reported the existence of Th2-type CD4$^+$ T cells directed against a single immunodominant epitope from mugwort pollen in allergic patients, which coexisted with Ag-specific IgE Abs and exhibited a large TCR repertoire (52). These observations led us to examine the TCR repertoire of the TRAG-3-specific CD4$^+$ T cells derived from PBL of either a melanoma patient with TRAG-3-expressing tumor or a normal donor. We observed that all 10 clones derived from the melanoma patient exhibited the same TCRβ CDR3 region, whereas 3 distinct TCRβ CDR3 regions were found among the 10 CD4$^+$ T cell clones derived from the normal donor. Although we cannot exclude a selective in vitro expansion of one specific CD4$^+$ T cell clone that occurred only for the melanoma patient, our data suggest an in vivo selection of one specific T cell clonotype in the melanoma patient with TRAG-3-expressing tumor. It is thus possible that the help provided by the TRAG-3-specific CD4$^+$ T cells is of limited specificity and less likely to promote a sustained effector B cell response and Ab production in patients with TRAG-3-expressing tumors. Further TCR studies of TRAG-3-specific CD4$^+$ T cells from a larger number of patients with TRAG-3-expressing tumors are needed to confirm our findings in one melanoma patient.

Whenever detectable, the TRAG-3-specific CD4$^+$ T cells obtained from patients with melanoma and breast cancers were either Th1-type or Th0-type CD4$^+$ T cells because they produced IFN-γ, or IFN-γ and IL-5, respectively. We have not identified TRAG-3-specific CD4$^+$ T cells or clones that produced only Th-2-type cytokines, suggesting no Th2 skewing of the spontaneous TRAG-3-specific CD4$^+$ T cell responses as previously reported for MAGE-6 (53). These findings are in line with our previous studies of NY-ESO-1-specific CD4$^+$ T cells in patients with active NY-ESO-1-expressing melanoma (12). They illustrate that Th2 skewing is not a phenomenon that is generalized for all CGA-specific CD4$^+$ T cell responses in cancer patients.

The majority of the breast cancer and lung cancer patients in this study have been treated with taxane-based chemotherapy, which might be expected to induce TRAG-3 expression by the patients' tumor. However, and as indicated in Table II, we have not found that taxane-based treatment induced spontaneous immune responses against TRAG-3 in vivo. Furthermore, none of the patients with stage IV melanoma in this study with TRAG-3-expressing tumors and TRAG-3-specific CD4$^+$ T cells had previously received taxanes. These findings strongly suggest that taxanes play no major role in the expression of TRAG-3 or in the induction of TRAG-3-specific CD4$^+$ T cells.

In summary, this study reports a novel immunological pattern of CGA-specific immune responses and adds to the diversity of spontaneous immune responses in patients with CGA-expressing tumors. Because of the common expression of TRAG-3 by melanoma and its strong spontaneous immunogenicity in patients with stage IV melanoma, the TRAG-3_{34-48} epitope represents a good candidate for the monitoring and the dissection of tumor Ag-specific spontaneous immune responses in patients with advanced melanoma.

Acknowledgments

We are grateful to the patients and their physicians for giving time and the blood samples for the performance of these experiments. We thank Drs. L. J. Old and G. Ritter (Ludwig Institute for Cancer Research, New York, NY) for providing the NY-ESO-1 protein. We also thank Peggy Chocarne and Aurelien Missonnier (CEA-Saclay, France) for technical help.

Disclosures

The authors have no financial conflict of interest.

References

1. Janssen, E. M., E. E. Lemmens, T. Wolfe, U. Christen, M. G. von Herrath, and S. P. Schoenberger. 2003. CD4$^+$ T cells are required for secondary expansion and memory in CD8$^+$ T lymphocytes. *Nature* 421: 852–856.
2. Sun, J. C., and M. J. Bevan. 2003. Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science* 300: 339–342.
3. Rocha, B. 2002. Requirements for memory maintenance. *Nat. Immunol.* 3: 209–210.
4. Rocha, B., and C. Tanchot. 2004. Towards a cellular definition of CD8$^+$ T-cell memory: the role of CD4$^+$ T-cell help in CD8$^+$ T-cell responses. *Curr. Opin. Immunol.* 16: 259–263.
5. 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: 1351–1354.
6. Wang, R. F., X. Wang, and S. A. Rosenberg. 1999. Identification of a novel major histocompatibility complex class II-restricted tumor antigen resulting from a chromosomal rearrangement recognized by CD4$^+$ T cells. *J. Exp. Med.* 189: 1659–1668.
7. Zarour, H. M., J. M. Kirkwood, L. S. Kierstead, W. Herr, V. Brusic, C. L. Slingluff, Jr., J. Sidney, A. Sette, and W. J. Storkus. 2000. Melan-A/MART-1(51–73) represents an immunogenic HLA-DR4-restricted epitope recognized by melanoma-reactive CD4$^+$ T cells. *Proc. Natl. Acad. Sci. USA* 97: 400–405.
8. Zarour, H. M., B. Maillere, V. Brusic, K. Coval, E. Williams, S. Pouvelle-Moratille, F. Castelli, S. Land, J. Bennouna, T. Logan, and J. M. Kirkwood. 2002. NY-ESO-1 119–143 is a promiscuous major histocompatibility complex class II T-helper epitope recognized by Th1- and Th2-type tumor-reactive CD4$^+$ T cells. *Cancer Res.* 62: 213–218.
9. Zarour, H. M., W. J. Storkus, V. Brusic, E. Williams, and J. M. Kirkwood. 2000. NY-ESO-1 encodes DRB1*0401-restricted epitopes recognized by melanoma-reactive CD4$^+$ T cells. *Cancer Res.* 60: 4946–4952.
10. Mandic, M., C. Almunia, S. Vicel, D. Gillet, B. Janjic, K. Coval, B. Maillere, J. M. Kirkwood, and H. M. Zarour. 2003. The alternative open reading frame of LAGE-1 gives rise to multiple promiscuous HLA-DR-restricted epitopes recognized by T-helper 1-type tumor-reactive CD4$^+$ T cells. *Cancer Res.* 63: 6506–6515.
11. Zeng, G., X. Wang, P. F. Robbins, S. A. Rosenberg, and R. F. Wang. 2001. CD4$^+$ T cell recognition of MHC class II-restricted epitopes from NY-ESO-1 presented by a prevalent HLA DP4 allele: association with NY-ESO-1 antibody production. *Proc. Natl. Acad. Sci. USA* 98: 3964–3969.
12. Mandic, M., F. Castelli, B. Janjic, C. Almunia, P. Andrade, D. Gillet, V. Brusic, J. M. Kirkwood, B. Maillere, and H. M. Zarour. 2005. One NY-ESO-1-derived epitope that promiscuously binds to multiple HLA-DR and HLA-DP4 molecules and stimulates autologous CD4$^+$ T cells from patients with NY-ESO-1-expressing melanoma. *J. Immunol.* 174: 1751–1759.
13. 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: 625–630.
14. 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: 767–778.
15. Schultz, E. S., B. Lethe, C. L. Cambiaso, J. Van Snick, P. Chaux, J. Corthals, C. Heirman, K. Thielemans, T. Boon, and P. van der Bruggen. 2000. A MAGE-A3 peptide presented by HLA-DP4 is recognized on tumor cells by CD4$^+$ cytolytic T lymphocytes. *Cancer Res.* 60: 6272–6275.
16. Jassim, A., W. Ollier, A. Payne, A. Biro, R. T. Oliver, and H. Festenstein. 1989. Analysis of HLA antigens on germ cells in human semen. *Eur. J. Immunol.* 19: 1215–1220.
17. Ayyoub, M., A. Merlo, C. S. Hesdorffer, D. Rimoldi, D. Speiser, J. C. Cerottini, Y. T. Chen, L. J. Old, S. Stevanovic, and D. Valmori. 2005. CD4$^+$ T cell responses to SSX-4 in melanoma patients. *J. Immunol.* 174: 5092–5099.
18. Gnjatic, S., D. Atanackovic, E. Jager, M. Matsuo, A. Selvakumar, N. K. Altorki, R. G. Maki, B. Dupont, G. Ritter, Y. T. Chen, A. Knuth, and L. J. Old. 2003. Survey of naturally occurring CD4$^+$ T cell responses against NY-ESO-1 in cancer patients: correlation with antibody responses. *Proc. Natl. Acad. Sci. USA* 100: 8862–8867.
19. Jager, E., Y. Nagata, S. Gnjatic, H. Wada, E. Stockert, J. Karbach, P. R. Dunbar, S. Y. Lee, A. Jungbluth, D. Jager, et al. 2000. Monitoring CD8 T cell responses to NY-ESO-1: correlation of humoral and cellular immune responses. *Proc. Natl. Acad. Sci. USA* 97: 4760–4765.
20. Feller, A. J., Z. Duan, R. Penson, H. C. Toh, and M. V. Seiden. 2000. TRAG-3, a novel cancer/testis antigen, is overexpressed in the majority of melanoma cell lines and malignant melanoma. *Anticancer Res.* 20: 4147–4151.
21. Wu, Y. Z., T. T. Zhao, B. Ni, L. Y. Zou, H. L. Liu, and B. Zhu. 2003. Expression of TRAG-3 in breast cancer. *Int. J. Cancer* 107: 167–168.
22. Chen, Z., B. Zhu, and Y. Wu. 2002. Expression of TRAG-3 antigen in non-small-cell lung carcinomas. *Lung Cancer* 38: 101–102.
23. Duan, Z., A. J. Feller, H. C. Toh, T. Makastorsis, and M. V. Seiden. 1999. *TRAG-3,* a novel gene, isolated from a taxol-resistant ovarian carcinoma cell line. *Gene* 229: 75–81.
24. Zhu, B., Z. Chen, X. Cheng, and Y. Wu. 2002. Induction of TRAG-3 expression in A549 lung adenocarcinoma cell line by 5-aza-2′-deoxyazacytidine. *Lung Cancer* 38: 321–322.
25. Meier, A., S. Reker, I. M. Svane, L. Holten-Andersen, J. C. Becker, I. Sondergaard, M. H. Andersen, and P. Thor Straten. 2005. Spontaneous T-cell responses against peptides derived from the taxol resistance-associated gene-3 (TRAG-3) protein in cancer patients. *Cancer Immunol. Immunother.* 54: 219–228.
26. Zhu, B., Z. Chen, X. Cheng, Z. Lin, J. Guo, Z. Jia, L. Zou, Z. Wang, Y. Hu, D. Wang, and Y. Wu. 2003. Identification of *HLA-A*0201*-restricted cytotoxic T lymphocyte epitope from TRAG-3 antigen. *Clin. Cancer Res.* 9: 1850–1857.
27. Turvy, D. N., and J. S. Blum. 1998. Detection of biotinylated cell surface receptors and MHC molecules in a capture ELISA: a rapid assay to measure endocytosis. *J. Immunol. Methods* 212: 9–18.
28. Watson, A. J., R. DeMars, I. S. Trowbridge, and F. H. Bach. 1983. Detection of a novel human class II HLA antigen. *Nature* 304: 358–361.
29. Zarour, H., C. De Smet, F. Lehmann, M. Marchand, B. Lethe, P. Romero, T. Boon, and J. C. Renauld. 1996. The majority of autologous cytolytic T-lymphocyte clones derived from peripheral blood lymphocytes of a melanoma patient recognize an antigenic peptide derived from gene *Pmel17/gp100*. *J. Invest. Dermatol.* 107: 63–67.
30. Texier, C., S. Pouvelle, M. Busson, M. Herve, D. Charron, A. Menez, and B. Maillere. 2000. HLA-DR restricted peptide candidates for bee venom immunotherapy. *J. Immunol.* 164: 3177–3184.
31. Texier, C., S. Pouvelle-Moratille, M. Busson, D. Charron, A. Menez, and B. Maillere. 2001. Complementarity and redundancy of the binding specificity of HLA-DRB1, -DRB3, -DRB4 and -DRB5 molecules. *Eur. J. Immunol.* 31: 1837–1846.
32. Castelli, F. A., C. Buhot, A. Sanson, H. Zarour, S. Pouvelle-Moratille, C. Nonn, H. Gahery-Segard, J. G. Guillet, A. Menez, B. Georges, and B. Maillere. 2002. HLA-DP4, the most frequent HLA II molecule, defines a new supertype of peptide-binding specificity. *J. Immunol.* 169: 6928–6934.
33. Atanackovic, D., M. Matsuo, E. Ritter, G. Mazzara, G. Ritter, E. Jager, A. Knuth, L. J. Old, and S. Gnjatic. 2003. Monitoring CD4$^+$ T cell responses against viral and tumor antigens using T cells as novel target APC. *J. Immunol. Methods* 278: 57–66.
34. 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: 1349–1354.
35. Genevee, C., A. Diu, J. Nierat, A. Caignard, P. Y. Dietrich, L. Ferradini, S. Roman-Roman, F. Triebel, and T. Hercend. 1992. An experimentally validated panel of subfamily-specific oligonucleotide primers (Vα1-w29/Vβ1-w24) for the study of human T cell receptor variable V gene segment usage by polymerase chain reaction. *Eur. J. Immunol.* 22: 1261–1269.
36. 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: 3363–3373.
37. Sercarz, E. E., P. V. Lehmann, A. Ametani, G. Benichou, A. Miller, and K. Moudgil. 1993. Dominance and crypticity of T cell antigenic determinants. *Annu. Rev. Immunol.* 11: 729–766.
38. Sant, A. J., F. A. Chaves, S. A. Jenks, K. A. Richards, P. Menges, J. M. Weaver, and C. A. Lazarski. 2005. The relationship between immunodominance, DM editing, and the kinetic stability of MHC class II:peptide complexes. *Immunol. Rev.* 207: 261–278.
39. Rosenthal, A. S. 1978. Determinant selection and macrophage function in genetic control of the immune response. *Immunol. Rev.* 40: 136–152.
40. Sinigaglia, F., M. Guttinger, J. Kilgus, D. M. Doran, H. Matile, H. Etlinger, A. Trzeciak, D. Gillessen, and J. R. Pink. 1988. A malaria T-cell epitope recognized in association with most mouse and human MHC class II molecules. *Nature* 336: 778–780.
41. Panina-Bordignon, P., A. Tan, A. Termijtelen, S. Demotz, G. Corradin, and A. Lanzavecchia. 1989. Universally immunogenic T cell epitopes: promiscuous

binding to human MHC class II and promiscuous recognition by T cells. *Eur. J. Immunol.* 19: 2237–2242.

42. Ferrari, C., A. Bertoletti, A. Penna, A. Cavalli, A. Valli, G. Missale, M. Pilli, P. Fowler, T. Giuberti, F. V. Chisari, et al. 1991. Identification of immunodominant T cell epitopes of the hepatitis B virus nucleocapsid antigen. *J. Clin. Invest.* 88: 214–222.
43. 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: 616–628.
44. Diepolder, H. M., J. T. Gerlach, R. Zachoval, R. M. Hoffmann, M. C. Jung, E. A. Wierenga, S. Scholz, T. Santantonio, M. Houghton, S. Southwood, A. Sette, and G. R. Pape. 1997. Immunodominant $CD4^+$ T-cell epitope within nonstructural protein 3 in acute hepatitis C virus infection. *J. Virol.* 71: 6011–6019.
45. Tangri, S., B. R. Mothe, J. Eisenbraun, J. Sidney, S. Southwood, K. Briggs, J. Zinckgraf, P. Bilsel, M. Newman, R. Chesnut, C. Licalsi, and A. Sette. 2005. Rationally engineered therapeutic proteins with reduced immunogenicity. *J. Immunol.* 174: 3187–3196.
46. Ayyoub, M., N. E. Souleimanian, E. Godefroy, L. Scotto, C. S. Hesdorffer, L. J. Old, and D. Valmori. 2006. A phenotype based approach for the immune monitoring of NY-ESO-1-specific $CD4^+$ T cell responses in cancer patients. *Clin. Immunol.* 118: 188–194.
47. Ayyoub, M., D. Rimoldi, P. Guillaume, P. Romero, J. C. Cerottini, D. Valmori, and D. Speiser. 2003. Tumor-reactive SSX-2-specific $CD8^+$ T cells are selectively expanded during immune responses to antigen-expressing tumors in melanoma patients. *Cancer Res.* 63: 5601–5606.
48. Bricard, G., H. Bouzourene, O. Martinet, D. Rimoldi, N. Halkic, M. Gillet, P. Chaubert, H. R. Macdonald, P. Romero, J. C. Cerottini, and D. E. Speiser. 2005. Naturally acquired MAGE-A10- and SSX-2-specific $CD8^+$ T cell responses in patients with hepatocellular carcinoma. *J. Immunol.* 174: 1709–1716.
49. Valmori, D., V. Dutoit, D. Lienard, D. Rimoldi, M. J. Pittet, P. Champagne, K. Ellefsen, U. Sahin, D. Speiser, F. Lejeune, J. C. Cerottini, and P. Romero. 2000. Naturally occurring human lymphocyte antigen-A2 restricted $CD8^+$ T-cell response to the cancer testis antigen NY-ESO-1 in melanoma patients. *Cancer Res.* 60: 4499–4506.
50. Busser, B. W., M. P. Cancro, and T. M. Laufer. 2004. An increased frequency of autoantibody-inducing CD4+ T cells in pre-diseased lupus-prone mice. *Int. Immunol.* 16: 1001–1007.
51. Peng, S. L., S. Fatenejad, and J. Craft. 1996. Induction of nonpathologic, humoral autoimmunity in lupus-prone mice by a class II-restricted, transgenic $\alpha\beta$ T cell: separation of autoantigen-specific and -nonspecific help. *J. Immunol.* 157: 5225–5230.
52. Jahn-Schmid, B., P. Kelemen, M. Himly, B. Bohle, G. Fischer, F. Ferreira, and C. Ebner. 2002. The T cell response to Art v 1, the major mugwort pollen allergen, is dominated by one epitope. *J. Immunol.* 169: 6005–6011.
53. Tatsumi, T., L. S. Kierstead, E. Ranieri, L. Gesualdo, F. P. Schena, J. H. Finke, R. M. Bukowski, J. Mueller-Berghaus, J. M. Kirkwood, W. W. Kwok, and W. J. Storkus. 2002. Disease-associated bias in T helper type 1 (Th1)/Th2 $CD4^+$ T cell responses against MAGE-6 in HLA-DRB10401$^+$ patients with renal cell carcinoma or melanoma. *J. Exp. Med.* 196: 619–628.