Spontaneous CD4⁺ T Cell Responses against TRAG-3 in Patients with Melanoma and Breast Cancers

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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-3₅₆₋₆₈ 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.

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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), 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-3₅₆₋₆₈ 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.

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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 article 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, 2 M urea, 50 mM NaCl, and 20 mM Tris-HCl, pH 8. The TRAG-3 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. The TRAG-3 recombinant protein was expressed 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 UPC1-Mel 285, UPC1-Mel 558, and UPC1-Mel 527.1 using the RNaseasy 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’-TGGTGGATGGGCT CATCCAAATTGTT-3’) and reverse primer, HZ27 (5’-AGCCGCG GTTCATTGGACG-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 product was phosphorylated and then run on a 1% agarose gel before purification using QIAquick (Qiagen), pTrEx4-neo eucaryotic expression vector (Novagen) was digested with SmaI restriction enzyme (New England Biolabs), dephosphorylated, and used for ligation with the TRAG-3 cDNA before transformation of Novablu 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 B721, 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-DP 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-DP 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 (IC50).

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 irritated 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-DR5 (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-3134–148 (4 h, 37°C), irradiated (3000 rads), washed, and used to stimulate 2 × 105 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, 5 µg each of purified anti-HLA mAbs were added in each ELISPOT well. The recognition of T-APCs pulsed with peptides by CD4+ T 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, 106 presensitized CD4+ T effector cells and 105 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 Na₂CO₃, 30 mM NaHCO₃, pH 9.6), with 0.02% NaN₃ 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, and plates were incubated for 2 h at room temperature. Following additional washes, 50 μl/well substrate solution (phosphatase substrate system; Kirkegaard & Perry Laboratories) was added, and plates were incubated for 25 min at room temperature and read immediately (MRX Microplate Reader; Dynatech). Sera were tested in duplicate and the results were 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.

TCRβ gene usage
Total RNA was isolated from 1 × 10⁶ 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-3₃₄–₄₈ 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-3cDNA; G instead of A at position 251; T instead of A at position 340 (23). These nucleotide differences result in a 3-aaa difference at the protein level: R instead of H at position 33; L instead of H at position 37; 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*1501, and HLA-DRB1*1501), three molecules encoded by the HLA-DRB3, HLA-DRB4, and HLA-DRB5 genes and the HLA-DPB1*0401 and -0402 molecules. The data are presented in Table I.

As compared with the IC₅₀ 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-3₁⁻₁₅ 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-3₃₄–₄₈ 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-DRS1).

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

Peptide TRAG-3₃₄–₄₈ 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
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*0701/DRB1*0101 and -DRB1*0401/DRB1*0401, and -DRB1*0101/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-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-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-34-48 but not with an irrelevant peptide (NY-ESO-1119–143) (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-34-48, peptide specifically recognized L.DR7 cells but not L.DR11 cells or L.DP4 cells pulsed with peptide TRAG-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-1119–143 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.

Bulk CD4+ T cells isolated from normal donor 2 and stimulated with peptide TRAG-34-48, peptide produced IFN-γ- and IL-5 in the presence of L.DR4 cells but not L.DP4 cells pulsed with peptide TRAG-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-1119–143 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 demonstration ability of peptide TRAG-34-48 to stimulate CD4+ T cells capable of recognizing peptide TRAG-34-48 in the context of the HLA-DRB1*0101, -DRB1*0401, and -DRB1*0701 molecules. Importantly, these TRAG-34-48 CD4+ T cells recognized autologous DCs loaded with the relevant protein.

**Peptide TRAG-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-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-34-48 and autologous DCs loaded with the TRAG-3 protein in IFN-γ ELISPOT assays (Fig. 3A). The activity 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-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-34-48 or loaded with the TRAG-3 protein but not with an irrelevant protein (NY-ESO-1) in IFN-γ-secretion assays (Fig. 3B). Autologous DCs pulsed with an irrelevant peptide (i.e., NY-ESO-1119–143), 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-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-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-34-48, was evaluated. Half-maximal stimulation of clone 62/4 required peptide loading concentrations of ~600 nM (Fig. 3E).

We also observed that the peptide sequence TRAG-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 1.** TRAG-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-1119–143 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 106 CD4+ T cells initially seeded per well.

![Figure 1](image-url)
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-334–48 and peptide HA60–73 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. 4A). 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 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.
3 patients (Fig. 4E). None of the 14 patients with stage IV NSLCC had detectable TRAG-3-specific CD4⁺ T cells (Fig. 4C), HA-specific CD4⁺ T cell responses were analyzed from nine patients and were detectable in only two patients (Fig. 4F). Of note and as controls, none of the nine normal donors had detectable TRAG-3-specific CD4⁺ T cells (Fig. 4A–C). HA-specific CD4⁺ T cells were detectable in seven of nine normal donors (Fig. 4D–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. 4G and H, we observed that 4 and 2 of the 12 melanoma patients had detectable CD4⁺ T cells against peptides NY-ESO-1157-170 and NY-ESO-1137-151, 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. 4G 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
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/H11001 T cells. Twelve of fourteen lung cancer patients were previously treated with taxanes; none of them developed TRAG-3-specific CD4/H11001 T cells. Most importantly, none of the nine melanoma patients with TRAG-3-expressing tumors and TRAG-3-specific CD4/H11001 T cells had received taxanes.

Altogether, our data demonstrate the existence of spontaneous TRAG-3-specific CD4/H11001 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/H11001 T cell responses that coexist with the presence of circulating anti-NY-ESO-1 Abs.

**TCR 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 TCR CDR3 gene region of 10 different CD4/H11001 T cell clones either from patient 1 or normal donor 1 as previously described in Materials and Methods. The data are presented in Table III.

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**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*    | T2N2M0          | Yes             | NA            | –         |
| 14      | 0701, 1302 | IV          | Yes             | NA            | –         |
| 15      | 1502, 1501 | IV      | Yes             | NA            | –         |
| 16      | 0101, 1401 | IV          | Yes             | NA            | –         |
| 17      | 0403, 0701 | T2N2M0      | No              | NA            | –         |
| 18      | 1302, 1301 | T2N2M0      | Yes             | NA            | –         |
| 19      | NA      | T2N2M0         | No              | NA            | –         |
| 20      | NA      | IV             | Yes             | NA            | –         |
| 21      | 0401    | IV             | Yes             | NA            | –         |
| 22      | 1104, 1501 | T2N2M0      | No              | NA            | –         |
| 23      | 0103    | T2N2M0         | No              | NA            | –         |
| 24      | NA      | T2N2M0         | No              | NA            | –         |
| 25      | 0103, 1501 | T2N2M0   | No              | NA            | –         |

| Lung cancer patients |
| 26      | 0301, 1301 | IV          | Yes             | NA            | –         |
| 27      | 0701, 1501 | IV          | Yes             | NA            | –         |
| 28      | 0401, 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 | –          | –              | –             | –         |

*NA, Not available; TNM, tumor-nodes-metastasis classification.*
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 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 cell responses was demonstrated by the use of IFN-γ and IL-2 ELISPOT assays, and the results are presented in Figure 4. The T-cell responses were measured against peptide TRAG-3_34-48, peptide HA60–73, peptide NY-ESO-1_87–111, and peptide NY-ESO-1_157–170, which were presented in the context of HLA-DR molecules.

In summary, our study provides evidence for the targeting of TRAG-3 by autologous CD4+ T cells in patients with TRAG-3-expressing tumors, suggesting potential therapeutic strategies for these malignancies.
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-334–48 (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 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, no major role in the expression of TRAG-3 by the patients’ cytokines, suggesting no Th2 skewing of the spontaneous TRAG-3-specific CD4+ T cell responses as previously reported for MAGE-A3 (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.

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.

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 | T-CRBY20-1 | CSAW | GGLN | TDTQYF | T-CRBJ2-3 |
| Normal donor 1 | 62/4, 62/52 | T-CRBY24-1 | CATSD | PGTG | TEKLF | T-CRBJ1-4 |
| 62/3, 62/25, 62/18, 62/39, 62/43 | T-CRBY19 | CASS | MQQYFKRP | YNEQF | T-CRBJ2-1 |
| 62/8, 62/19, 62/25 | T-CRBY4-1 | CASSQ | ET | YGYTF | T-CRBJ1-2 |
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 peptide represents a good candidate for the monitoring and the dissection of tumor Ag-specific spontaneous immune responses in patients with advanced melanoma.

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Disclosures

The authors have no financial conflict of interest.

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