Detection of Spontaneous CD4+ T-Cell Responses in Melanoma Patients against a Tyrosinase-Related Protein-2–Derived Epitope Identified in HLA-DRB1*0301 Transgenic Mice

Annette Paschen,1 Mingxia Song,1 Wolfram Osen,1 Xuan Duc Nguyen,2 Jan Mueller-Berghaus,1 Daniela Fink,1,3 Nadine Daniel,4,5 Mariel Donzeau,7 Wolfgang Nagel,7 Annette Paschen,1 Mingxia Song,1 Wolfram Osen,1 Xuan Duc Nguyen,2 Jan Mueller-Berghaus,1 Daniela Fink,1,3 Nadine Daniel,4,5 Mariel Donzeau,7 Wolfgang Nagel,7 Harald Kropshofer,4,6 and Dirk Schadendorf1

Abstract

Purpose: The frequently expressed differentiation antigen tyrosinase-related protein-2 (TRP-2) has repeatedly been described as a target of spontaneous cytotoxic T-cell responses in melanoma patients, suggesting that it might be an ideal candidate antigen for T-cell–based immunotherapy. As a prerequisite for immunization, T-cell epitopes have to be identified. Whereas a number of HLA class I–presented TRP-2–derived epitopes are known, information about HLA class II–presented antigenic ligands recognized by CD4+ T helper (Th) cells is limited.

Experimental Design: The search for TRP-2–derived Th epitopes was carried out by competitive in vitro peptide binding studies with predicted HLA-DRB1*0301 ligands in combination with peptide and protein immunizations of HLA-DRB1*0301 transgenic mice. In vivo selected candidate epitopes were subsequently verified for their immunogenicity in human T-cell cultures.

Results: This strategy led to the characterization of TRP-260-74 as an HLA-DRB1*0301–restricted Th epitope. Importantly, TRP-260-74–reactive human CD4+ Th cell lines, specifically recognizing target cells loaded with recombinant TRP-2 protein, could be established by repeated peptide stimulation of peripheral blood lymphocytes from several HLA-DRB1*0301 melanoma patients. Even short-term peptide stimulation of patients’ peripheral blood lymphocytes showed the presence of TRP-260-74–reactive T cells, suggesting that these T cells were already activated in vivo.

Conclusion: Peptide TRP-260-74 might be a useful tool for the improvement of immunotherapy and immune monitoring of melanoma patients.

T cells isolated from the peripheral blood and tumor tissue of melanoma patients are known to recognize epitopes derived from melanoma differentiation antigens. These melanoma differentiation antigen–specific T cells exhibit cytolytic activity against autologous tumor cells in vitro and, most importantly, in vivo, as impressively shown by recent clinical studies of adoptive T-cell transfer (1, 2). Antigens belonging to the melanoma differentiation antigen group include gp100, MelanA/MART-1, tyrosinase, tyrosinase-related protein (TRP)-1, and TRP-2. A study by Takeuchi et al. (3) showed that elevated mRNA copy levels of some of these antigens, specifically tyrosinase and TRP-2, significantly correlate with an improved overall survival of stage IV melanoma patients. Determination of TRP-2 expression by quantitative reverse transcription-PCR in human melanomas revealed the presence of TRP-2 mRNA in up to 90% of metastatic tumor specimens (3), indicating that this protein might be an ideal candidate antigen for vaccination. Indeed, TRP-2 is a frequently described in vivo target of tumor-reactive cytotoxic CD8+ T lymphocytes from melanoma patients (4–6). Interestingly, several studies revealed the protective and therapeutic antitumor capacity of TRP-2–specific murine CTLs in the B16 melanoma model (7–9), suggesting that TRP-2 might function as an autologous tumor rejection antigen also in humans.

Although CTLs constitute essential mediators of antigen-specific tumor destruction, it is now well accepted that effective antitumor immunity requires the participation of both antigen-specific CTLs and T helper (Th) cells. The CD4+ Th cells are involved in several steps of the induction and maintenance of CTL immunity: The interaction of CD40 ligand on activated antigen-specific CD4+ Th cells with CD40 on dendritic cells, the most potent activators of naïve T cells, greatly increases the capacity of dendritic cells to effectively prime CD8+ T cells (10, 11), and signals delivered by activated CD4+ Th cells are essentially needed for the generation of CD8+ T-cell memory
(12). Consequently, vaccination of tumor patients should target both T-cell subsets which in most cases can only be achieved if knowledge about HLA class I and class II-presented ligands from tumor antigens is provided. With respect to TRP-2, a number of CTL epitopes presented by HLA-A*02 (6, 13–15), HLA-A*31/HLA-A*33 (4, 16), and HLA-Cw*8 (5) molecules, respectively, have been identified, but information about HLA class II–restricted epitopes is limited, except for an HLA-DRB1*1502 binding sequence described by Robbins et al. (17).

In the present work, we screened for TRP-2–derived antigenic ligands presented by the HLA-DRB1*0301 molecule, which is expressed in 14% to 31%8 of the Caucasians depending on the population analyzed. We combined in vitro peptide binding studies with immunization experiments of HLA-DRB1*0301 transgenic (HLA-DR3tg) mice to preselect candidate epitope sequences and subsequently evaluated the most promising ligands for their T-cell target function in melanoma patients. This strategy led to the identification of the HLA-DRB1*0301–presented epitope TRP-2 560–74, a potential tool for immunotherapy and immune monitoring of melanoma patients.

**Materials and Methods**

**Cells and media.** Murine lymph node cells were cultured in αMEM (Sigma, Taufkirchen, Germany) containing 10% FCS (Biochrom, Berlin, Germany), 2 mmol/L glutamine (Gibco-Invitrogen, Karlsruhe, Germany), and 50 μmol/L 2-mercaptoethanol. Human T2 DR3 cells, kindly provided by Dr. F. Momburg (German Cancer Research Center, Heidelberg, Germany), were generated by transfection of human HLA class II–deficient T2 cells with cDNA encoding HLA-DRB1*0301 and were maintained in RPMI 1640/HEPES/2 mmol/L glutamine (PAA Laboratories, Göbel, Germany) supplemented with 10% FCS (PAA Laboratories), 100 units/mL penicillin, 100 μg/mL streptomycin, and 500 μg/mL gentamicin (Gibco-Invitrogen).

**Mice.** HLA-DRB1*0301tg mice lacking expression of endogenous MHc class II molecules (18) were kindly provided by C.S. David (Mayo Clinic, Rochester, MN). Mice were bred in isolators or individually ventilated cages and transgene expression was tested by flow cytometry of peripheral blood lymphocytes stained with the FITC-conjugated HLA-DR–specific monoclonal antibody (mAb) L243 (Becton Dickinson, Heidelberg, Germany). Experimental protocols with recombinant mice were approved by a national animal care committee.

**Synthetic peptides.** Peptides were synthesized by Fmoc chemistry and analyzed by high-performance liquid chromatography at the Division of Peptide Synthesis of the German Cancer Research Center: TRP-2 560–74 (QCTEVRADTRPWSGP), TRP-2 123–137 (HLLCLERDQIKANKSKFIGITE). Lyophilized peptides were dissolved in DMSO at 50 mg/mL and stored at -20°C.

**Peptide binding assay.** The relative binding affinity of peptides was determined in a chimerimunencescence-based competition assay, as previously described (19). Briefly, affinity-purified HLA-DRB1*0301 molecules (10 μg/mL), isolated from T2 DR3 cells, were coincubated with a biotinylated reporter peptide (1 μmol/L) and increasing doses of the TRP-2 derived peptide (0–100 μmol/L) to be analyzed for 24 hours at 37°C, pH 5.5. Binding of the biotinylated reporter peptide was analyzed by a sandwich ELISA, using anti–HLA-DR mAb L243 as a capture antibody and streptavidin-europium as the detection reagent. Plates were developed by incubation for 45 minutes with 0.1 μg/mL streptavidin-europium (Perkin-Elmer, Rodgau-Jügesheim, Germany) according to the protocol of the manufacturer and time-resolved fluorescence was quantified using the VICTOR multilabel counter (Perkin-Elmer).

**Expression and purification of recombinant tyrosinase-related protein-2.** The cDNA encoding TRP-2 30–387 (corresponding to GenBank accession no. S69231 except for an G to A transition at position 233 of the coding region) was cloned into the vector pTrcHisTOPO (Gibco-Invitrogen), mediating expression of a His-Xpress–tagged TRP-2 fusion protein under control of an isopropyl-1-thio-β-D-galactopyranoside (IPTG)–inducible trc promoter. The resulting plasmid was transformed into Escherichia coli BL21-CodonPlus™(RIL). Bacteria were grown overnight at 37°C in SOB medium (Gibco-Invitrogen). Cultures were diluted 1:100 and incubated for 3 hours before recombinant protein expression was induced by adding 1 mmol/L IPTG (Peglabs, Erlangen, Germany). Three hours after induction, bacteria were pelleted and lysed in buffer A (6 mol/L GuHCl, 100 mmol/L NaH₂PO₄, 10 mmol/L Tris-Cl, pH 8.0). Cellular debris was removed by centrifugation, cleared lysates were incubated with Ni-NTA agarose Superflow (Qiagen, Hilden, Germany) for 1 hour, and the beads were then poured in a fast protein liquid chromatography column and sequentially washed with buffer A and buffer B (8 mol/L urea, 100 mmol/L NaH₂PO₄, 10 mmol/L Tris-Cl, pH 8.0). The bound protein was eluted with buffer E (8 mol/L urea, 100 mmol/L NaH₂PO₄, 10 mmol/L Tris-Cl, pH 4.5). Detection of the eluted TRP-2 protein was carried out with an anti-X-press mAb (Gibco-Invitrogen) by Western blot analysis; purity was >50% as determined by SDS-PAGE. The proteins were dialyzed twice against 0.1× PBS. The precipitated protein was washed twice with PBS and resuspended in complete Freund’s adjuvant for immunization experiments.

**Construction and isolation of bacteriophages.** To generate recombinant bacteriophages, cDNA encoding TRP-2 30–387 was amplified by PCR and cloned into the phagemid vector SurZAP (Stratagene, La Jolla, CA) in frame with DNA encoding the M13 major coat protein (cpVIII) and transformed into E. coli TG-1. After overnight growth in 2×YT medium [2% (w/v) glucose], bacteria suspension was diluted 1:10 and cultivated to A₅₉₀ 0.5. M13K07 helper phages (Amsbers Biosciences, Freiburg, Germany) were added at a multiplicity of infection of 10 and incubated for 30 minutes at 37°C. After centrifugation, bacteria were resuspended in 2×YT medium supplemented with 1 mmol/L IPTG and incubated for 18 hours at 37°C. Bacteria were pelleted by centrifugation and phages were precipitated from supernatant by addition of 0.2 volume of 5×polyethylene glycol 8000/NaCl [30% (w/v) polyethylene glycol 8000, 1.5 mol/L NaCl]. To prevent proteolytic digestion, 1 mmol phenylmethylsulfonyl fluoride was added. After a 4-hour incubation at 4°C, phages were pelleted by centrifugation (12,000×g). Hybrid phage particles were purified under native conditions by affinity chromatography using Ni-NTA agarose (Qiagen). TRP-2 hybrid phages were eluted with PBS (pH 7.4), 100 mmol/L EDTA, and endotoxin content of the eluates was monitored with Limulus amoebocyte lysate assay (Bio-Whittaker, Walkersville, MD). Remaining endotoxin was removed by standard methods using polymyxin B-agarose (Pierce, Rockford, IL) or Triton X-114 extraction. Expression of TRP-2 30–387 on hybrid phages was verified by immunoblot employing a mouse anti-polyHis mAb. For generation of wild-type phages, TG-1 bacteria were infected with M13K07 helper phages. The purification was carried out according to the previous procedure, omitting the affinity chromatography step. Endotoxin content of all phage preparations was lower than 1 endotoxin unit/mL.

**Immunization of mice and analysis of T-cell responses.** Mice received 100 μg synthetic peptide or 15 μg recombinant TRP-2 emulsified 1:1 in complete Freund’s adjuvant s.c. into hind foot pads. Ten to 12 days later, single-cell suspensions prepared from draining lymph nodes were tested for specificity in 96-well flat-bottomed microtiter plates by incubation of 4×10³ lymph node cells with 50 μg/mL peptide. Unspecific T-cell stimulation was obtained by adding 5 μg/mL concanavalin A per well. After 72 hours, the amount of IFN-γ in the supernatant was determined by ELISA according to the instructions of the manufacturer (Becton Dickinson Biosciences). Depletion of CD4⁺ T cells from total lymph node cells was achieved by magnetic cell separation using anti-CD4 specific mAb conjugated microbeads.
according to the instructions of the manufacturer (Miltenyi Biotec, Bergisch-Gladbach, Germany).

**HLA typing of blood donors.** HLA type of peripheral blood mononuclear cells from healthy donors and melanoma patients was determined by high-resolution PCR typing. Blood donations from patients were approved by the Institutional Review Board and an informed consent was given by all patients.

**In vitro activation of human T cells.** Frozen peripheral blood mononuclear cells from normal blood donors were thawed and used as a source of CD4+ T cells and as a source of monocytes for the generation of dendritic cells following a routine protocol (15). Briefly, plastic-adherent monocytes were incubated with IL-4 (800 units/ml; R&D Systems, Wiesbaden-Nordenstadt, Germany) and granulocyte macrophage colony-stimulating factor (1,000 units/ml; Novartis, Nuremberg, Germany) in X-VIVO 15 medium (BioWhittaker, Verviers, Belgium) over 5 days to obtain immature dendritic cells. On day 6, IL-1β (10 ng/ml; R&D Systems), IL-6 (1,000 units/ml; R&D Systems), MPE2 (1 μg/ml; Pharmacia Upjohn, Erlangen, Germany), and tumor necrosis factor-α (10 ng/ml; Boehringer Ingelheim, Germany) were added to the cultures to induce dendritic cell maturation. In parallel, peptide (10 μg/ml) was added. After 24 hours, dendritic cells were harvested, resuspended in T-cell medium consisting of RPMI 1640/HEPES/2 mmol/L glutamine medium supplemented with 10% heat-inactivated human AB serum (PAA Laboratories), and used to stimulate peripheral blood mononuclear cells at a ratio of 1:20 (dendritic cells/peripheral blood mononuclear cells) in the presence of IL-6 (1,000 units/ml) and IL-12 (1 ng/ml). Responder T cells were restimulated at 14-day intervals with peptide (10 μg/ml)–loaded autologous peripheral blood mononuclear cells inactivated with mitomycin (0.1 mg/ml) for 30 minutes. Restimulated T cells were maintained in T-cell medium supplemented with interleukin (IL)-2 (20 IU/ml) and IL-7 (10 ng/ml).

**Specificity analysis of human T-cell lines.** Reactivity of in vitro induced T-cell lines was determined against peptide- or protein-loaded stimulator cells. For peptide loading, stimulators (T2.DR3) were incubated with 10 μg/ml peptide for 3 hours. For protein loading, stimulators (T2.DR3) were incubated overnight with 13 μg/ml recombinant TRP-2 or wild-type bacteriophages. Responder cells consisted either of bulk T cells or positive-selected CD4+ T cells. Selection was carried out against human CD4 specific mAb conjugated microbeads according to the instructions of the manufacturer (Miltenyi Biotec). T cells were analyzed for their specificity by IFN-γ ELISPOT assays as described (20), by incubation of 103 responder T cells with 5 × 104 stimulator cells. All determinations were done at least in duplicates. Spots were imaged using the Bioreader 3000 (Bio-Sys, Karpen, Germany). The data are presented as mean IFN-γ spots per 104 T cells.

**Detection of in vivo primed T-cell responses.** Screening for in vivo sensitized TRP-2α0.74–reactive T cells was done as follows: Frozen peripheral blood mononuclear cells from normal donors and melanoma patients were thawed and seeded at 2 × 106 cells/ml per well of a 24-well plate in T-cell medium. Peptide was added at a concentration of 10 μg/ml; control wells were incubated with DMSO only. No cytokine was added to the cultures. After 10 days, cells were harvested and screened for their peptide reactivity by IFN-γ ELISPOT assay. Therefore, 105 peripheral blood mononuclear cells per well of 96-well microfiltration plates were seeded and synthetic peptides (10 μg/ml) were added to appropriate wells. Controls were incubated with DMSO only. The assay was done as described (20). Data are presented as mean IFN-γ spots per 105 peripheral blood mononuclear cells.

Students’s t test for unpaired samples was used for statistical evaluation of T-cell responses. Differences in T-cell responses were considered significant at P < 0.05.

**Results**

**Peptide selection and HLA-binding studies.** Screening of human TRP-2 for potential HLA-DRB1*0301-binding peptides by computer algorithms led to the selection of three putative epitopes: TRP-2α0.74, TRP-2α223-237, and TRP-2α298-413. The capacity of these peptides to bind to the HLA-DRB1*0301 molecule was analyzed in a competitive binding assay employing TTα298-413 peptide as a reporter peptide known to bind to HLA-DRB1*0301 with high affinity (Fig. 1). Sequence TRP-2α298-413 did not exhibit any detectable binding capacity for the HLA-DRB1*0301 molecule, even at high peptide concentrations. In contrast, the 15-mer TRP-2α223-237 competed against TTα223-237 peptide with moderate potency; whereas peptide TRP-2α223-237 revealed an IC50 of about 5 μmol/L, the IC50 value for TRP-2α298-413 was ~20 μmol/L.

**Analysis of peptide immunogenicity and antigenicity in HLA-DR3 transgenic mice.** On the basis of their HLA-DRB1*0301 binding capacities, the 15-mer TRP-2α223-237 and TRP-2α223-237 were selected for peptide immunizations of HLA-DR3tg mice (18). These mice do not express endogenous murine MHC class II molecules and therefore serve as an ideal tool to determine HLA-DRB1*0301–restricted peptide immunogenicity and antigenicity. Mice received a s.c. injection of the TRP-2-derived candidate peptides emulsified in complete Freund’s adjuvant; control animals were treated with complete Freund’s adjuvant plus PBS. After 10 days, draining lymph nodes were harvested and specificity of T cells was measured by IFN-γ ELISA after stimulation with peptides (Fig. 2). T lymphocytes from control mice did not respond to any peptide whereas peptide-specific T-cell responses could be detected in mice immunized with TRP-2α20.74, indicating its immunogenicity. In contrast, lymph node cells from mice immunized with TRP-2α223-237 did not exhibit any specific T-cell reactivity.

To prove that IFN-γ secretion was mediated by CD4+ Th cells, lymph node cultures from two TRP-2α20.74–immunized mice were depleted from CD4+ T cells and subsequently analyzed for
their peptide reactivity. As shown in Fig. 3, depletion of CD4\(^+\) T cells completely abrogated TRP-2\(^{60-74}\)–specific IFN-\(\gamma\) secretion by residual lymph node cells whereas total lymph node cell cultures from both mice still exhibited peptide-specific reactivity, indicating that the cytokine release was mediated by CD4\(^+\) Th cells.

To finally show that the TRP-2\(^{60-74}\) peptide can be processed from the TRP-2 antigen in vivo and that T cells did not respond to a contamination in the peptide preparation, HLA-DR3tg mice were immunized with recombinant TRP-2 protein emulsified in complete Freund’s adjuvant and control animals were again treated with complete Freund’s adjuvant plus PBS. Twelve days later, lymph node T cells from protein-vaccinated and control mice were screened for their specificity by IFN-\(\gamma\) ELISA after stimulation with peptides (Fig. 4). T lymphocytes from control mice did not respond to any of the peptide sequences. Comparably, incubation of lymph node cells from protein-vaccinated mice with peptide TRP-2\(^{223-237}\) did not lead to any detectable cytokine release. In contrast, stimulation with peptide TRP-2\(^{60-74}\) induced IFN-\(\gamma\) secretion in lymph node cultures from protein-immunized mice, indicating that an epitope located in the TRP-2\(^{60-74}\) sequence was processed from global TRP-2 antigen and that epitope-specific T cells were activated by protein immunization. None of the mice analyzed by peptide and protein immunizations developed any sign of skin depigmentation.

TRP-2\(^{60-74}\) epitope recognition by CD4\(^+\) T cells from melanoma patients. To determine if TRP-2\(^{60-74}\) is also a relevant epitope in humans, peripheral blood lymphocytes from three HLA-DRB1*03\(^+\) melanoma patients were employed to generate peptide-reactive T-cell lines. After primary stimulation with TRP-2\(^{60-74}\)–loaded autologous dendritic cells and restimulation with peptide-loaded autologous peripheral blood mononuclear cells, T cells from these donors were analyzed for their peptide reactivity by IFN-\(\gamma\) ELISPOT assays. As shown in Fig. 5A, TRP-2\(^{60-74}\)–induced IFN-\(\gamma\) secretion by T cells could be detected in peptide stimulation cultures from two of the three donors tested. The use of T2.DR3 target cells within these assays showed that the TRP-2\(^{60-74}\)–specific T-cell reactivity was restricted to the HLA-DRB1*0301 molecule. Furthermore, this peptide-specific activity was clearly mediated by the positive-selected CD4\(^+\) T-cell population whereas no specificity could be detected in the CD4\(^-\) T-cell fraction (Fig. 5B and data not shown). Importantly, TRP-2\(^{60-74}\)–reactive CD4\(^+\) Th cells also responded specifically to T2.DR3 target cells loaded with recombinant TRP-2-bacteriophages as a source of exogenous TRP-2 protein (Fig. 5B), demonstrating processing of the epitope by tumor antigen–loaded human antigen-presenting cells. Further peptide stimulation experiments revealed that TRP-2\(^{60-74}\)–reactive T cells could also be induced in vitro from peripheral blood lymphocytes of HLA-DRB1*0301\(^+\) healthy individuals (data not shown).

This led to the question if the TRP-2\(^{60-74}\) epitope might also be a target of spontaneous T-cell responses in HLA-DRB1*03\(^+\) melanoma patients. To analyze this, peripheral blood mononuclear cells from two donors were seeded in the presence or

![Fig. 2. Peptide immunization of HLA-DR3tg mice. Mice were immunized s.c. with peptide TRP-2\(^{60-74}\) or TRP-2\(^{223-237}\) emulsified in complete Freund’s adjuvant. Control mice were treated with complete Freund’s adjuvant plus PBS. Ten days after immunization, lymph node cells were harvested and analyzed for their specificity by incubation for 72 hours with medium, an irrelevant peptide, the specific peptide used for immunization, and concanavalin A (lectin mitogen, positive control), respectively. Culture supernatants were then analyzed for the presence of IFN-\(\gamma\) secreted by stimulated T cells. Columns, mean of triplicate determination; bars, SD. Data obtained for each mouse are presented separately.

![Fig. 3. Depletion of CD4\(^+\) T cells abrogates TRP-2\(^{60-74}\)–specific T-cell responses. Mice were immunized s.c. with peptide TRP-2\(^{60-74}\) emulsified in complete Freund’s adjuvant. Ten days after immunization, lymph node cells were harvested and analyzed for their specificity by incubation for 72 hours with medium, an irrelevant peptide (TT829-843), peptide TRP-2\(^{60-74}\) used for immunization, and concanavalin A (lectin mitogen, positive control), respectively. Culture supernatants were then analyzed for the presence of IFN-\(\gamma\) secreted by stimulated T cells. Lymph node cells depleted of CD4\(^+\) T cells (CD4\(^{-}\) T cells) lost their capacity to respond to peptide TRP-2\(^{60-74}\). Columns, mean of triplicate determination; bars, SD. Data obtained for each mouse (recipient) are presented separately.](https://www.aacrjournals.org/clin-cancer-research/article-pdf/11/14/5244/2509837/clincancerres.aacrjournals.11_2005_05_14_5244爱尔兰)}
absence of the candidate peptide TRP-2 60-74 as well as peptide TRP-2 223-237, with the last supposed to be an additional negative control in this assay. Cells were incubated in medium without cytokines over a period of 10 days. This single round of \textit{in vitro} stimulation has been shown to efficiently recall memory T-cell responses (21, 22), but it should not generate specific T cells from naïve precursors. On day 11, cells were analyzed for peptide-specific cytokine release by IFN-\(\gamma\) ELISpot assay. Interestingly, melanoma patient BH clearly exhibited TRP-2 60-74–specific T-cell reactivity after short-term stimulation (Fig. 6), suggesting that this T-cell response was already induced \textit{in vivo}. However, this strategy did not lead to the detection of TRP-2 60-74–reactive T cells in donor BI, indicating that these specific T cells were either of low frequency and/or require potent stimulation under \textit{in vitro} priming conditions to become detectable as shown in Fig. 5A.

Corresponding to the results obtained in HLA-DR3tg mice, no T-cell stimulation was obtained with peptide TRP-2 223-237. Furthermore, no peptide-specific activity was detectable in short-term stimulated peripheral blood mononuclear cell cultures from four HLA-DRB1*0301* healthy donors (data not shown).

**Discussion**

The therapeutic effectiveness of antigen-specific immunotherapies against malignant melanoma is now being evaluated in many clinical studies, but, thus far, objective clinical responses are observed infrequently. Several mechanisms have been postulated to be responsible for this: (a) tumors have been described to escape from immune recognition (e.g., by down-regulation or even total loss of surface expression of HLA molecules or by secretion of immunosuppressive cytokines; refs. 23, 24); (b) inadequate T-cell stimulation during vaccination leading to the induction of only weak T-cell responses might contribute to therapeutic ineffectiveness. Although the critical role of CD4+ Th cells in primary activation and maintenance of CTL immunity is already known for some time (10–12), the majority of clinically applied immunotherapies still target only the CTL arm of the immune system. This might be due to the fact that only a few peptide sequences containing Th epitopes from a limited number of tumor antigens have been described thus far, but great efforts are now being taken to identify these targets (25–28).

Proteins involved in melanogenesis are well-characterized antigens of malignant melanoma (29). Because these proteins are autoantigens, central and peripheral tolerance mechanisms act on T cells specifically recognizing these structures. Indeed, a
whereas control cells were again left without peptide (five-well BH and four-well BI determinations; bars, SD). Compared with the presence of the indicated peptides (10 μg/mL), peripheral blood from HLA-DRB1*03+ melanoma patient BH. Peripheral blood BH (HLA-DRB1*03, HLA-DRBI*08) and BI (HLA-DRB1*0301, HLA-DRB1*0801) were incubated for 10 days without or with 10 μg/mL peptides (TRP-260-74, TRP-2223-237), then harvested and analyzed for peptide-induced IFN-γ secretion by ELISpot assay. Cells were incubated in the presence of the indicated peptides (10 μg/mL) already used for primary stimulation whereas control cells were again left without peptide (w/o). Columns, mean of five-well (BH) and four-well (BI) determinations; bars, SD. Compared with the control (w/o), T cells from patient BH were clearly stimulated by peptide TRP-260-74 but no reactivity could be observed against peptide TRP-2223-237.

Fig. 6. In vivo sensitized, TRP-260-74-specific T cells can be detected in the peripheral blood from HLA-DRB1*03+ melanoma patient BH. Peripheral blood mononuclear cells from melanoma patient BH (HLA-DRB1*03, HLA-DRBI*08) and BI (HLA-DRB1*0301, HLA-DRB1*0801) were incubated for 10 days without or with 10 μg/mL peptides (TRP-260-74, TRP-2223-237), then harvested and analyzed for peptide-induced IFN-γ secretion by ELISpot assay. Cells were incubated in the presence of the indicated peptides (10 μg/mL) already used for primary stimulation whereas control cells were again left without peptide (w/o). Columns, mean of five-well (BH) and four-well (BI) determinations; bars, SD. Compared with the control (w/o), T cells from patient BH were clearly stimulated by peptide TRP-260-74 but no reactivity could be observed against peptide TRP-2223-237.

recent report by Gotter et al. (30) showed melanoma differentiation antigen expression in purified human medullary thymic epithelial cells playing an essential role in the induction of self-tolerance. Interestingly, expression of antigens belonging to the group of germ line–related antigens (e.g., MAGE, NY-ESO), which had long been postulated to be precluded from tolerance, was also shown in these cells, suggesting that a certain degree of negative selection might also act on T cells specifically recognizing these antigens. However, irrespective of tolerance mechanisms, the remaining T-cell repertoire specific for melanoma differentiation antigen can be mobilized in vivo and has been shown to be therapeutically effective against the tumor (1, 2).

We concentrated our efforts on the identification of T epitopes from the TRP-2 antigen for the following reasons: First, an elevated expression level of TRP-2 mRNA (beside tyrosinase mRNA) in tumors has been reported to be associated with increased overall survival of late-stage melanoma patients (3). Second, expression of TRP-2 is detectable in up to 90% of metastatic tumor specimens (3), indicating that the majority of tumor patients might be candidates for TRP-2–specific vaccination. Third, cytotoxic CD8+ T cells specific for TRP-2 have repeatedly been isolated from patients’ tumor tissue and peripheral blood, demonstrating that TRP-2–specific immunity develops spontaneously (4–6). Fourth, TRP-2 has also recently been described as an antigen expressed in human glioma cells which were killed by TRP-2–specific CTLs (31). Mouse studies already showed that TRP-2–based vaccines can confer protective T cell–mediated immunity against gliomas (32, 33), suggesting that TRP-2–specific immunotherapies might be applicable to melanoma and glioma patients.

We employed HLA-DR3tg mice for the identification of TRP-2–derived Th epitopes to limit time- and cost-intensive in vitro culturing of human T cells. Accordingly, these mice had already been used for the identification of epitopes from candidate antigens involved in HLA-DR3–associated autoimmune diseases (34–36). These animals do not express endogenous MHC class II molecules but express the murine TRP-2 that exhibits ~87% amino acid identity to the human sequences, leading to the speculation that a certain level of tolerance towards human TRP-2 might also exist in these mice, making them an even more interesting tool for epitope characterization.

Via peptide immunization with in silico predicted epitope sequences, we showed immunogenicity of peptide TRP-260-74 in HLA-DR3tg mice and subsequent protein immunizations revealed its antigenicity. Previous work in HLA-DR4tg mice already showed that tumor antigen epitopes from gp100 (37), NY-ESO (38), and TRP-1 (39) identified in the murine model system were indeed relevant epitopes also in humans, as it was also shown for TRP-260-74 in our study: Peptide-reactive T cells could be induced after repeated in vitro stimulation of peripheral blood lymphocytes from different HLA-DRB1*03+ melanoma patients. These CD4+ T cells clearly responded specifically to target cells loaded with exogenous TRP-2 peptide, demonstrating processing of the TRP-260-74 epitope. Interestingly, T cells from a melanoma patient responded to peptide TRP-260-74 even after short-term in vitro peptide stimulation, suggesting in vivo sensitization of these T cells. Based on these results, we postulate that the TRP-260-74 epitope might be employed as a therapeutic and diagnostic tool for melanoma and probably also for glioma patients.

Acknowledgments

We thank Angela Funk for supporting animal studies and Antje Sucker and Claudia Sterzik for preparation of patient material.

References


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