Therapeutic Effectiveness of Recombinant Cancer Vaccines Is Associated with a Prevalent T-Cell Receptor α Usage by Melanoma-specific CD8⁺ T Lymphocytes

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ABSTRACT

Definition of immune variables that correlate with the antitumor activity of vaccine is critical for monitoring immunotherapy protocols. To define surrogate endpoints predictive of the therapeutic efficacy of recombinant vaccines based on melanoma antigen tyrosinase-related protein (TRP)-2, we evaluated several properties of antigen-specific CD8⁺ T lymphocytes in single mice undergoing either prophylactic or therapeutic immunization. Predictive markers for the efficacy of genetic vaccination were identified in the prophylactic model used. Interestingly, the number of tetramer⁺ CD8⁺ T lymphocytes expanded in vitro after a single cycle of stimulation with the immunodominant TRP-2 peptide was of the highest predictive value. In the therapeutic model, no variable examined at a single mouse level predicted the long-term therapeutic effect. Mice that survived did not show the highest expansion of antigen-specific lymphocytes or the more functionally active effectors, ex vivo or after in vitro culture with the peptide antigen. Successful therapy correlated strictly with the skewing of the T-cell receptor repertoire of tetramer-specific TRP-2-specific CD8⁺ T lymphocytes, which showed a preferential α chain usage with a common CDR3 region.

INTRODUCTION

Tyrosinase-related protein (TRP)-2 is a melanosomal enzyme defined as a melanocyte differentiation antigen because it is expressed in most mammalian melanocytes and melanomas. TRP-2 is the main antigenic target of the immune response elicited in mice by immunization with genetically modified B16 melanoma vaccine (1, 2). Although slight differences have been described under therapeutic vs. prophylactic conditions (2), the mouse immune response to TRP-2 is dominated by the generation of CD8⁺ T lymphocytes responsible for both the antitumor response and autoimmunity, i.e., skin depigmentation (vitiligo) due to normal melanocyte destruction (3). Tumor-infiltrating lymphocytes isolated from melanoma patients also recognize TRP-2 peptides in the context of different class I HLA alleles. Mouse and human TRP-2 have about 80% homology at the protein level, and the peptide SVYDFFVWL (TRP-2,180–188) is presented in association with mouse H-2 MHC class I molecules Kᵇ and human MHC molecule A2 (1, 4, 5).

Peripheral tolerance, which seems to limit the anti–TRP-2 response in the C57BL/6 strain, can be broken by xenoinmunization, or immunization with an altered source of antigen. Immunization with a recombinant adenovirus (rAd) encoding human TRP-2, in fact, elicited an immune response against the respective mouse homologue and completely protected C57BL/6 mice from a lethal melanoma challenge (6). Tolerance could be also broken by linking the mouse self-antigen with a foreign immunogenic protein providing strong CD4 helper sequences, such as the fusion protein between the mouse TRP-2 gene product and the enhanced green fluorescent protein (EGFP) of jellyfish Aequorea victoria (7). Whereas the induction of an effective immune response against TRP-1/gp75 was often accompanied by vitiligo, immunity to TRP-2, delivered by either plasmid DNA or recombinant viruses, was not always associated with widespread vitiligo (8, 9). These interesting findings delineate a window of opportunity between proautoimmune and therapeutic activity that could be exploited to promote the therapeutic effects of recombinant vaccines based on TRP-2.

Based on encouraging preclinical studies, melanoma-specific vaccines were designed and evaluated in clinical trials (10). Unfortunately, partial or complete tumor regression was reported only in a minority of patients (11). This was somewhat expected because most initial trials were conducted in patients with advanced metastatic disease. These trials were designed to identify surrogate endpoints with respect to vaccine efficacy in the absence of overt tumor regression. The most confounding results of cancer vaccine trials concern the absence of a clear-cut correlation between the clinical responses and the antigen-specific immune response detected in patient-derived T lymphocytes. In fact, vaccination with class I major histocompatibility complex (MHC)-restricted peptides can easily generate tumor-specific CD8⁺ T cells among the circulating lymphocytes of immunized patients, but there is no assay that unambiguously identifies those patients who will respond clinically to immunotherapy (11, 12).

To simultaneously analyze multiple aspects of the TRP-2-specific, CD8⁺ T lymphocyte-dependent response, we designed an experimental protocol to evaluate different immune variables in single mice after prophylactic or therapeutic immunization. In prevention experiments, after immunization with recombinant vaccines encoding TRP-2 antigen, mice underwent splenectomy. After recovery from surgery, they were challenged with a lethal intravenous inoculum of B16 melanoma cells to monitor tumor development. In the therapeutic model, splenectomy was performed in mice that had been previously inoculated with tumor cells and then vaccinated with recombinant vaccines. Fresh splenocytes were used to quantify the number of TRP-2-specific CD8⁺ T cells by cytometry and to enumerate the antigen-specific effectors releasing interferon (IFN)γ in an enzyme-linked immunosorbent spot (ELISPOT) assay. Moreover, by stimulating the antigen-specific T lymphocytes with the Kᵇ-restricted, TRP-2,180–188 Peptide for 5 days, peptide-stimulated mixed leukocyte cultures (MLPCs) were designed to expand limited numbers of TRP-2-reactive T lymphocytes. Finally, we studied the T-cell repertoire usage in tetramer-sorted, TRP-2-specific T lymphocytes recovered from peptide-stimulated cultures.

MATERIALS AND METHODS

Mice and Cell Lines. C57BL/6 (H-2ᵇ) mice (8 weeks old) were purchased from Charles River (Calco, Como, Italy). Animal care and procedures followed
in vivo tumor growth experiments were conducted in mice randomized before tumor injection or before treatment for the therapeutic model. Mice were bred in filtered cages placed inside biohazard closets. For the adoptive transfer, B6 mice were inoculated with $10^6$ B16U8 tumor cells via tail vein injection on day 0. On day 3, $5 \times 10^6$ TRP-2–specific CTLs were administered intravenously, and 30,000 IU of recombinant interleukin (IL)-2 were given intraperitoneally twice a day for 3 days.

**Synthesis of Major Histocompatibility Complex/Peptide Tetrameric Complexes.** Soluble H-2–peptide tetramers were produced using a previously described method (14). Soluble purified complexes were biotinylated using BirA enzyme (Avidity, Denver, CO). Phycoerythrin (PE)–labeled tetramers were produced by mixing the biotinylated complexes with Extravidin–PE (Sigma, St. Louis, MO) and validated by staining CTL clones with the appropriate specificity. Each tetramer batch was titrated and used at the optimum concentration (5 μg/mL) of K$^b$ heavy chain.

**Cell Staining, Flow Cytometry, and Cell Sorting.** Fresh or in vitro stimulated splenocytes (10$^6$ per sample) were resuspended in 50 μL of fluo- rescence-activated cell sorting (FACS) buffer (0.9% NaCl solution containing 2% bovine serum albumin and 0.02% NaN$_3$; both from Sigma) with anti-mouse Fc–γ receptor 2.4G2 mAb (ATCC HB-197) for 10 minutes at room temperature

**DNA Immunization.** Preparation of the VR1055-P15 plasmid encoding the p15E portion of the env gene has been described previously (14). Plasmid amplification was performed with the Endofree plasmid mega kit (Qiagen, Hilden, Germany), following the manufacturer’s instructions. Plasmids were purified with Qiagen columns using an endotoxin-free DNA preparation (Qiagen GmbH, Hilden, Germany). For DNA immunization, C57BL/6 mice were anesthetized by avertn injection and inoculated intramuscularly with 100 pmol/L cardiotoxin (Latoxan, Rosans, France). Five days later, mice received intramuscular injection with 100 μg of plasmid DNA in 100 μL of saline.

**In vitro Antitumor Treatment.** Mice were depleted of either CD4$^+$ or CD8$^+$ T cells by four intraperitoneal injections of 200 μg of affinity chromatography–purified GK1.5 (anti-CD4) or 2.43 (anti-CD8) monoclonal antibody (mAb). Depleting mAbs were administered 2 days before and 0, 4, and 8 days after subcutaneous challenge with tumor cells. In therapy experiments, the mAbs were administered 1 day before and 0, 4, and 8 days after vaccination with rAd. Depletion was consistently $>$98%. As a control, anti-Esherichia coli β-galactosidase (GL117.14; rat IgG2a) was used at the same dose.

**Mixed Leukocyte Peptide Culture.** Three weeks after plasmid DNA inoculation, spleens were removed, and 2.5 × 10$^7$ splenocytes were stimulated in vitro in a MLPC with 1 μg/mL of a nonamer peptide corresponding to amino acids 180 to 188 of TRP-2 protein (SYVDFFVWL). K$^b$–restricted peptides corresponding to amino acids 604 to 611 of p15E protein (KSPW-2-mercaptoethanol, 150 units/mL streptomycin, 200 units/mL penicillin, and 10% heat-inactivated fetal bovine serum (Invitrogen). TRP-2–specific cyto- toxic T lymphocyte (CTL) clones 8 and 24 were obtained from a C57BL/6 mouse immunized with pcDNA3-trp-2 peptide, as described in ref. 8, by several in vitro restimulations with syngeneic splenocytes pulsed with TRP-2$_{181–188}$ peptide (10 μM/mL) and after limiting dilution cloning.

**Viruses.** Adenoviruses used in this study were constructed through Cre-lox recombination with reagents generously provided by Dr. S. Hardy (Somatics, Alameda, Alameda, CA). Adenoviruses were propagated on 293 cells, purified by cesium chloride density gradient centrifugation, dialyzed according to standard protocols (6, 7), and then stored at −70°C. All vectors used express only the antigen of interest under the cytomegalovirus immediate early promoter. The recombinant vaccinia virus (v) encoding the human gp100 epitope as env–galactosidase (GL117.14; rat IgG2a) was used at the same dose.

**Cell Staining, Flow Cytometry, and Cell Sorting.** Three weeks after immunization, mice were challenged subcutaneously with a lethal dose of B16 melanoma cells (2 × 10$^7$) and then monitored for 100 days after tumor injection. Tumor growth was monitored every 3 days by caliper measurement. Alternatively, C57BL/6 mice were inoculated intravenously with $10^6$ B16U8 cells. Lungs were removed 14 days after challenge, and pulmonary metastases were counted in a blind fashion. In therapy experiments, the same dose of tumor cells was used, but tumor was injected 3 days before immunization. All the in vivo experiments were conducted in mice randomized before tumor injection or before treatment for the therapeutic model. Mice were bred in filtered cages placed inside biohazard closets. For the adoptive transfer, B6 mice were inoculated with $10^6$ B16U8 tumor cells via tail vein injection on day 0. On day 3, $5 \times 10^6$ TRP-2–specific CTLs were administered intravenously, and 30,000 IU of recombinant interleukin (IL)-2 were given intraperitoneally twice a day for 3 days.

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RNA and Complementary DNA Preparation for T-Cell Repertoire Analysis. RNA was extracted using guanidine hydrochloride-containing Trizol reagent (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer’s instructions. First-strand cDNA synthesis was performed using oligo(dT) as a primer for reverse transcription of 1 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) as described previously (15).

Polymerase Chain Reaction Amplification for T-Cell Receptor Genes. Polymerase chain reaction (PCR) amplification was performed as reported in detail elsewhere (15). Briefly, after titration of the different templates, cDNAs were amplified for 30 cycles under nonsaturating PCR conditions. The T-cell receptor (TCR) repertoire was analyzed using a panel of TCR BV and AV family-specific primers by PCRs as described previously (15, 16). In each PCR reaction, the common TCR-α or -β primer was labeled at the 5′ end with 5′-6-carboxyfluorescein. In addition to the TCR BV or AV family-specific and independent discrete random variables. All

Recognizing Mouse TRP-2180–188 Peptide. trp-2 (rAd-hTRP-2) Induces Therapeutic CD8+ T cells, but not CD4+ T cells, abrogated the preventive activity (Fig. 1A). The vaccination protocol generates an antigen-specific CTL response against the immunodominant Kb-restricted TRP-2180–188 peptide. In fact, splenocytes from mice immunized with rAd-hTRP-2 and stimulated in vitro with syngeneic cells infected with the same virus showed a strong cytotoxic activity against B16 cells and 293Kb pulsed with the TRP-2180–188 peptide, but not toward control cells pulsed with the irrelevant β-gal peptide or the natural killer target YAC-1 cells (Fig. 1B), suggesting that the TRP-2-specific CTLs were responsible for all or most of the lytic activity of the cultures stimulated with the whole rAd-hTRP-2. Even after tumor rejection, the immune response in mice vaccinated with rAd-hTRP-2 did not spread significantly toward other antigens expressed by the melanoma cell line B16. Splenocytes of mice that had rejected B16 pulmonary metastases after preventive vaccination with rAd-hTRP-2 were stimulated in MLPCs with the immunodominant peptides of the mouse melanoma antigens TRP-2, gp100, or p15E (13, 14). These peptide-stimulated splenocytes were tested for the ability to recognize B16 cells by releasing IFN-γ, an assay useful to detect T lymphocytes with high avidity toward the tumor antigen (see below). Only the MLPCs stimulated with TRP-2 peptide contained effectors that recognized B16 cells, provided that the mice had been immunized with rAd-hTRP-2 but not with the control rAd-EGFP. This suggests that tumor growth in the host did not generate per se an immune reactivity to different melanoma antigens including TRP-2 (Fig. 1C). No reactivity against either gp100 or p15E was detected in mice that rejected the melanoma after immunization with rAd-hTRP-2, whereas antimelanoma reactivity directed to gp100 and p15E antigens could be easily induced by immunizing mice with either a plasmid DNA encoding p15E or a rVV encoding the altered peptide of gp100 (13, 14). These results are in agreement with previously published data. In fact, immune response elicited by irradiated B16 melanoma cells engineered to produce granulocyte macrophage colony-stimulating factor and given in conjunction with anti-CTLA-4 was directed exclusively to TRP-2 antigen, with no evidence of reactivity to melanocyte antigens gp100, tyrosinase, Melan-A/MART-1, or TRP-1 (2). Analysis of Preventive Vaccination with Recombinant Adenovirus Encoding trp-2. To evaluate the efficacy of different immunogens, the rAds encoding either green fluorescent protein fused to mouse TRP-2 [mTRP (rAd-EGFP-mTRP-2)] or murine TRP-2 (rAd-mTRP-2) were compared with rAd-hTRP-2. Mice underwent splenectomy 14 days after vaccination with different rAds. Splenocytes were used for staining with H-2 Kb tetramers assembled with the TRP-2180–188 peptide (TRP-2-TET) as effectors in the ELISPOT assay and for MLPC. After 5 days, we performed tetramer staining and IFN-γ release from the MLPC. On day 21, these mice were challenged with an intravenous lethal inoculum of B16 melanoma cells. Fourteen days later, lungs were removed, and pulmonary metastases were counted in a blind fashion (Supplemental Fig. S1A).

As expected, immunization with rAd-mTRP-2 did not prevent tumor take (Fig. 2A). After immunization with rAd-hTRP-2 but not with rAd-mTRP-2, all mice showed significant changes in the immunologic variables examined (ELISPOT assay, TRP-2-TET+ cells ex vivo and in MLPC, and IFN-γ released by MLPC-derived lymphocytes, Fig. 2B–D). The fusion product, EGFP-mTRP-2, delivered by rAds also conferred protection from challenge. However, whereas MLPC-derived T lymphocytes released IFN-γ when cocultured with
the mice receiving the control rAd and those depleted of CD8 T lymphocytes of B16LU8 melanoma were counted in a blind fashion in lungs removed 14 days after injection of C57BL/6 mice inoculated with rAd-hTRP-2 were surgically removed 14 days after injection (Fig. 3A). These mice will be referred to as regressors. Depletion of CD8 T cells before vaccination with rAd-hTRP-2 adversely affected the therapeutic activity of the vaccine because tumor development at the same speed as the negative control, confirming a prevalent role of CD8 T lymphocytes in tumor eradication (Fig. 3B). Interestingly, CD4 T cells appear to be required in the later phase of tumor rejection because mice depleted of this lymphocyte population developed tumor, but with slower kinetics (Fig. 3B).

Analysis of the immune response in single mice (Supplemental Fig. S1B) did not reveal any characteristic that predicted this long-term therapeutic effect. Complete regressors did not have the highest TRP-2-TET cell expansion or the more functionally active effectors, ex vivo or after in vitro stimulation with the TRP-2180–188 peptide (Table 1), a situation similar to that observed in melanoma patients. The overall analysis indicated that the mice rejecting the tumor in the therapy model presented intermediate levels of TRP-2-TET cell expansion, as compared with the group that did control tumor growth for some time but did not reject the tumor completely (Table 1).

Analysis of T-Cell Repertoire. To further characterize the immune response in this therapeutic model, we analyzed the TCR repertoire usage of the mice combining tetramer sorting with T-cell repertoire examination by means of CDR3 length analysis, a technique known as spectratyping or immunoscope. The nucleotidic sequence of each CDR3 rearrangement defines a T-cell clonotype. Spectroscopy of splenocytes from mice treated with rAd-hTRP-2 were cultured with TRP-2180–188 peptide and the triple-positive lymphocytes (TRP-2-TET+/CD3+/CD8+) separated by high-speed cell sorting. Tetracycline-guided analysis of TCR α- and β-chains revealed a severe restriction in TCR BV and TCR AV gene segment usage and a difference in repertoire between regessor and regressor mice. In Fig. 4A, we show a synopsis of the complete TCR BV and TCR AV repertoire in two animals, one regressor and one regressor, after therapy with rAd-hTRP-2 (for actual spectratypies, see Supplemental Fig. S2). The mouse that was cured by therapeutic immunization showed a severely

**Fig. 1.** Immune response elicited by rAd-hTRP-2 is dominated by CD8 T lymphocytes recognizing the mouse epitope. A. Depletion of CD8 T lymphocytes prevents the prophylactic activity of vaccination with rAd-hTRP-2. Groups of five C57BL/6 mice were immunized intraperitoneally with 107 plaque-forming units (PFU) per mouse of either rAd-hTRP-2 or the control rAd-EGFP. Mice immunized with rAd-hTRP-2 were further depleted of various immune cells with specific mAbs, as described in Materials and Methods. Pulmonary metastases of B16 melanoma were counted in a blinded fashion in lungs removed 14 days after challenge and plotted as mean ± SE. No significant statistical difference was found between the mice receiving the control rAd and those depleted of CD8 T cells. For all other groups, P < 0.001 versus Ad-EGFP. B. CTL response against mouse TRP-2180–188 epitope. Splenocytes of C57BL/6 mice inoculated with rAd-hTRP-2 were surgically removed 14 days after vaccination, and splenocytes were cultured for 6 days in the presence of γ-irradiated syngeneic tumor cells infected with rAd-hTRP-2. Splenocytes were then tested in a 6-hour 3HCr release assay against 293Kc cells pulsed with 5 μg/mL of either TRP-2180–188 or β-gal–gal96–103 peptide or against B16 melanoma cells (left panel). Effector to target cell ratios are indicated; spontaneous release never exceeded 20%. Titrated numbers of cold TRP-2–pulsed 293Kc cells, β-gal–loaded 293Kc cells, the natural killer target YAC-1 cells, and B16 cells were admixed to hot B16 cells and tested in a standard cytotoxicity assay with the same effectors(right panel). Values are expressed as means of triplicates of the percentages of lysis inhibition of B16 cells at an effector to target cell ratio of 100. The data shown are representative of at least three independent experiments. C. Absence of epitope spreading after tumor rejection. Five C57BL/6 mice in each group were immunized with rAd-mTRP-2, rAd-EGFP, plasmid DNA encoding p15E (p15E-DNA), or rVV expressing the altered (human) peptide of gp100. No Tumor. For all comparisons, the B16 control group was set as 100%, and each value is expressed as a percentage versus the control wells containing equal volume of medium alone.
Fig. 2. Multivariable analysis of T-cell response in prophylactic vaccination with rAd. A, C57BL/6 mice (n = 10; n = 9 only for mice immunized with rAd-EGFP-mTRP-2) were vaccinated with rAd encoding different antigens. The mice were challenged intravenously with 10^7 B16LU8 melanoma cells 25 days after vaccination. The reduction in metastasis number observed in mice immunized with rAd-hTRP-2 and rAd-EGFP-mTRP-2 was significant (P < 0.0001) in comparison with the control group treated with rAd-EGFP. Data are from two cumulated experiments. B, The same mice described in A were splenectomized 21 days after rAd vaccination (before tumor challenge) for functional analysis. This panel shows IFN-γ release by whole splenocytes stimulated in vitro with TRP-2_180–188 peptide for 24 hours in ELISPOT assay. The number of spots differs significantly (P < 0.01) in the group treated with either rAd-hTRP-2 or rAd EGFP-mTRP-2 compared with those treated with rAd-EGFP control virus and rAd-mTRP-2. C, Splenocytes isolated 21 days after immunization with rAd were cultured in MLPCs, and the cultures were tested for IFN-γ release against B16 cells, MBL-2 pulsed with TRP-2_180–188 peptide, and unpulsed MBL-2. The difference between IFN-γ released against pulsed and unpulsed MBL-2 is reported. Statistics for pulsed MBL-2 targets are as follows: rAd-EGFP-mTRP-2 versus rAd-EGFP, P = 0.027; and rAd-mTRP-2 versus rAd-EGFP, P < 0.0001. Statistics for B16 target are as follows: rAd-EGFP-mTRP-2 versus rAd-EGFP, P = 0.17; and rAd-hTRP-2 versus rAd-EGFP, P = 0.008. D, Cytofluorometric analysis of CD3⁺/CD8⁺/TRP-2-TET⁺ cells in mice described above. The splenocytes were stained ex vivo, as soon as the spleens were removed, and after 5 days of culture with TRP-2_180–188 peptide (MLPC). Statistics for ex vivo stained cells are as follows: rAd-EGFP-mTRP-2 versus rAd-EGFP, P < 0.0001; and rAd-hTRP-2 versus rAd-EGFP, P < 0.0001. Statistics for splenocytes derived from MLPCs are as follows: rAd-EGFP-mTRP-2 versus rAd-EGFP, P = 0.004; and rAd-hTRP-2 versus rAd-EGFP, P = 0.003. E, Analysis of correlation between the number of metastases in mice immunized with rAd-EGFP-mTRP-2 and the number of CD3⁺/CD8⁺/TRP-2-TET⁺ MLPC splenocytes of the same mice. A correlation was detected according to a nonparametric statistic test (Spearman’s rank, correlation = −0.955; P = 0.001). The best curve fitting the data (Rsqr = 0.996) is the two-parameter exponential curve described by the formula Y = 0.44e⁻^−X, in which Y = metastasis fraction, and X = percentage of Tet⁺ cells. One outlier was excluded from the analysis.

Skewed repertoire; in particular, the TCR AV compartment presented a more marked skewing in comparison with TCR BV usage. Similar results were found in all animals studied (data not shown). Even more strikingly, we observed the expression of high message levels for a given α family (TCR AV5) in all of the survivors. By spectratype analysis using a combination of AV and AJ primers, this family was resolved as a single peak (Fig. 4B). These peaks were directly sequenced using an internal primer (data not shown) and found to contain a major CDR3 sequence originally described in CTL clone 24. Interestingly, the CTL clones 8 and 24, which were characterized ex vivo, as soon as the spleens were removed, and after 5 days of culture with TRP-2_180–188 peptide (MLPC), expressed the CDR3 sequence that revealed the T-cell clonotype mentioned above, which contained a major CDR3 sequence originally described in CTL clone 24 and shared a common AV gene (GenBank accession numbers AY089787 and AY089788, respectively). This allowed us to investigate the properties of TRP-2–specific clones that differed only in the TCR β-chain. Both clones 8 and 24 were able to lyse a syngeneic cell line pulsed with TRP-2_180–188 (Fig. 5A). However, a titration assay performed to evaluate the functional TCR avidity for the TRP-2_180–188 K^b antigenic complexes clearly indicated that CTL clone 24 possessed a somewhat higher avidity (Fig. 5A). In fact, clone 24 exhibited an EC_{50} (2 × 10^{-11} mol/L) that was about 10 times lower than the EC_{50} of clone 8 (2.5 × 10^{-10} mol/L). In agreement with these results, both clones released similar levels of INF-γ when stimulated with peptide-pulsed syngeneic cells, but only clone 24 was able to secrete INF-γ against B16 parental melanoma and the variant B16LU8 (Fig. 5B). Taken together, these data suggest that TCR-α usage is predominantly shaped by the recognition of TRP-2. However, the β-chain contributes considerably in determining the function and avidity of specific T cells.

We speculated that the higher avidity clone 24 might better recognize the few antigen complexes exposed on B16 tumor cells also in vivo. C57BL/6 mice inoculated intravenously with B16LU8 cells...
for Ad-EGFP, Ad-hTRP-2, and rAd-EGFP were used as a negative control group. The appearance of tumors greater than 2 mm was plotted as a function of time. The Mantel-Haenszel test gave $P > 0.05$; rAd-EGFP versus rAd-hTRP-2. $P = 0.0001$; rAd-hTRP-2 versus rAd-mTRP-2, $P = 0.0016$. B. To identify the effector population responsible for the antitumor effect, mice were depleted by intraperitoneal injections of either anti-CD4, anti-CD8, or control mAb (as described in Materials and Methods) before immunization with rAd-hTRP-2. Mice that received the rAd-EGFP were used as a negative control group. The appearance of tumors greater than 2 x 2 mm was plotted as a function of time. The Mantel-Haenszel test gave $P = 0.026$ for Ad-EGFP versus Ad-hTRP-2 + control mAb, $P = 0.044$ for the Ad-EGFP versus Ad-hTRP-2 + anti-CD4 mAb, and $P = 0.25$ (nonsignificant) for Ad-EGFP versus Ad-hTRP-2 + anti-CD8 mAb. Duplicate experiments confirmed these results.

received an inoculum of $5 \times 10^6$ cells of the different CTL clones 3 days later. IL-2 was given intraperitoneally for 3 days after adoptive transfer. A group of mice treated only with IL-2 was used as a control. The IL-2 treatment alone reduced the number of metastases in comparison with untreated C57BL/6 mice ($P = 0.007$). The adoptive transfer of clone 8 marginally increased the antitumor effects exerted by IL-2 treatment alone ($P = 0.11$). On the other hand, the adoptive transfer of clone 24 significantly reduced the total number of pulmonary metastases (clone 24 versus IL-2, $P = 0.006$). These results clearly reveal a strict association of the ability of CTLs to recognize melanoma in vitro with the TCR avidity for the peptide-MHC complexes and with the antitumor effects in adoptive transfer experiments.

DISCUSSION

In the present study, a multivariable analysis of the CD8$^+$ T-cell response in mice vaccinated with different recombinant vaccines encoding the melanoma antigen TRP-2 delineated three main situations. The simplest finding involved the activity of the powerful rAd-hTRP-2 vaccine in prophylaxis of tumor challenge. All of the mice mounted a vigorous immune response against the mouse K$b$-restricted epitope, as indicated by the significant changes in the number and functions of TRP-2–specific T lymphocytes. The immune response was so strong that correlation with the number of metastases was not feasible because the majority of the mice did not present countable metastases after challenge with B16 (7 of 10 mice had no metastases). T lymphocytes stimulated with the TRP-2$_{180-188}$ peptide in vitro also recognized the wild-type melanoma. Nevertheless, it must be noted that prophylactic vaccination with the xenogeneic form of the antigen, although valuable for several experimental mouse tumor antigens, would be difficult to implement and interpret in a clinical setting.

The second finding involved formulation of a clinically applicable immunogen (chimeric protein between an immunogenic protein and mouse TRP-2 expressed in rAd) that was shown to have some protective effect on tumor challenge, but with a thwarted immune response. TRP-2–specific T lymphocytes, which proliferated after in vitro stimulation, efficiently recognized the immunodominant peptide, but not the B16 melanoma, which displays a low number of K$b$-peptide complexes on the surface due to down-regulation of class I H-2 molecules (21). This finding suggests that T lymphocytes with low avidity TCR were the main effectors elicited by these vaccines. The percentage [and the absolute number (data not shown)] of TRP-2–TET$^+$ lymphocytes in peptide-stimulated cultures correlated directly with the antimitastic activity of the vaccine. Moreover, rAd-EGFP-mTRP-2 vaccination was successful in eliciting effector lymphocytes detected by an ELISPOT assay for IFN-$\gamma$, and the number of effector lymphocytes also correlated with the prophylactic efficacy (data not shown).

The third finding involved the model most similar to the clinical setting. Therapy of established tumors required a strong vaccine formulation, and yet only about half of the treated mice completely rejected the tumor. Even with this small tumor burden, therapy appeared to be a stochastic event, as in human melanoma patients. All mice, in fact, developed an easily detectable immune response against TRP-2, but the breadth of the response did not allow us to distinguish tumor progressors from regressors. Qualitative rather than quantitative differences were thus suspected. Individual differences in the orientation of the CTL response to a tumor antigen were previously explained by the stochastic timing of recruitment of different epitope-specific T cells, a sort of “first come, first served” hypothesis (22). Indeed, it has been elegantly proven that T cells that encounter the antigen at early time points can account for a significant part of the specific response, even though they are not the most frequent in the preimmune repertoire (23). Our results might reflect a scenario in which only those mice that present an in vivo expansion of a selected population of T cells bearing particular TCR AV chains are able to reject the preexisting tumor. The properties and dynamics of these “fittest” T cells are currently not known, although we can speculate that lymphocytes possessing the AV5 chain (together with a few other AV clonotypes) might include high avidity TRP-2–specific T cells, whose prototype is represented by clone 24.

Combining the sorting of TRP-2–specific CD8$^+$ T cells and quantitative PCR-based T-cell repertoire analysis greatly improves the accuracy in detecting T-cell clones that could not otherwise be detected by using functional assays, semiquantitative TCR analysis, or tetramer sorting alone (19). Functional assays based on the estimation of the overall immune reactivity against the melanoma antigen could not be sufficiently predictive in therapeutic vaccination because they reflect an oligoclonal expansion that might or might not include the therapeutic CTL clones. In a prophylactic setting, on the other hand, this oligoclonal response might be sufficient to control the growth of
Fig. 4. T-cell repertoire analysis of TRP-2–specific lymphocytes. A. Three-day–old melanoma-bearing mice were immunized with rAd-hTRP-2 and monitored for survival. Three weeks after immunization, spleens were removed, splenocytes stimulated with TRP-2180–188 peptide, and CD3/H11001/CD8/H11001/TRP-2-TET/H11001 cells were isolated by high-speed flow cytometry sorting. T-cell repertoire analysis for BV and AV gene usage in sorted cells was analyzed by spectratyping. A shows a synopsis of AV and BV gene usage in two animals, one tumor regressor and one progressor. White boxes indicate that the corresponding TCR family (AV or BV) has a normal, Gaussian-like repertoire; the incomplete boxes indicate the absence of the given family; right-hatched boxes indicate a skewed TCR family, characterized by a severe alteration in the Gaussian trend; and black boxes indicate a given TCR family in which a predominant peak is found by CDR3-length analysis. Similar results were found in all of the animals studied (shown in B). B. Detailed analysis of the TCR AV5 family in all animals studied. Amplification was performed using a TCR AV5-specific primer paired with a primer amplifying AJ41 in place of a primer amplifying for the Constant/H9251 region. Left histograms show TCR AV5 in the animals that survived for >90 days after therapy with rAd-hTRP-2. The predominant peak, corresponding to a PCR product of 172 bp, was further characterized by sequencing analysis and found to be composed mainly of the sequence found in the TRP-2–specific CTL clone 24. Right histograms show the peaks for progressor mice. Numbers indicate the day of death after tumor challenge.

Table 1 Multivariable analysis of T-cell response in therapeutic vaccination with rAd

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<th>Mouse no.</th>
<th>Day of death*</th>
<th>ELISPOT†</th>
<th>Tetramer‡ cells ex vivo</th>
<th>IFN-γ release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>B16§</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tetramer† cells in MLPC¶</td>
</tr>
<tr>
<td>Ad-EGFP</td>
<td></td>
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</tr>
<tr>
<td>975</td>
<td>Alive</td>
<td>34.5</td>
<td>0.09</td>
<td>4.930</td>
</tr>
<tr>
<td>Ad-hTRP-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>973</td>
<td>54</td>
<td>17</td>
<td>0.32</td>
<td>3.259</td>
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<tr>
<td>974</td>
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<td>934</td>
<td>43</td>
<td>3</td>
<td>0.19</td>
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<tr>
<td>804</td>
<td>69</td>
<td>ND</td>
<td>1.82</td>
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<tr>
<td>802</td>
<td>35</td>
<td>ND</td>
<td>0.98</td>
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<tr>
<td>890</td>
<td>Alive</td>
<td>ND</td>
<td>0.97</td>
<td>3,281</td>
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<td>893</td>
<td>Alive</td>
<td>ND</td>
<td>0.41</td>
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<td>805</td>
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<td>ND</td>
<td>0.63</td>
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<tr>
<td>951</td>
<td>Alive</td>
<td>25</td>
<td>0.91</td>
<td>5,327</td>
</tr>
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</table>

NOTE. Mice immunized with different rAds (described in Fig. 3) were splenectomized 14 days after immunization to simultaneously evaluate different immune variables. Data are presented as the mean ± SE for the group of mice immunized with the control Ad-EGFP; single values were reported for the all mice treated with Ad-hTRP-2 and for the only mouse cured by Ad-mTRP-2 inoculation.

Abbreviation: ND, not done.

* Mice were euthanized when the tumor was > 1 cm².
† Number of spots (IFN-γ–releasing cells) per 10⁶ fresh splenocytes.
‡ Percentage of TRP-2–specific splenocytes evaluated ex vivo as the difference between TRP-2-TET+ and unrelated β-gal-TET+ splenocytes among the CD3+ and CD8+ T cells.
§ Amount (pg/mL) of IFN-γ released by MLPC splenocytes against B16 cells (ELISA assay).
¶ Percentage of TRP-2–specific splenocytes evaluated in MLPCs as the difference between the amounts released in the presence of peptide-pulsed MBL-2 after subtraction of background release against unpulsed MBL-2 (ELISA assay).
the limited number of B16 melanoma cells inoculated in mice regardless of the clonal composition of the TRP-2-responsive population.

Preferential AV usage in tumor regressors was not entirely surprising because antigen recognition by CTLs was shown to require a specific α-chain pairing with a variety of TCR β-chains (24), suggesting a biased TCR AV usage in peptide recognition that has been confirmed by analysis of TCR-MHC-peptide complex crystals (25, 26). Moreover, a restricted TCR-α repertoire has been described in CTLs recognizing the Melan-A/MART-1 melanoma antigen in HLA A2 context (27–29), although in these studies, different CDR3 rearrangements were found in the presence of the same AV gene usage.

In one report, Melan-A-specific T cells isolated from melanoma patients were found to have a frequent usage of the AV 2.1 chain but a large BV chain repertoire (29). This preferential usage is not related to an antigen-driven narrowing of the TCR affinities or peripheral homeostatic expansion of selected clones in tumor-bearing hosts but rather reflects a constraint already present in the preimmune repertoire. Our data are apparently discrepant; however, some important differences need to be highlighted. We analyzed the TCR repertoire on a T-cell population able to bind TRP-2 tetramers. AV preferential usage was found in animals that regressed the tumor, thus it is possible that we preferentially selected the lymphocytes that recognize TRP-2 with higher efficiency. Moreover, the Melan-A/MART-1 antigenic system is unique in that a sizeable pool of naïve Melan-A/MART-1-specific CD8 T cells is generated during thymic selection (30, 31).

Although studies with melanoma patients failed to reveal a correlation between AV 2.1 usage and avidity of antigen recognition, some CDR3 public or homologous sequences within the AV 2.1-AJ 35 rearrangements were more frequently found in CTLs derived from different donors that recognized the tumor with high avidity (29). Recurrent or homologous AV sequences appeared also to pair preferentially with BV 14, suggesting that additional factors such as the specific CDR3 loop or the pairing with some BV chains could influence the overall avidity. In this regard, the mouse AV5 chain with a conserved CDR3 region described in this article was found in at least four clones isolated from the same bulk culture of mice immunized with pcDNA3-TRP-2: paired with BV7 in clones CTL24 and CTL20 and paired with BV8.2 in clones CTL7 and CTL8. As shown in Fig. 5, these CTL clones possessed different avidities toward TRP-2-Kb complexes, thus confirming the relevance of the α-chain to guide antigen recognition and the requirement for α- and β-chain pairs to shape the strength of interaction with the antigen-MHC complex.

Our data strongly support the concept that the presence of specific T-cell clonotypes is a requirement in breaking peripheral tolerance and mounting a therapeutic immune response in tumor-bearing hosts toward a tumor-associated antigen such as TRP-2. Additional studies are needed to investigate whether an efficient expansion of the protective T-cell clonotype requires a preimmune bias in the T-cell repertoire of single animals, as proposed in human studies (28, 29, 31), or is due to a stochastic usage of unselected repertoire. However, the possibility of identifying a close correlation between a particular TCR usage or a given T-cell clonotype and the efficacy of the immune response against a tumor antigen may open new scenarios in the immunotherapy of tumors.

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REFERENCES


Therapeutic Effectiveness of Recombinant Cancer Vaccines Is Associated with a Prevalent T-Cell Receptor $\alpha$ Usage by Melanoma-specific CD8 $^+$ T Lymphocytes

Raffaele De Palma, Ilaria Marigo, Francesco Del Galdo, et al.


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