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Genetic Vaccination with "Self" Tyrosinase-related Protein 2 Causes Melanoma Eradication but not Vitiligo[1]

Vincenzo Bronte[2], **Elisa Apolloni**, **Roberto Ronca**, **Paola Zamboni**, **Willem W. Overwijk**, **Deborah R. Surman**, **Nicholas P. Restifo**, and **Paola Zanovello**

Department of Oncology and Surgical Sciences University of Padova, 35128 Padova, Italy [V. B., E. A., R. R., P. Z., P. Z.], and Surgery Branch, National Cancer Institute, NIH, Bethesda, Maryland 20892 [W. W. O., D. R. S, N. P. R.]

Abstract

"Self" melanocyte differentiation antigens are potential targets for specific melanoma immunotherapy. Vaccination against murine tyrosinase-related protein (TRP)-1/gp75 was shown recently to cause melanoma rejection, which was accompanied by autoimmune skin depigmentation (vitiligo). To further explore the linkage between immunotherapy and autoimmunity, we studied the response to vaccination with a related antigen, TRP-2. i.m. inoculation of plasmid DNA encoding murine *trp-2* elicited antigen-specific CTLs that recognized the B16 mouse melanoma and protected the mice from challenge with tumor cells. Furthermore, mice bearing established s.c. B16 melanomas rejected the tumor upon vaccination with a recombinant vaccinia virus encoding *trp-2*. Depletion experiments showed that $CD8^+$ lymphocytes and natural killer cells were crucial for the antitumor activity of the *trp-2*-encoding vaccines. Mice that rejected the tumor did not develop generalized vitiligo, indicating that protective immunity can be achieved in the absence of widespread autoimmune aggression.

Introduction

Tumor-specific human $CD8^+$ and $CD4^+$ lymphocytes respond to molecular complexes formed by MHC molecules and short peptides derived from the processing of tumor Ags[3] expressed from either mutated or normal genes (1-3). Several melanoma Ags recognized by lymphocytes of melanoma patients in association with class I MHC molecules belong to the category of MDAs, which includes gp100/pmel-17, MART-1/Melan-A, tyrosinase, TRP-1/gp75, and TRP-2. Active immunization against these widely shared Ags represents an attractive and simple strategy for the therapy of cancer, and initial studies using mouse models have produced encouraging results. Thymic, central tolerance to MDAs is not complete, and peripheral tolerance can be broken by xenoimmunization or immunization with an altered source of the Ag. Administration of a rVV encoding human gp100/pmel-17 or with a plasmid encoding human TRP-1/gp75 elicited an immune response against the respective murine homologues (4,5). Tolerance to murine TRP-1/gp75 was also broken by giving the murine Ag in an altered form; exposure to the protein expressed in insect cells or produced during *in vivo* infection with a rVV (6,7) was able to trigger an immune response that fully recognized the naturally

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[2]To whom requests for reprints should be addressed, at Department of Oncology and Surgical Sciences, Oncology Section, University of Padova, Via Gattamelata 64, 35128 Padova, Italy. E-mail: vbronte@ux1.unipd.it.

[3]The abbreviations used are: Ag, antigen; TRP, tyrosinase-related protein; MDA, melanocyte lineage differentiation antigen; rVV, recombinant vaccina virus; NK, natural killer; mAb, monoclonal antibody; MLPC, mixed leukocyte peptide culture; PFU, plaque-forming unit(s).

processed form of the native Ag and led to tumor eradication. The therapeutic effect required $CD4^+$ lymphocytes, antibodies, NK1.1 cells, and the Fc receptor γ-chain, suggesting a scenario in which melanoma cells targeted by specific antibodies were eliminated by antibody-dependent cellular cytotoxicity-mediated lysis (6-9). Induction of an effective immune response against TRP-1/gp75 was associated with autoimmune manifestations consisting of diffuse depigmentation of skin patches (vitiligo) attributable to the destruction of normal melanocytes sharing the TRP-1/gp75 Ag. Additional studies are required to understand the association between vitiligo and melanoma therapy, because the mechanisms inducing vitiligo could be uncoupled from those causing tumor regression in one study but not in another (5, 7).

Although the immune response to TRP-1/gp75 is dominated by production of anti-TRP-1/gp75 antibodies, TRP-2 Ag is the main target of murine CTLs generated after immunization with irradiated melanoma cells (10); CTL lines raised from splenocytes by repeated *in vitro* stimulation with the "self," immunodominant TRP-2 peptide showed therapeutic activity against established pulmonary metastases. On the other hand, active immunization with DNA encoding murine *trp-2* administered using a gene gun in association with a plasmid encoding interleukin 12 was capable of eliciting CTLs recognizing B16 melanoma but induced only a weak protective response (11). The present study demonstrates that i.m. immunization with plasmid DNA encoding murine *trp-2* conferred protection to challenge with B16 melanoma cells; moreover, administration of a rVV encoding murine *trp-2* was therapeutic in tumor-bearing mice. In contrast to mice immunized with TRP-1/gp75, the main effector populations induced by DNA immunization with the *trp-2* gene and involved in tumor protection were $CD8^+$ lymphocytes and $NK1.1^+$ cells. Furthermore, mice that rejected the tumor did not develop generalized vitiligo during the observation period but exhibited depigmentation and hair loss localized at the site of tumor inoculation. Active immunization with *trp-2*-encoding vectors may thus represent a promising immunotherapy strategy against melanoma.

Materials and Methods

Mice and Cell Lines

C57BL/6 × BALB/c mice, 5– 6 weeks of age, were purchased from Charles River (Calco, Como, Italy). Procedures involving animals and their care conformed with institutional guidelines that comply with national and international laws and policies [EEC Council Directive 86/609, OJ L 358, 1, Dec. 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication 85-23, 1985; UKCCR Guidelines for Welfare of Animals in Experimental Neoplasia (12)]. Mice used for the *in vivo* tumor growth experiments were examined every other day and were euthanized when the tumor became ulcerated or reached a diameter >50 mm^2.

Cell Lines

MBL-2 is a leukemia cell line ($H\text{-}2^b$) derived from a Moloney murine leukemia virus-infected B6 mouse; C57BL/6 is a melanoma line ($H\text{-}2^b$) spontaneously growing in C57BL/6 mice (kindly provided by Dr. I. J. Fidler, M. D. Anderson Cancer Center, Houston, TX). The cell lines were cultured in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 2 mM L-glutamine, 10 mM HEPES, 20 μM 2-mercaptoethanol, 10 units of ampicillin/ml, and 5 or 10% heat-inactivated fetal bovine serum (Life Technologies).

Plasmid and Viruses

The cDNA coding for murine *trp-2* was a kind gift of Dr. V. J. Hearing (Laboratory of Cell Biology, NIH, Bethesda, MD; Ref. 13). The *trp-2* gene was cloned into the eukaryotic expression plasmid pcDNA3 (Invitrogen BV, Leek, the Netherlands), resulting in plasmid

pcDNA3- *trp-2*, in which expression of the mouse *trp-2* gene is driven by the cytomegalovirus promoter/enhancer. Endotoxin-free plasmids were purified by anion exchange chromatography (Qiagen GmbH, Hilden, Germany). rVV-*trp-2* was generated by insertion of the *trp-2* gene by homologous recombination as described previously by Moss and Earl (14) and was derived using plasmid pSC65, in which the completely synthetic early/late promoter $pS_{E/L}$ drives expression of the Ag and the early/late promoter $p7.5_{E/L}$ drives expression of the *LacZ* gene (14). Expression of TRP-2 protein by rVV-*trp-2* and by pcDNA3-*trp-2* was confirmed by immunostaining of infected/transfected cells using anti-TRP-2 serum (anti-PEP8 serum, kindly provided by Dr. V. J. Hearing).

DNA and Recombinant Virus Immunization Protocols

DNA immunization was performed according to commonly used protocols available at the "DNA vaccine web" site.[4] Briefly, mice were anesthetized by ethyl ether inhalation and injected i.m. with 100 μl of 10 μ_M cardiotoxin (Latoxan, Rosans, France). Five to 9 days later, mice were injected i.m. with 100 μg of plasmid DNA in 100 μl of saline. rVV (5×10^6 PFU/mouse) was inoculated i.v. 5 days after tumor injection.

MLPC

Three weeks after plasmid DNA inoculation, spleens were removed, and 2.5×10^7 splenocytes were stimulated *in vitro* in a MLPC with 1 μ_M of a nonamer peptide corresponding to amino acids 180–188 of TRP-2 (SVYDFFVWL; Refs. 10 and 11); the peptide was synthesized and purified by Neosystem (Strasbourg, France). The cultures were set up in DMEM-10% fetal bovine serum, maintained for 5 days at 37°C under 5% CO_2, and then tested in ^{51}Cr and IFN-γ release assays. Cytotoxic activity of cultured splenocytes was tested in a short-term incubation ^{51}Cr release assay by mixing 2×10^3 ^{51}Cr-labeled target cells with the effector cells at various E:T cell ratios in 96-well microplates; after 5 h of incubation at 37°C, supernatants were harvested, and radioactivity was counted in a microplate scintillation counter (Packard Instruments Co., Meriden, CT). For peptide pulsing, 10^6 ^{51}Cr-labeled cells were incubated for 30 min at 37°C with 1 μ_M peptide and then washed before use. The IFN-γ release assay was carried out by restimulating 10^5 splenocytes from MLPC for 24 h in triplicate wells with an equal number of target cells; the supernatant was then harvested and tested for released IFN-γ in a sandwich ELISA assay (Endogen, Boston, MA).

Tumor Protection and Therapy

Three weeks after DNA inoculation, mice were challenged s.c. with a lethal dose of B16 melanoma cells (*i.e.*, 10^5 cells, 10-fold greater than the minimum tumorigenic dose), and then monitored for 120 days after tumor injection. Mice inoculated with mock plasmid and uninoculated mice were used as control groups. For tumor therapy experiments, 10^5 B16 cells were injected s.c. 5 days before rVV inoculation.

In Vivo Antibody-mediated Depletion

Mice were depleted of either $CD4^+$, $CD8^+$, or NK cells by four i.p. injections of 200 μg of GK1.5 (anti-CD4), 2.43 (anti-CD8), or PK136 (anti-NK1.1) monoclonal antibodies (mAb) prepared in 200 μl of endotoxin-free PBS (Sigma). Depleting mAb were given on days −2, 0, 4, and 8 with respect to the s.c. challenge with B16 melanoma cells. The mAbs were produced from hybridomas (obtained from American Type Culture Collection, Manassas, VA) grown in ascites and purified by ammonium sulfate precipitation, followed by protein-G Sepharose affinity chromatography (Pharmacia, Uppsala, Sweden). Depletion was monitored by cytofluorimetry of peripheral lymphocytes isolated from mouse blood and stained with FITC-

[4]Internet address: http://www.genweb.com/Dnavax/dnavax.html.

or PE-conjugated anti-CD4, anti-CD8, or anti-NK1.1 (clones RM4-5, 53.6-7, and 2B4, respectively; all from PharMingen, San Diego, CA). Depletion was consistently >98%.

Statistical Analysis

The Wilcoxon-Mann-Whitney *U* test was used to examine the null hypothesis of rank identity between two sets of data. Kaplan-Maier plots and the Mantel-Haenszel test were used to compare survival of mice belonging to different treatment groups. All *P*s presented are two sided.

Results

DNA Immunization Can Elicit an Immune Response against the Self Melanoma Ag TRP-2

To break tolerance against self MDAs, we constructed the eukaryotic expression vector pcDNA3-*trp-2*, coding for murine *trp-2*. After verifying the expression of TRP-2 from pcDNA3-*trp-2* in transiently transfected cells by immunofluorescence with an antibody that recognized a COOH-terminal region of this protein (data not shown), we injected mice with the plasmid according the protocol reported in "Materials and Methods" and evaluated whether TRP-2 genetic immunization could elicit Ag-specific CTLs. Three weeks after DNA inoculation, splenocytes from injected mice were stimulated in a MLPC using the immunodominant, H-2K^b-restricted, TRP-2 peptide (TRP-2$_{180–188}$). Five days later, cytotoxic activity against target cells was evaluated as shown in Fig. 1A. CTL activity against MBL-2 cells pulsed with TRP-2$_{180–188}$ peptide was present only in mice injected with DNA expressing TRP-2. Peptide-specific CTLs were not elicited in control mice that received cardiotoxin alone or cardiotoxin followed by plasmid pcDNA3. Although CTLs specifically recognized the TRP-2 immunodominant peptide, they were not able to efficiently lyse B16 tumor cells.

B16 melanoma cells express very low amounts of K^b molecules (<30% at low intensity in fluorescence-activated cell sorting analysis; data not shown). Such limited expression of K^b molecule can compromise the sensitivity of the ^{51}Cr assay, which allows for a brief incubation of CTLs with their targets. One strategy to overcome this limitation is to expose the B16 cells to IFN-γ prior to the ^{51}Cr assay (11); alternatively, the levels of IFN-γ released in the supernatant of CTL cultures stimulated for 24 h can be measured. Fig. 1B shows IFN-γ release assay of MLPC set up with splenocytes from mice immunized with pcDNA3-*trp-2* or pcDNA3 and either B16 tumor cells or TRP-2$_{180–188}$ peptide-pulsed MBL-2 cells as targets. Results showed that elevated amounts of IFN-γ were detected in the supernatants of lymphocytes cultured in the presence of either target, thus indicating that the DNA protocol is indeed capable of eliciting CTLs specifically recognizing the B16 melanoma.

DNA Immunization with pcDNA3-trp-2 Protects from Challenge with B16 Melanoma Cells

The release of IFN-γ by CTLs as an indicator of immune response was shown previously to correlate with the *in vivo* antitumor activity of the CTLs upon adoptive transfer (15). We thus asked whether the Ag-specific response demonstrated in mice immunized with pcDNA3-*trp-2* could protect them from tumor challenge. Fig. 2 summarizes results of five experiments in which naïve mice or mice previously immunized with pcDNA3-*trp-2* were injected with a lethal dose of B16 melanoma cells. Although control mice left untreated died within 3 weeks after challenge, almost complete protection against the lethal challenge was achieved in mice vaccinated with the plasmid DNA encoding *trp-2*. Data accumulated from the five experiments yielded an overall rate of tumor prevention of 86% (*i.e.*, 36 of 42 mice were protected). Unexpectedly, untreated mice and mice injected with the empty pcDNA3 vector also showed a significant difference in survival, suggesting a marginal, Ag-independent effect of DNA immunization in prevention of tumor growth. This effect was related to the pcDNA3 vector,

because it was not observed with a second construct derived from the plasmid VR1012 (Ref. 16; data not shown).

CD8$^+$ Lymphocytes and NK Cells Are Involved in Tumor Eradication after DNA Immunization

To explore the role of T cells in the antitumor efficacy of the DNA vaccine, mice that had been immunized with pcDNA3-*trp-2* received depleting doses of mAbs specific for either CD4$^+$ lymphocytes, CD8$^+$ lymphocytes, or NK cells. The inoculation schedule assured that the corresponding population was completely absent at the moment of tumor challenge and during the first 10 days of tumor growth, as assessed by cytofluorimetry (data not shown). As shown in Fig. 3A, DNA immunization increased the survival of both normal mice and mice depleted of CD4$^+$ lymphocytes, indicating that CD4$^+$ cells were not required to control B16 melanoma growth. In contrast, mice depleted of either CD8$^+$ lymphocytes or NK cells were no longer protected and succumbed from tumor challenge. Thus, both CD8$^+$ lymphocytes and NK cells acted in concert to eradicate melanoma cells in immune mice.

Local Depigmentation and Alopecia but not Vitiligo Are Associated with Tumor Regression

On the basis of observations made after immunization with TRP-1/gp75 Ag (7,8), the induction of a strong CTL and NK reactivity against TRP-2 was expected to give rise to melanocyte destruction and manifestations of vitiligo. However, mice that had rejected the tumor after challenge did not develop vitiligo during the follow-up period of >1 year, even when given two consecutive i.m. injections of pcDNA3-trp-2 (data not shown). Localized hair loss and depigmentation in the area surrounding the site of tumor inoculation was evident in some mice, including those that had been depleted of CD4$^+$ lymphocytes by antibody treatment (Fig. 3B). Haircoat loss was clearly evident 3 weeks after tumor challenge when the hair grew back in the shaved left flank, with the exception of the tumor injection site. The haircoat alterations were observed in ~40% (15 of 36) of all of the mice that survived the challenge, and they remained stable for the observation period of 1 year.

Immunization with rVV Encoding *trp*-2 Can Cure Established Tumors

The high rate of protection against B16 melanoma conferred by a DNA vaccine coding for TRP-2 Ag prompted us to investigate its therapeutic ability against established tumors. However, application of the same DNA immunization protocol used in the protection experiment did not result in tumor regression (data not shown). We speculated that the time required for optimal CTL generation after DNA immunization (2 weeks, not shown) was too long to allow a successful treatment of a swiftly growing tumor such as B16 melanoma, which is able to kill the host in 3–4 weeks. Therefore, we turned to an rVV vector as a means of expressing the TRP-2 Ag. In the hope that it might reduce the lag phase between vaccination and CTL production, mice were injected with a lethal dose of B16 tumor cells and 5 days later received 5×10^6 PFU of either mock rVV or rVV-*trp-2* (Fig. 4). As expected, negative control mice that were untreated or injected with the mock rVV died within 3–4 weeks after tumor challenge. In contrast, 50% of the mice that were treated with rVV-*trp-2* remained tumor free 3 months after inoculation with B16 cells. These mice did not show any signs of vitiligo and did not exhibit the changes in pigmentation and haircoat noted in pcDNA3-*trp-2*-vaccinated mice. The reasons for these differences are not clear. We hypothesize that NK cell activation induced by DNA immunization might help in causing more extensive tissue destruction. Moreover, immunization after tumor implantation might affect a population of CTL preprimed by the encounter with the tumor cells that is already highly specific for TRP-2. Immunization before tumor challenge might expand largely cross-reactive CTLs responsible for an higher degree of cross-killing and tissue devastation.

Discussion

Immune recognition of melanoma cells by T and B lymphocytes has been studied extensively, and several melanoma Ags have been defined (1-3). These Ags belong to three main categories: (*a*) mutated or aberrantly expressed Ags (CDK4, *β*-catenin, and Casp8); (*b*) cancer/testis-specific Ags (MAGE, BAGE, GAGE, PRAME, and NYESO-1); and (*c*) MDAs, which include tyrosinase, Melan-A/MART-1, gp100, TRP-1/gp75 and TRP-2. This last group comprises the most prevalent Ags recognized by T lymphocytes on human melanomas. The development of an immune response to MDAs is quite surprising, because it occurs despite the mechanisms of central and peripheral tolerance to self Ags. Indeed, the immune response to these MDAs is presumably limited by the existence of tolerance mechanisms that spare only T lymphocytes with low affinity T-cell receptors (17). Low-affinity lymphocytes can nonetheless be used therapeutically, and among the various MDAs, TRP-1/gp75 and gp100 were clearly shown to be tumor-regression Ags in mice and humans (4,7,18). The present study broadens the number of potential targets of specific immunotherapy by showing that TRP-2 represents a melanoma rejection Ag in mice.

The choice of the vaccine is as important as the choice of the tumor Ag, because the formulation and the route of delivery may profoundly influence the immune responses. We sought to understand whether the therapeutic responses induced by rVV or by naked DNA were qualitatively different. DNA vaccines are thought to be less efficient than recombinant viruses in the therapy of established tumors expressing a model tumor Ag (19,20). However, we urge caution in interpreting the finding of the reduced efficacy of DNA vaccination in a therapeutic setting (as described in the present report). Clearly, the decreased efficacy after vaccination with DNA could be entirely attributable to the long time required to elicit TRP-2-specific CTLs.

The association between autoimmune disorders, *i.e.*, vitiligo and tumor regression described in mice immunized with TRP-1/gp75 Ag and in melanoma patients undergoing therapy with high doses of interleukin 2, has led to the suggestion that a deliberate induction of autoimmunity against tissue Ags may be an acceptable side effect of tumor therapy (21), especially in the case of tumors arising from nonessential tissues. Our study indicates that diffuse vitiligo is not invariably associated with tumor eradication caused by immunization with MDAs and instead appears to depend on the specific Ag used.

Some mice immunized with TRP-2 showed localized hair loss and depigmentation, likely sequelae to the inflammatory response and residual fibrosis accompanying the immune destruction of tumor cells and adjacent tissues. The observation that the presence of TRP-2-specific CTLs did not lead to generalized destruction of normal melanocytes supports the hypothesis that the pathogenesis of human vitiligo in melanoma patients is related more to antibodies recognizing melanoma proteins such as tyrosinase or TRP-1/gp75 rather than CTL activity (8,22). Cutaneous lesions similar to those described in the present study were described recently in mice treated with a protocol consisting of immunization with a granulocyte/macrophage-colony stimulating factor-expressing melanoma vaccine, followed by inoculation with an mAb blocking the activity of the CTL-associated antigen 4; the combined treatment caused melanoma rejection which, analogous to our findings, was dependent on $CD8^+$ and $NK1.1^+$ cells but independent of $CD4^+$ T cells (23). About half of the mice surviving tumor challenge after combination treatment developed depigmentation, starting at the site of vaccination or challenge and spreading to distant sites; the initial lesions were similar to those observed after immunization with TRP-2-encoding plasmids. Because we did not observe vitiligo progression, it is conceivable that the CTL-associated antigen 4 blockade used in the previous study enhanced T-cell activation and triggered a massive proliferation of autoreactive T cells, the numbers of which are normally contained by the mechanisms of peripheral tolerance. Thus, although unleashing the mechanisms controlling the magnitude of the immune

response may be relevant for enhancement of therapeutic efficacy, it must be weighed against the cost of an autoimmunity sequela.

We used CB6 F1 mice in the present experiments for several reasons: (*a*) C57BL/6 mice lack a portion of the genome that contains the α chain of the I-E molecule, a condition that related to the human beings would translate in the loss of the *DR* locus and might affect the immune response to melanoma Ags; and (*b*) F1 hybrids are heterozygous at the H-2 locus and thus closer to patients who display an extreme polymorphism in HLA loci. Experimentally, F1 mice showed anti-TRP-2 immune responses stronger than that observed in C57BL/6 mice after DNA immunization. The autoimmune and anti-tumor responses are currently being compared in C57BL/6 and CB6F1 mice. Preliminary results indicate that differences in T-lymphocyte repertoire directed against the K^b-TRP-2 peptide complex might explain the different magnitudes of the immune responses in the two strains.

In contrast to the highly efficient protection afforded by immunization with artificial model tumor-associated antigens such as β-galactosidase (19,20), protection against tumor challenge was not observed in the totality of the mice immunized with the *trp-2*-expressing plasmid. These differences almost certainly reflect the stronger immunogenicity of the model tumor Ags as compared with self Ags. Similar findings were made in our previous studies with the murine Ag P1A, which is naturally expressed in P815 mastocytoma cells (24); although vaccination of mice with a plasmid expressing P1A considerably increased their survival rate after challenge with P815 cells, it did not confer complete protection. Several strategies to increase the efficacy of DNA immunization are currently being evaluated. Although repeatedly boosting the immune response is the simplest approach, it cannot be based on repeated i.m. inoculation of DNA in cardiotoxin-pretreated muscles. In fact, we did not observe any increase in the percentage of mice rejecting a B16 challenge after two DNA inoculations instead of a single immunization (data not shown). A promising approach is the use of a DNA vaccine for priming and the modified vaccinia virus Ankara for boosting, a protocol that has been used successfully to increase the CTL response against malaria Ags (25). Alternatively, combining different weak, self Ags in the same immunization protocol might increase the overall immune response against the tumor. This hypothesis is supported by preliminary results of a study, indicating that a mixture of two plasmids encoding *gp100* and *trp-2* increases the rate of protection in C57BL/6 mice from challenge with B16 melanoma cells.[5]

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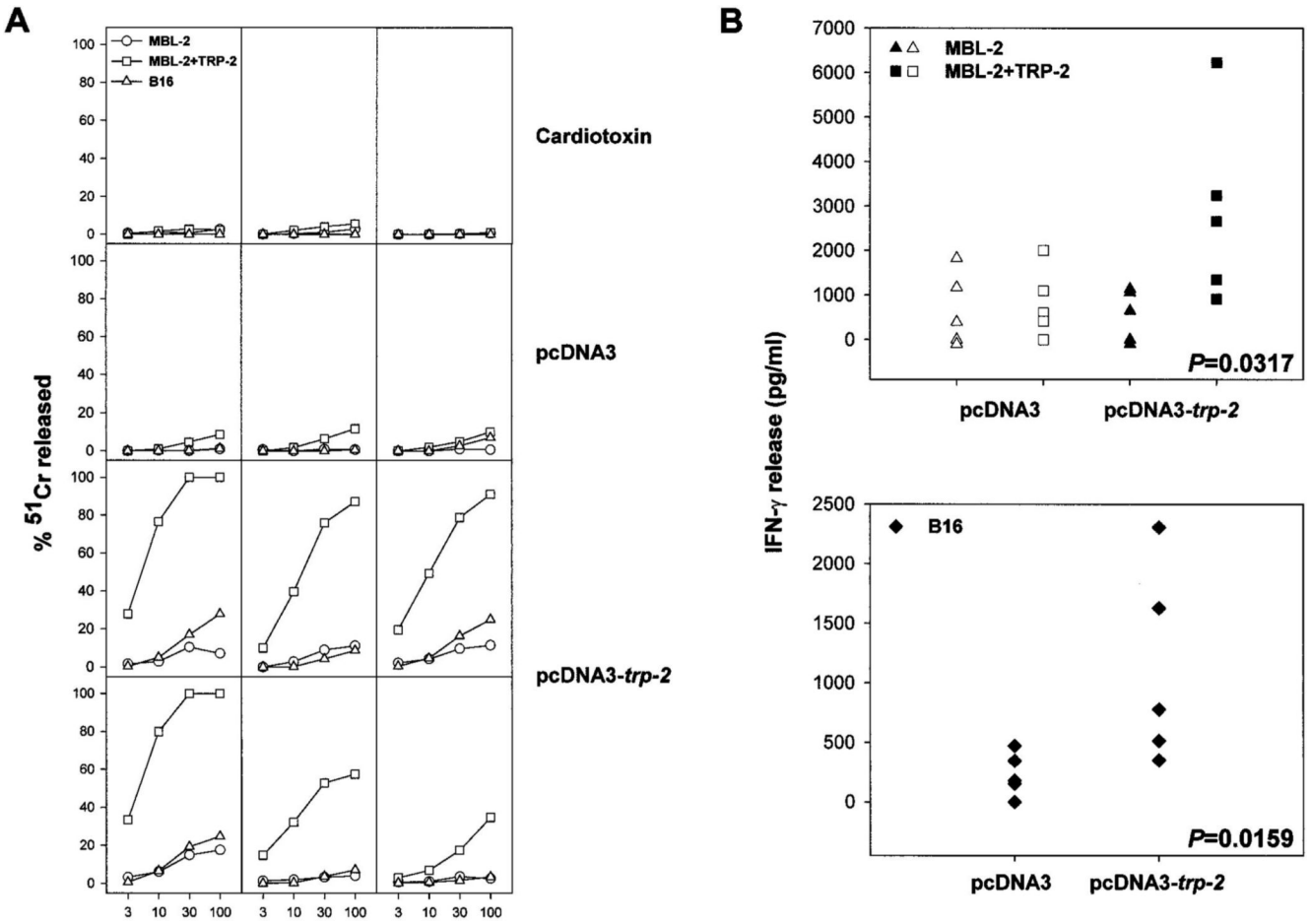

Fig. 1.

Plasmid DNA encoding TRP-2 induces Ag-specific CTL. *A*, 3 weeks after i.m. inoculation of plasmid DNA, spleens were removed, and splenocytes were stimulated *in vitro* in an MLPC with 1 μ_M of TRP-$2_{180–188}$ peptide. After 5 days of culture, cytotoxic activity of the splenocytes was tested in a ^{51}Cr release assay carried out for 5 h against the following target cells: MBL-2, MBL-2 pulsed with TRP-$2_{180–188}$ peptide, and B16. Each panel shows the CTL response generated in a single mouse after inoculation of either cardiotoxin alone or cardiotoxin followed by either the empty pcDNA3 vector or pcDNA3-*trp-2*. Results are representative of five experiments. The SD of the triplicate determinations for each effector/target cell ratio (E:T ratio) was <10%, and spontaneous release never exceeded 20%. *B*, splenocytes (10^5 cells) from MLPCs were restimulated for 24 h in triplicate wells with an equal amount of MBL-2 cells, MBL-2 cells pulsed for 1 h with 1 μ_M TRP-$2_{180–188}$ peptide, or B16 melanoma cells; the supernatant was then harvested and tested for released IFN-γ in a sandwich ELISA assay. The SD of the triplicate determinations for each effector/stimulator combination was <10%, and IFN-γ measured in control wells containing either effectors or stimulators alone did not exceed the lowest amount of IFN-γ detectable in our assay (*i.e.*, 547 pg/ml, determined using serial dilutions of IFN-γ). Two-tailed *P*s calculated by the Mann-Whitney test are reported in the figure for those groups showing significant differences over their respective controls (*filled symbols*).

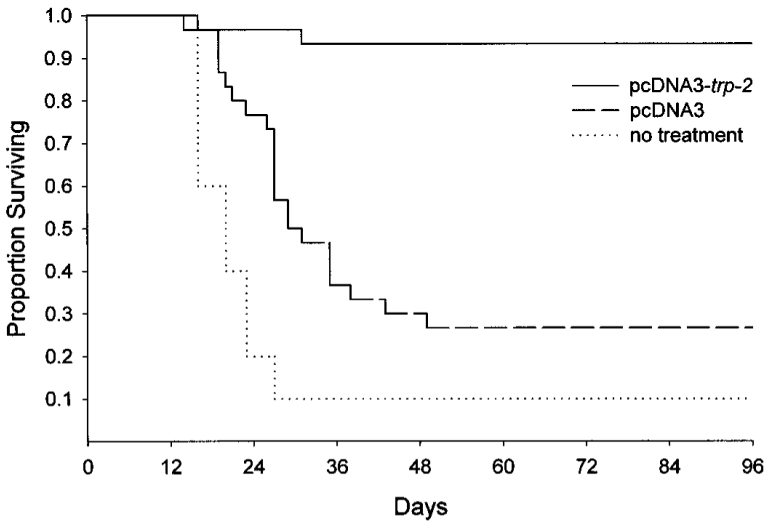

Fig. 2.

Immunization with a plasmid encoding TRP-2 prolongs survival of mice challenged with B16 melanoma cells. Three weeks after vaccination with pcDNA3-*trp-2*, mice were challenged s.c. in the right flank with 10^5 B16 melanoma cells. Untreated mice or mice immunized with the empty pcDNA3 vector were used as negative controls. Results represent the sums of two independently performed experiments with 5–10 mice in each treatment group. Immunization with pcDNA3-*trp-2* resulted in a significant prolongation of survival ($P < 0.0001$). Inoculation of the empty pcDNA3 vector also caused a significant prolongation of survival ($P = 0.006$) as compared with untreated mice.

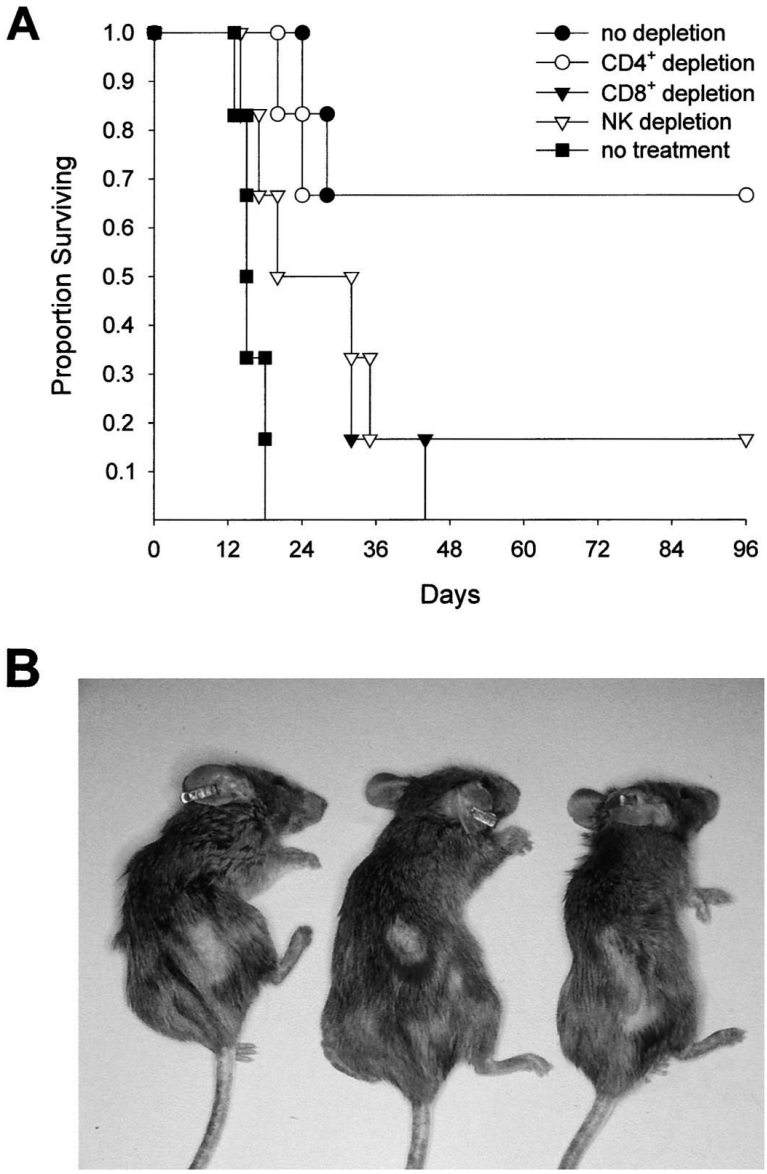

Fig. 3.

Protection against tumor challenge induced by DNA immunization depends on $CD8^+$ lymphocytes and NK cells and is associated with local depigmentation and alopecia. *A,* to identify the effector population responsible for the antitumor effect, mice immunized with pcDNA3-*trp-2* were depleted by i.p. injections of either anti-CD4, anti-CD8, or anti-NK1.1 mAbs as described in "Materials and Methods." Mice not immunized with pcDNA3-*trp-2* were used as controls (no treatment). Each treatment group contained six mice. The Mantel-Haenszel test gave $P = 0.025$ for the $CD8^+$-depleted *versus* no depletion group and $P = 0.09$ for the NK-depleted *versus* no depletion group. Duplicate experiments confirmed these results. *B,* mice were routinely shaved on the right flank in preparation for the s.c. tumor challenge. Although the hair normally grew back in 3 weeks, some mice showed an area of persistent hair loss and depigmentation at the site of tumor injection. The photograph was taken 4 months after tumor challenge and shows mice (anesthetized for the photo) belonging to the "no depletion" and "$CD4^+$ depletion" groups described in *A*.

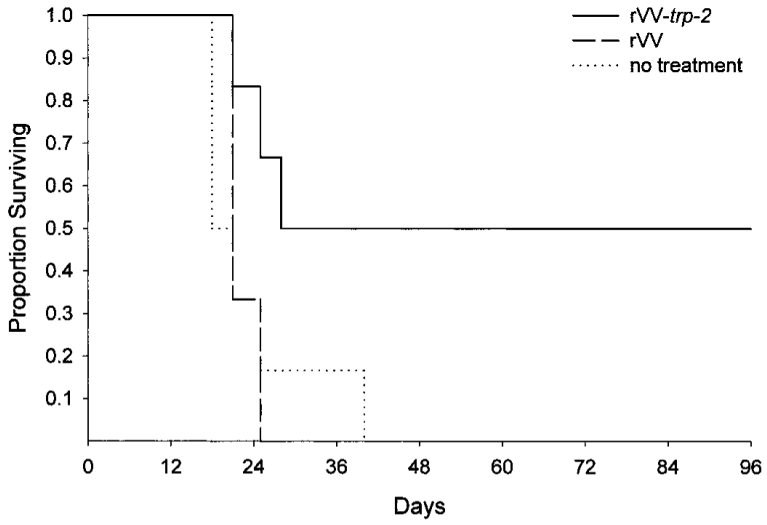

Fig. 4.

rVV encoding TRP-2 has a therapeutic effect on tumor-bearing mice. Mice were injected s.c. with 10^5 B16 melanoma cells. Five days later, they received a single i.v. inoculation of 5×10^6 PFU of an rVV encoding TRP-2 (rVV-*trp-2*). Untreated mice and mice injected with the same amount of mock virus (rVV) were used as negative controls. Results represent the sums of two independently performed experiments with five to six mice in each treatment group. The statistical analysis was performed according to the Mantel-Haenszel test ($P = 0.04$, no treatment *versus* rVV-*trp-2*; $P = 0.001$, rVV *versus* rVV-*trp-2)*.