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Advances in Brief

Therapeutic Tumor Immunity Induced by Polyimmunization with Melanoma Antigens gp100 and TRP-2

Sanjeev Kumar Mendiratta, Gerald Thai, Nima K. Eslahi, Nikolyn M. Thull, Majed Matar, Vincenzo Bronte,[1] and Federica Pericle[2]

Valentis, Inc., The Woodlands, Texas 77381-4248 [S. K. M., G. T., N. K. E., N. M. T., M. M., F. P.], and Department of Oncology and Surgical Sciences, 35128 Padova, Italy [V. B.]

Abstract

To improve the immunogenicity of melanoma self-antigens, we used a novel strategy of nonviral genetic vaccination coupled with muscle electroporation. Electroporation-enhanced immunization with plasmids encoding either human gp100 or mouse TRP-2 antigens induced only partial rejection of B16 melanoma challenge. However, immunization with a combination of these two antigens caused tumor rejection in 100% of the immunized mice. Splenocytes from combination-immunized animals killed syngeneic targets loaded with peptides derived from both gp100 and TRP-2. Immune cell depletion experiments identified $CD8^+$ T lymphocytes as the primary effectors of antitumor immunity. Most importantly, polyimmunization led to the generation of a therapeutic immune response that significantly improved the mean survival time of mice bearing established lung metastases. These results validated the usefulness of electroporation-enhanced, nonviral genetic immunization for the active immunotherapy of cancer and indicated that using a combination of different tumor antigens may be a decisive strategy for a successful therapeutic vaccination.

Introduction

Recent advances in the molecular identification of melanoma-specific antigens have given a significant boost to the study of novel cancer vaccines (1, 2). Development of an immune response against these antigens may seem arduous because many of them are nonmutated, differentiation antigens. Although self-reactive T cells in the host should be predominantly deleted during thymic education (3), individuals with malignant melanomas still carry $CD8^+$ T cells against these antigens, raising the possibility that immune response against some differentiation antigens may not be impossible (1, 2). Several immunization strategies have been tested in mice and humans to break tolerance to melanoma differentiation antigens. These include the use of viral (4) or bacterial (5) vectors expressing cancer antigens; transfected, peptide-, or tumor lysate-loaded dendritic cells (6, 7); and HLA-binding peptides derived from tumor-specific antigens (8). Despite the various limitations associated with the transfer of these approaches to the clinic, such as the potential immunogenicity of viral and bacterial vectors (9) and the labor-intensive task of either producing dendritic cells from each patient or characterizing a variety of tumor antigen-derived peptides capable of binding several HLA molecules, all these studies, taken together, provided convincing evidence on the possibility of eliciting an immune response to melanoma self-antigens. However, these studies also revealed that most cancer vaccines possess only a limited therapeutic efficacy, which constitutes a serious obstacle to their widespread use in tumor-bearing patients.

We have used a novel approach to improve the immunogenicity of cancer vaccines. We delivered tumor antigens i.m. as polymer-based formulations of a plasmid DNA encoding the antigens, followed by electroporation of the injected muscle. The polymer PVP[3]-based formulations were previously shown to allow a level of gene expression in muscle tissues higher than that of unformulated DNA (10) and thus might increase the immunogenicity by amplifying the antigen load. We also speculated that a further advantage would be conferred by electroporation of the injected muscle, which has been shown to augment protein expression by 100-1000-fold via a transient rise in permeability of muscle cell membranes (11, 12). We found that PVP-based hgp100 plasmid formulations, coupled with muscle electroporation, were effective in inducing tumor protection in about 40% of the immunized mice. To further improve the antitumor activity, we set up a protocol of polyimmunization by combining two cancer antigens in the same vaccine formulation. Using the method described above, mice immunized simultaneously with two melanosome antigens, hgp100 and mTRP-2, could be completely protected from a lethal challenge with B16 melanoma. Even more interestingly, when the above polyantigen vaccine was tested in a therapeutic setting, it was able to significantly increase the mean survival time of mice bearing established lung metastases.

Materials and Methods

Construction and Formulation of Tumor Antigen Expression Plasmids. The expression plasmid for hgp100 (pgp1399) was constructed by excising the *hgp100* gene from pgp1377 (a gift from Dr. Nicholas P. Restifo, NIH, Bethesda, MD) and subcloning it into pVC1157. Similarly, expression vector for mTRP-2 (pTR1494) was constructed by first deriving the *mTRP-2* gene (clone 3.6) from pTR1495 (13) by PCR and then cloning it into pVC1157. Expression of gene-specific transcripts from these two expression vectors was determined by reverse transcription-PCR with RNA preparations from transiently transfected cell lines (data not shown). Expression vector pVC1157 lacked its own coding region and served as the EV control. Plasmids were grown in DH5α *Escherichia coli* and purified by alkaline lysis and proprietary chromatographic methods (Valentis, Inc.). All plasmids used for immunization contained <50 endotoxin unit/mg endotoxin. Purified plasmids were formulated with either a 5% PVP solution (10) or suspended in 150 mM NaCl (unformulated plasmid).

Cell Lines. B16F10, a melanoma cell line of C57BL/6 ($H\text{-}2^b$) origin expressing mouse gp100 and TRP-2 gene products, was kindly provided by Dr. Nicholas P. Restifo. EL-4, a lymphoma cell line of C57BL/6 ($H\text{-}2^b$) origin, was a gift of Dr. Luc Van Kaer (Vanderbilt University, Nashville, TN). All cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 22 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, and 55 μM β-mercaptoethanol (all from Life Technologies, Inc., Gaithersburg, MD) at 37°C in a humidified 5% CO_2 environment.

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[2] To whom requests for reprints should be addressed, at Valentis, Inc., The Woodlands, TX 77381-4248. Phone: (281) 364-1150; Fax: (281) 364-0858; E-mail: pericf@valentis.com.

[3] The abbreviations used are: PVP, polyvinyl-pyrrolidone; hgp100, human gp100; mTRP-2, mouse TRP-2; EV, empty vector; NK, natural killer.

Mice. Eight-week-old female C57BL/6 mice were purchased from Charles River Laboratories (Raleigh, NC). Mice were maintained on *ad libitum* rodent feed and water at 23°C, 40% humidity, and a 12-h light/12-h dark cycle. All mice were acclimated for at least 4 days before the start of the study. All studies were performed in accordance with Institutional Animal Care and Use Committee acceptable animal use guidelines.

Immunizations. Mice were immunized i.m. with 100 μl of formulation containing 100 μg of plasmid DNA. In the case of mice that were simultaneously immunized with both pgp1399 and pTR1494, 50 μg of each plasmid were incorporated into the same formulation. To enhance i.m. gene expression further, immunization was followed 2 min later by electroporation of the injected area (375 V/cm, two pulses of 25 ms each) using an Electro Square Porator (T820; BTX, San Diego, CA). In the tumor protection assay, mice were immunized on days 0, 14, and 28, followed 2 weeks later by a s.c. challenge with 1.5×10^5 B16 cells. Tumor volumes were measured twice a week for 40–50 days. Mice that developed tumors of ≥ 1 cm^3 were sacrificed for humane reasons. In the therapeutic assay, lung metastases were first established in mice by injecting 2.5×10^4 B16 cells in 100 μl of HBSS (HBSS without Ca^{2+} or Mg^{2+}; Life Technologies, Inc.) into the tail vein. Treatments with formulated plasmids were started 3 days after tumor implantation. Mice were boosted on days 13 and 23. Mice were observed daily for signs of morbidity.

CTL Assay. A standard 6-h ^{51}Cr release assay was performed after 5 days of *in vitro* antigen-specific stimulation of splenocytes. Single cell suspensions of splenocytes were cleared of RBCs using hypotonic shock. *In vitro* stimulation cultures contained 100×10^6 splenocytes in 40 ml of complete media containing 10 units/ml recombinant murine interleukin 2 (Genzyme, Cambridge, MA) and 1 μg/ml peptide KVPRNQDWL (hgp100; Ref. 4) or SVYDFFVWL (TRP-2; Refs. 14 and 15), previously demonstrated to bind mouse K^b class I molecules. After 5 days at 37°C, effector cells were counted and mixed with ^{51}Cr-labeled targets at various E:T ratios in a 96-well U-bottomed plate (Costar/Corning, Cambridge, MA). EL-4 (H-2^b) targets were labeled by incubating them at 2×10^6 cells/ml in complete RPMI 1640 with 150 μCi of ^{51}Cr (Amersham) and 25 μg of peptide (KVPRNQDWL or SVYDFFVWL) for 1–1.5 h. After mixing effectors and targets (in triplicate wells), plates were placed at 37°C for 6 h. Supernatants were then collected from each well with the Skatron Harvesting Press and Supernatant Collection System (Skatron Instruments, Norway). ^{51}Cr release was determined using a WALLAC 1470 Wizard automatic gamma counter (WALLAC Inc., Gaithersburg, MD). Specific release was determined using the following equation: (experimental cpm − spontaneous cpm)/(total cpm − spontaneous cpm $\times$ 100. Spontaneous release from the targets was less than 10%.

***In Vivo* Antibody-mediated Depletion of Leukocytes.** Ascites of the following antibodies were used at the appropriate concentrations: (*a*) anti-CD4 (GK1.5 and TIB207; American Type Culture Collection), 1:10; (*b*) anti-CD8 (2.43 and TIB210; American Type Culture Collection), 1:100; (*c*) anti-asialo-GM1 (anti-NK cells; Wako, Osaka, Japan), 50 μl; and (*d*) anti-Gr-1 (anti-polymorphonuclear cells, RB6-8C5; PharMingen, San Diego, CA), 100 μg. Antibody dose and treatment regimens were determined previously (16) and found to completely deplete the desired population of cells for the entire duration of the study. All antibody injections were given in a volume of 500 μl. The first injection of the antibody was given i.v. 2 weeks after the last immunization and 1 day before tumor challenge. The second antibody injection of the same concentration was given i.p. 1 week after the first antibody injection.

Statistical Analyses. Tumor protection data were analyzed by cross-tabulation analysis using the χ^2 test, and Kaplan-Meier's test was used to analyze the survival experiments.

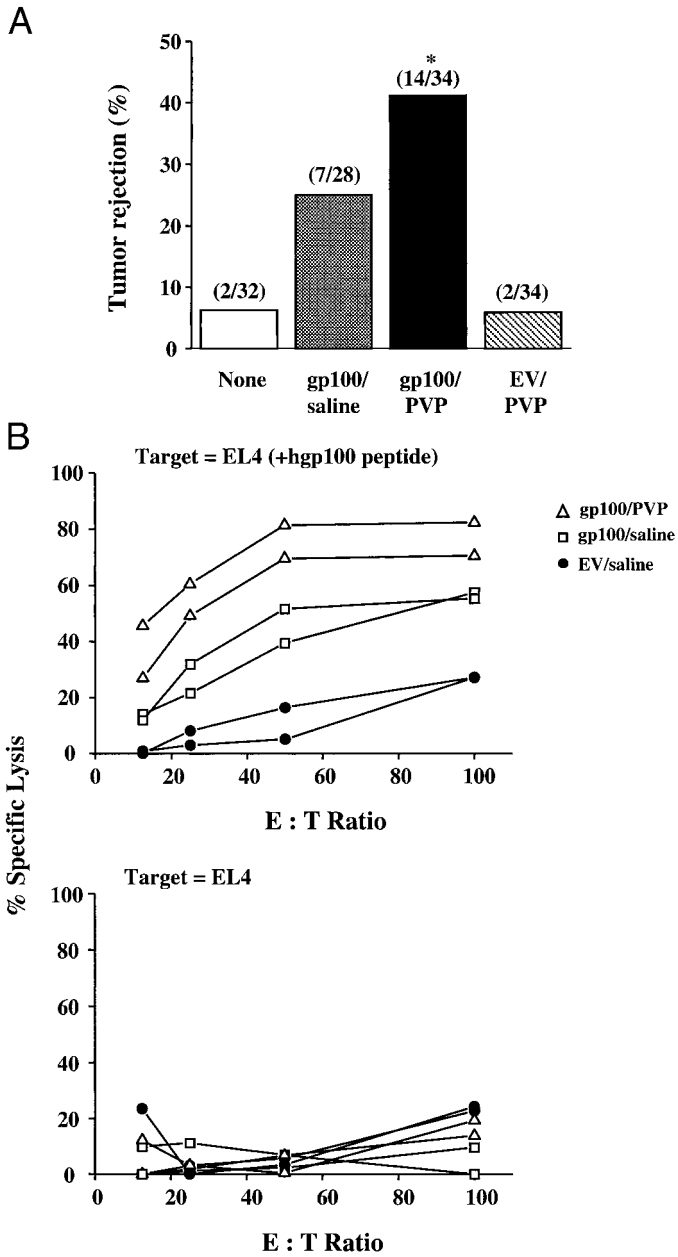

Fig. 1. Antitumor response induced by immunization with plasmid encoding hgp100. *A*, C57BL/6 mice were immunized by i.m. electroporation every 2 weeks (a total of three treatments) with unformulated hgp100 plasmid or PVP-formulated hgp100 or EV plasmids. Untreated mice were used as controls. Two weeks after the last treatment, mice were challenged with B16 melanoma cells. *Numbers in parentheses* represent the number of mice rejecting tumors/total number of mice challenged. Each *bar* is the result of four independent experiments with at least six mice/group/experiment. *, $P = 0.285$ *versus* EV/PVP (χ^2 test). *B*, splenocytes from two representative tumor-rejecting mice in each of the above-mentioned groups were stimulated *in vitro* with a hgp100-derived K^b-binding peptide plus interleukin 2, as described in "Materials and Methods." Effector cells thus generated were mixed with either hgp100-peptide-loaded [*target = EL-4 (+ hgp100 peptide)*] or nonloaded (*target = EL-4*) and ^{51}Cr-labeled EL-4 cell targets at various E:T ratios. Results are shown as the percentage of specific cytotoxicity after a 6-h ^{51}Cr release assay. Spontaneous release from the targets was less than 10%.

Results

Electroporation-enhanced Immunization with hgp100-expressing Plasmid Confers Partial Protection from a Challenge with B16 Melanoma. To study the ability of a pgp1399/PVP formulation to elicit an antitumor response, mice were immunized i.m. with either unformulated or PVP-formulated pgp1399 plasmid, followed by electroporation of the injected area. Fig. 1*A* shows pooled tumor rejection data from four different experiments. About 40% of C57BL/6 mice immunized by i.m. electroporation with pgp1399/PVP rejected B16 tumor challenge, a number significantly higher than that seen in mice immunized with EV/PVP (9%) or in untreated mice (6%). Tumor rejection in pgp1399/PVP-immunized animals was 1.6-fold higher than that in mice immunized similarly with unformulated pgp1399 plasmid. Whereas this increase was not statistically significant ($P = 0.28$), the PVP-based formulation always showed higher protection than the saline solution and was therefore selected for additional experiments.

Splenocytes from mice immunized by i.m. electroporation with pgp1399/PVP or unformulated pgp1399 plasmid effectively killed target cells pulsed with the immunodominant K^b-restricted peptide of hgp100, whereas splenocytes from mice immunized with EV showed only background levels of killing (Fig. 1*B*). Because CTL assays were performed on tumor-rejecting mice in various treatment groups, and EV-treated, tumor-rejecting animals did not show a CTL response above the background, the occasional protection observed in EV-treated animals is likely to be independent from $CD8^+$ T lymphocytes.

Tumor Rejection Mediated by Immunization with a Combination of Cancer Antigens, hgp100 and mTRP-2. Because the maximum protection obtained after genetic immunization with pgp1399 formulated in any fashion was only 41%, we asked whether cancer vaccine efficacy could be further improved by polyimmunization, *i.e.*, simultaneous vaccination of mice with two melanoma differentiation antigens instead of one. C57BL/6 mice were immunized with pgp1399 and pTR1494, PVP formulated and delivered as described previously for pgp1399. Mice immunized by i.m. electroporation with the two PVP-formulated plasmids showed tumor rejection in 100% of challenged mice, a percentage significantly higher than that achieved with either of the single plasmids alone [pgp1399 (0%) or pTR1494 (17%); Fig. 2*A*]. Mice that were immunized with the EV showed no protection.

To test the ability of i.m. immunization with the two plasmids to raise cytotoxic T-cell responses against each of the immunizing antigens, CTL assays against peptide-pulsed targets were performed on immunized mice. Mice that were immunized with the pgp1399 and pTR1494 combination/PVP formulation demonstrated cytotoxicity against EL-4 targets pulsed with the K^b-restricted gp100- or TRP-2-

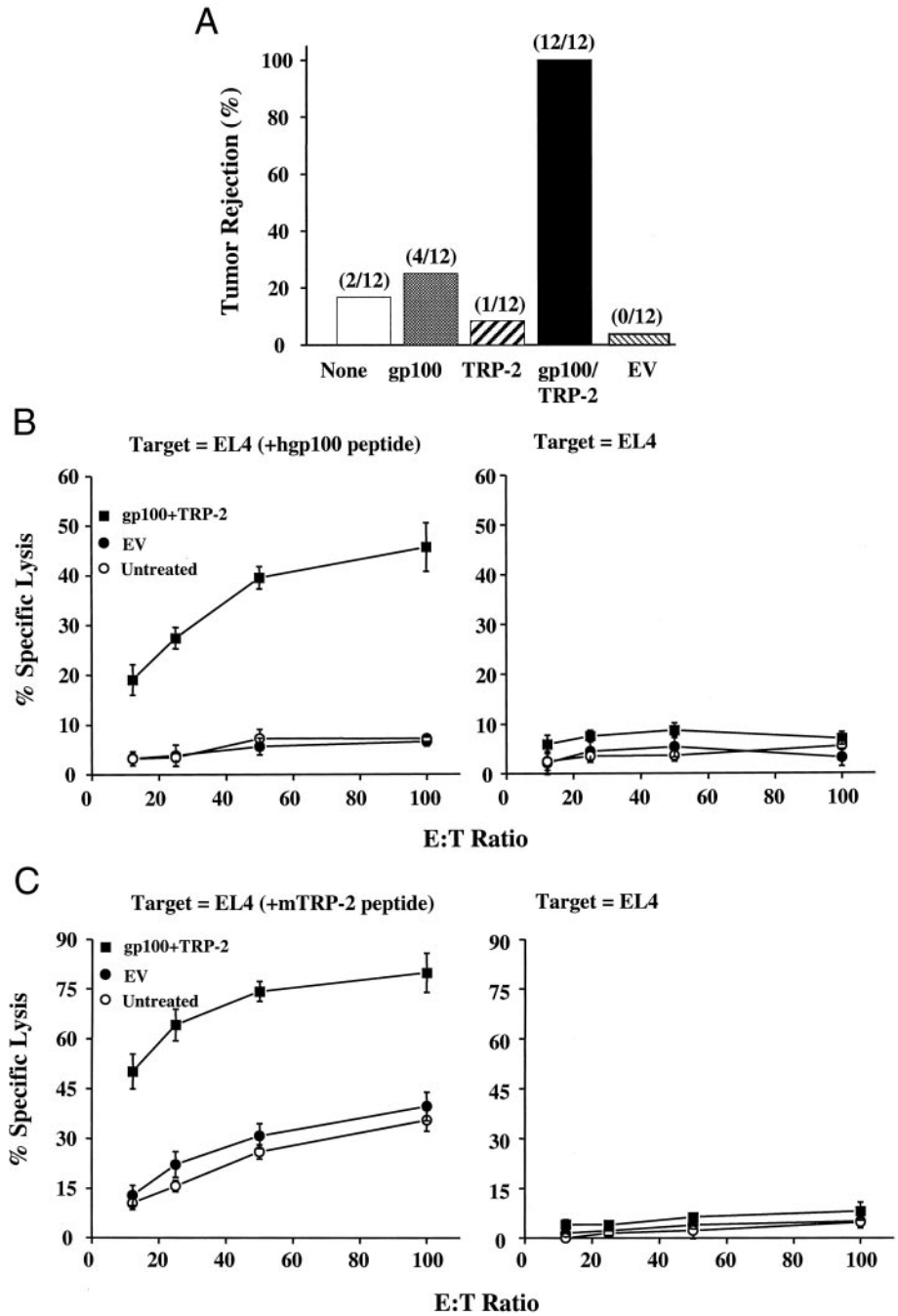

Fig. 2. Antitumor immune response mediated by combination immunization with hgp100- and mTRP-2-expressing plasmids. *A*, C57BL/6 mice were untreated or immunized with PVP-formulated hgp100, mTRP-2, hgp100 + mTRP-2, or EV plasmids (see Fig. 2 for dose regimen). Two weeks after the last immunization, mice were challenged s.c. with B16 melanoma cells. *Numbers in parentheses* represent the number of mice rejecting tumors/total number of mice challenged. This experiment is representative of a total of three experiments with similar results. *B*, splenocytes from the above-mentioned tumor-rejecting mice were used to perform a CTL assay with hgp100-derived peptide as described above for Fig. 1*B*. Effector cells were mixed with either hgp100 peptide-loaded [*target = EL-4 (+ hgp100 peptide)*] or nonloaded (*target = EL-4*) ^{51}Cr-labeled EL-4 cell targets at various E:T ratios. Results are shown as the percentage of specific cytotoxicity after a 6-h ^{51}Cr release assay. Spontaneous release from the targets was less than 10%. *C*, splenocytes from the above-mentioned tumor-rejecting mice were used to perform a CTL assay with mTRP-2-derived peptide. Effector cells were mixed with either mTRP-2 peptide-loaded [*target = EL-4 (+ TRP-2 peptide)*] or nonloaded (*target = EL-4*) ^{51}Cr-labeled EL-4 cell targets at various E:T ratios. Results are shown as the percentage of specific cytotoxicity after a 6-h ^{51}Cr release assay. Spontaneous release from the targets was less than 10%.

derived peptides (Fig. 2, *B* and *C*, respectively). On the other hand, unimmunized or EV/PVP-immunized mice showed only background killing against each of these targets. As observed previously by others (17), background anti-TRP-2 CTL responses were generally higher in naïve or EV/PVP-immunized mice, unlike the anti-gp100 responses. The significance of this finding is unknown, but these CTLs are certainly not sufficient to cause tumor rejection of B16 melanoma.

Mechanism of Tumor Protection after Immunization with hgp100 and mTRP-2. Cytotoxicity data suggested that tumor protection was mediated in C57BL/6 mice by the expansion of tumor-specific $CD8^+$ T cells, possibly with the contribution of NK cells, as suggested by previous studies (13). To better clarify the mechanism of protection and to identify the key immune effectors mediating it, we depleted combination-immunized mice of $CD4^+$ T, $CD8^+$ T, polymorphonuclear cells, or NK cells by treating them *in vivo* with specific monoclonal antibodies. These selectively immunosuppressed mice were then challenged with B16 tumor cells to analyze the role of each cell population in tumor rejection. Fig. 3 shows that tumor rejection was significantly abrogated only in those animals that were depleted of $CD8^+$ T cells. All other mouse groups treated with depleting antibodies showed only insignificant changes in the percentages of tumor rejection.

Therapeutic Effect of Polyimmunization with hgp100 and mTRP-2. Encouraged by our observations in the protection experiment, we asked whether immunization by i.m. electroporation with formulations of gp100- and TRP-2-expressing plasmids would be beneficial in the treatment of pre-established tumors. Mice with 3-day-old established B16 lung metastases were variously immunized, as shown in Fig. 4. Mice immunized with a combination of hgp100 and mTRP-2 had significantly increased mean survival times compared with untreated or EV-treated mice. On the other hand, mean survival times of mice treated with single antigens gp100 and TRP-2 were not significantly different from those of control mice.

Discussion

Data shown in this study demonstrate that polyimmunization with a mixture of tumor differentiation antigens can have a synergistic effect in the treatment of experimental melanoma. Because the goal of cancer vaccination is to generate a strong and effective antitumor immune response in tumor-bearing patients, the use of a powerful immunization strategy is critical to achieve efficacy. Nonviral genetic immunization is a very promising vaccination strategy that offers the advantage of a cheap and stable source of antigen and is virtually unable to elicit undesirable immune responses to other vaccine components, a limitation of bacterial- and viral-based vaccines (9). However, DNA vaccines are not considered strong immunogens and require multiple inoculations or the use of adjuvants to improve efficacy. In this study, we exploited our proprietary Protective Interactive Non-Condensing (PINC™) gene delivery system (10) coupled with electroporation as a cancer vaccination strategy. Inoculation with hgp100/PVP was only effective as a vaccine when it was followed by electroporation of the injected muscle (data not shown). Electroporation is known to increase protein expression by 2–3 log order, and this higher dose of antigen may be responsible for the increased antigenicity (11). The choice of B16 melanoma and gp100 as tumor/antigen model to set up our protocol was dictated by published data suggesting that gp100 immunogenicity had to be improved to result in the active immunotherapy of B16 melanoma (4, 5, 18, 19).

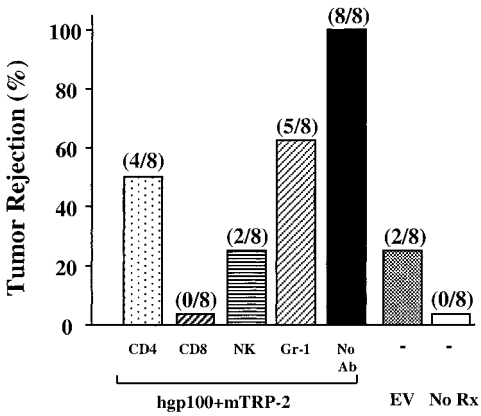

Fig. 3. Identification of immune effector cells responsible for tumor rejection mediated by vaccination with hgp100 and mTRP-2. Groups of eight mice were immunized by i.m. electroporation every 2 weeks for a total of three treatments with a plasmid combination expressing cancer antigens hgp100 and mTRP-2, EV, or nothing, as described in "Materials and Methods." One day before and 6 days after the tumor challenge, mice were depleted of various immune cells with specific monoclonal antibodies. *Numbers in parentheses* represent the number of mice rejecting tumors/total number of mice challenged.

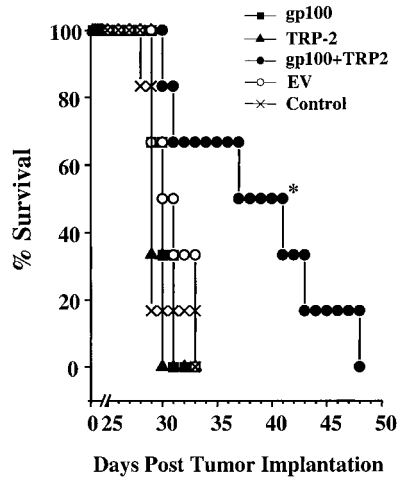

Fig. 4. Immunization with a combination of hgp100 and mTRP-2 plasmids is therapeutic. C57BL/6 mice bearing 3-day-old established lung metastases were immunized every 10 days with nothing, PVP-formulated EV or plasmids expressing hgp100, or mTRP-2, a PVP-formulated combination of plasmids expressing hgp100 and mTRP-2. Death was used as an end point. Data represent the percentage of surviving mice in every group, each day after tumor implantation. *, $P = 0.0297$ *versus* control, EV- and single antigen-immunized mice (Kaplan-Meier's analysis). This experiment is representative of a total of three experiments with similar results.

Electroporation of muscles inoculated with hgp100/PVP led to a partial tumor protection associated with the development of antigen-specific immunity, a result superior to that obtained by similar immunization with unformulated DNA. Following the initial set up, we sought to improve the antitumor activity of our immunization protocol by simultaneously using two cancer antigens. We chose mTRP-2 as the second cancer antigen for several reasons. It is relatively easy to raise low-affinity CTLs recognizing this self-antigen (14, 17), and, in contrast to other melanoma differentiation antigens, CTLs but not antibodies were found to be the primary effectors for rejection of B16 melanoma after immunization with TRP-2-based vaccines (13, 20). Interestingly, immunization with mTRP-2 was not able to induce antitumor activity in C57BL/6 mice, but it was successful in a different mouse strain, CB6 F1 (13, 20). Using genetic immunization coupled with electroporation, we demonstrated that a mixture of hgp100 and mTRP-2 DNA expression vectors had a straightforward advantage over vaccination with each of the single antigens. Polyimmunization, in fact, was not only able to confer protection in all of the C57BL/6 mice challenged with B16 melanoma tumor cells but was also effective in prolonging the survival of mice bearing established lung metastases.

We hypothesized that polyimmunization would lead to the expan-

sion of immune responses against both antigens. This was indeed confirmed when we observed that mice immunized with these two cancer antigens developed CTL responses against both hgp100- and TRP-2-derived peptides. These two findings (the absence of any interference in the generation of CTLs specifically recognizing both antigens and the synergistic effect of the two antigens on tumor protection) have an immediate impact on cancer vaccine formulation for clinical use. Antigen-encoding DNA can apparently be admixed in the same formulation with PVP and inoculated i.m. without the need to construct polycystronic vectors, a clear advantage when multiple antigens are concerned. Moreover, the safety of the electroporation method has been already demonstrated in cancer patients because it was applied to tumor masses to increase the delivery of chemotherapeutic drugs after their i.v. (21) or intralesional (22) administration. In both cases, the treatment was well tolerated with minimal side effects, suggesting that electroporation-enhanced polyimmunization could soon be moved to the clinic.

We do not have enough data to conclude whether PVP formulation plus electroporation is effective because it allows for the development of high-affinity CTLs toward either gp100 or TRP-2 or whether low-affinity CTLs recognizing the two tumor antigens on the same target act synergistically in delivering a lethal hit, whereas a single tumor-specific CTL fails to reach the threshold to efficiently recognize and lyse all of the melanoma cells. In any case, polyimmunization can be extremely important for human melanomas because tumor cell lines stabilized from different explants were found to express extremely variable levels of differentiation antigens, and a correlation appeared to emerge between threshold levels of gene expression and recognition by T lymphocytes (23).

Complete inhibition of tumor rejection in only those mice that were depleted of $CD8^+$ T cells suggests that these were the primary mediators of immunity. The fact that NK cells were less important in the antitumor response was unexpected in light of a previous report on the immunization of CB6 F1 mice with mTRP-2 (13). It is possible that the role of NK cells may be limiting when a particular mouse strain is concerned or that their requirement could be overcome by the synergistic activity of the different populations of antigen-reactive $CD8^+$ T lymphocytes.

Although we did not investigate this in detail, localized or widespread vitiligo was not observed in mice that rejected the tumor challenge. Our data are thus another example that the association between vitiligo and antimelanoma response is far from being conclusively understood, and additional studies are required to clarify whether autoimmunity sequelae are invariably associated with active immunization against self-antigens or rather depend on the type of antigen, the immunization protocol, or the genetic background of the host.

Vaccination by nonviral genetic immunization is a relatively new method, and there are very few examples of polyimmunization protocols in experimental mouse models. Interestingly, DNA vaccines encoding the tuberculosis antigens MPT-63 and MPT-83 evoked only a partial protection against an aerogenic challenge with *Mycobacterium tuberculosis*, a model of pulmonary tuberculosis, whereas immunization with a combination of DNA constructs encoding four different antigens (ESAT-6, MPT-64, MPT-63, and KatG) elicited a strong, protective response superior to that evoked by live Bacillus Calmette-Guerin vaccine (24). We can speculate that, analogously to pathogens causing chronic infections, the best cancer vaccines could be represented by a mixture of the lowest number of antigens sufficient to elicit a protective immunity. We are currently testing the possibility of further increasing the therapeutic potential of PVP and electroporation by mixing four melanosome antigens (TRP-1, TRP-2, gp100, and tyrosinase).

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