

Comparison of Two Cancer Vaccines Targeting Tyrosinase: Plasmid DNA and Recombinant Alphavirus Replicon Particles

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Abstract Purpose: Immunization of mice with xenogeneic DNA encoding human tyrosinase-related proteins 1 and 2 breaks tolerance to these self-antigens and leads to tumor rejection. Viral vectors used alone or in heterologous DNA prime/viral boost combinations have shown improved responses to certain infectious diseases. The purpose of this study was to compare viral and plasmid DNA in combination vaccination strategies in the context of a tumor antigen.

Experimental Design: Using tyrosinase as a prototypical differentiation antigen, we determined the optimal regimen for immunization with plasmid DNA. Then, using propagation-incompetent alphavirus vectors (virus-like replicon particles, VRP) encoding tyrosinase, we tested different combinations of priming with DNA or VRP followed by boosting with VRP. We subsequently followed antibody production, T-cell response, and tumor rejection.

Results: T-cell responses to newly identified mouse tyrosinase epitopes were generated in mice immunized with plasmid DNA encoding human (xenogeneic) tyrosinase. In contrast, when VRP encoding either mouse or human tyrosinase were used as single agents, antibody and T-cell responses and a significant delay in tumor growth *in vivo* were observed. Similarly, a heterologous vaccine regimen using DNA prime and VRP boost showed a markedly stronger response than DNA vaccination alone.

Conclusions: Alphavirus replicon particle vectors encoding the melanoma antigen tyrosinase (self or xenogeneic) induce immune responses and tumor protection when administered either alone or in the heterologous DNA prime/VRP boost approaches that are superior to the use of plasmid DNA alone.

The immune system can recognize self-antigens on human cancer (1, 2), with differentiation antigens representing frequent targets (3–5). Differentiation antigens are too weak to elicit effective immune responses, being expressed by both normal cells and their malignant counterparts. Immune recognition has been intensively studied in melanoma, a cancer

arising from melanocytes (6). There are multiple known melanocytic differentiation antigens, including tyrosinase (*albino* locus), TYRP-1/gp75 (*brown* locus), TRP-2/DCT (*slaty* locus), and gp100 (*silver* locus). These antigens are recognized by antibodies and CD4⁺ and CD8⁺ T cells in patients with melanoma (3–5). Tyrosinase was one of the first self-proteins identified as being recognized by CD8⁺ T cells in patients with cancer (5). Several studies have shown that it is possible to overcome immunologic ignorance and/or tolerance to these otherwise poorly immunogenic antigens by immunizing with xenogeneic DNA encoding the orthologue of a given differentiation antigen. Specifically, we have shown that immunization of mice with plasmid DNAs encoding the human melanocytic differentiation antigens gp75, DCT, and gp100 induces an immune response against the respective syngeneic proteins, leading to tumor rejection (7–10). Each of these related antigens induces antitumor immunity with unique cellular requirements for priming and effector function (11).

Immunization with plasmid DNA encoding differentiation antigens offers several advantages over other means of vaccination, such as peptides and peptide-pulsed dendritic cell vaccines. First, the presence of the full-length cDNA provides multiple potential MHC class I-restricted and MHC class II-restricted epitopes that are recognized by T cells. Second, the bacterial plasmid DNA itself contains unmethylated CpG motifs (immunostimulatory sequences) that may act as potent

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immunologic adjuvants through TLR9-dependent signaling (12). Finally, DNA is relatively simple to produce and purify in large quantities. Despite these advantages, and the immune responses seen using xenogeneic plasmid DNA immunization in mouse models, DNA vaccines alone have not been sufficient to induce complete or sustained immunity and have had suboptimal potency in humans and nonhuman primates (13, 14). Recombinant viral vectors have been investigated as a means of vaccination because they can carry full-length antigen-encoding genes, have the capacity to produce these antigens in large quantities, and may contain helper epitopes. Recombinant vaccinia virus (rVV), a double-stranded DNA virus, is one such vector that has been used to deliver antigen in both infectious disease and tumor antigen models (15, 16). Vaccinia-based vaccines have been able to break tolerance to syngeneic TYRP-1 in mice (15).

Despite the advantages of live viral vectors, their efficacy has been shown to decrease with repeated dosing (17, 18), possibly due to the development of antivector immunity (e.g., neutralizing antibodies against the viral vector). Heterologous prime/boost immunization programs have been investigated in an effort to avoid the effects of antivector-neutralizing immunity. In this type of vaccination, distinct vectors are used for the initial (prime) and subsequent (boost) immunizations. Pre-clinical experiments using the prime/boost programs for pathogen-derived antigens have been encouraging (18–21). These studies showed that priming with plasmid DNA followed by boosting with a replication-deficient viral vector encoding the same antigen produced the most potent pathogen protection and T-cell responses (17, 19, 20). Reversing the order of immunization or using the same viral vector both to prime and to boost provided less protection compared with the DNA prime/viral boost strategy (19, 20, 22).

Alphaviruses are positive-stranded RNA viruses, and several have been evaluated as replicon vaccine vector systems for HIV and other diseases (23). An attenuated variant of one member of this family, the Venezuelan equine encephalitis virus (VEEV), has been developed as a propagation-defective virus-like replicon particle (VRP) vector system (24, 25). This class of virus is particularly attractive because the double-stranded RNA created during viral replication stimulates the innate immune response through interaction with TLR3 (23). Moreover, the efficacy of VRP vaccines based on VEEV has been attributed to the expression of heterologous proteins at high levels, to targeting expression to dendritic cells, and to the ability to induce both humoral and cellular immune responses against the product of the gene carried by the vector (24, 26).

We present the first study to our knowledge that directly compares plasmid DNA and recombinant alphavirus replicon particles in their ability to immunize against a tumor antigen. Specifically, we immunize against tyrosinase in a mouse model. Mice are generally tolerant to syngeneic tyrosinase (27). We show that compared with plasmid DNA vaccination, which requires the use of xenogeneic (human) tyrosinase to overcome immunologic ignorance and/or tolerance to this self-molecule, alphavirus constructs containing either syngeneic (mouse) or human tyrosinase are immunogenic. In addition, although heterologous prime/boost immunization with plasmid DNA followed by VRP is more immunogenic than plasmid DNA alone, repetitive doses of VRP can be given without detrimental consequences of antivector immunity. In the course of

performing these experiments, we have also defined novel MHC class I-restricted epitopes in mouse tyrosinase. These findings have implications for the design of future clinical trials evaluating novel cancer vaccines.

Materials and Methods

Plasmid constructs. Human tyrosinase cDNA was previously cloned from human melanoma cells and sequenced at Memorial Sloan-Kettering Cancer Center (28). Wild-type mouse tyrosinase was amplified by PCR from mouse B16F10 (B16) melanoma cells described below. The sequence was confirmed to be correct when compared with mouse tyrosinase genes in the National Center for Biotechnology Information database. Both constructs were inserted into the pING vector as previously described (29).

Vaccinia virus constructs. All rVVs used in this study were generated, purified, and quantified as previously described (30, 31). rVV mouse tyrosinase (MTyr rVV) was a kind gift of Dr. P. Greenberg (University of Washington, Seattle, WA). Wild-type vaccinia virus (rVV) and the pUV 1 plasmid were generously provided by Dr. Bernard Moss (NIH, Bethesda, MD). To create the human tyrosinase vaccinia construct (HTyr rVV), the human tyrosinase gene was cloned into the plasmid pUV 1. This plasmid contains a strong late p11 promoter that drives expression of the antigen and a weaker early/late p7.5 element that drives expression of the *Escherichia coli* galactosidase gene (32). Expression of tyrosinase from the rVVs was confirmed by immunostaining and Western blotting of transfected and infected cells with anti-pep7 and anti-pep7h antisera, which were kindly provided by Dr. V.J. Hearing (Laboratory of Cell Biology, NIH; ref. 33).

Alphavirus virus-like replicon particle vaccines. Mouse and human tyrosinase cDNAs described above were inserted into the replicon vector phosphorylated extracellular signal-regulated kinase, and VRP were prepared as previously described (24). The VEEV-based VRP constructs were produced at AlphaVax, Inc. (Research Triangle Park, NC). To confirm expression of human and mouse tyrosinase, Vero cells were infected with the respective VRP preparation, and expression was detected by immunofluorescence as described previously (24). The mouse tyrosinase monoclonal antibody T311 (Lab Vision Corp., Fremont, CA) was used as the primary antibody, and goat anti-mouse IgG Alexa Fluor 488 (Molecular Probes, Eugene, OR) was used as the secondary antibody.

DNA Immunization. C57BL/6 mice were immunized with plasmid DNA by particle bombardment using a gene gun device kindly provided by PowderMed Vaccines (Oxford, United Kingdom; refs. 8, 34). The method for DNA immunization has been reported (8, 34). Briefly, plasmid DNA was purified and coated onto 2.1- μ m gold bullets, and mice were anesthetized by isoflurane inhalation (Henry Schein, Port Washington, NY). Hair was removed with Nair depilatory cream (Carter-Wallace, New York, NY), exposing the abdominal skin of the mice for immunization. Animals were immunized by delivering gold/DNA complexes via helium-driven gene gun into each abdominal quadrant for a total of four injections per mouse per treatment. The gene gun was operated at a helium pressure of 400 lbs. p.s.i. Each bullet contained 1 μ g of DNA; therefore, each mouse received 4 μ g of DNA per immunization. DNA immunizations were given once a week for a total of three to five doses.

Virus-like replicon particle and recombinant vaccinia virus immunization. VRP injections were given as a 20- μ L s.c. injection into the plantar surface of the left rear footpad using a 28-gauge needle. Mice receiving rVV were vaccinated i.p. with 10^7 plaque-forming units of the different variants of rVV tyrosinase or control virus and boosted 1 week later.

Antibody response. Sera were collected 5 to 7 days after the last immunization. Immunoprecipitation of tyrosinase antibodies was done using B16 melanoma cell lysate (as a source of mouse tyrosinase) and protein A-Sepharose beads. The B16 lysate was prepared at a concentration of 5×10^7 cells/mL lysis buffer [10 mmol/L Tris-HCl (pH 7.8),

5 mmol/L EDTA, 1% NP40, 0.5% deoxycholate]. Serum (20 μ L) was combined with 20 μ L of lysate and 15 μ L of protein A-Sepharose (Pierce Biotechnology, Rockford, IL), and immunoprecipitation was conducted by rocking the mixture at 4°C overnight. After washing, 250 μ L of 0.2% L-3,4-dihydroxy-L-phenylalanine (L-DOPA) solution was added as substrate for tyrosinase. The L-DOPA solution was prepared fresh each time by dissolving L-DOPA (Sigma-Aldrich, St. Louis, MO.) in 0.1 mol/L sodium phosphate buffer (pH 6.8). Samples were incubated at 37°C for 2 to 4 hours until the positive control sample turned black. Anti-pep7 rabbit polyclonal anti-mouse tyrosinase antibody (described above) served as a positive control (28). Samples positive for anti-mouse tyrosinase antibodies retained the antibody-bound tyrosinase that was still enzymatically active and thus converted the substrate to insoluble melanin.

Peptide selection. Mouse tyrosinase peptides that were predicted to bind MHC class I molecules (K^b and D^b) were selected by estimated peptide/MHC I half-life. We used applied virtual quantitative matrices of Parker et al. (35) to predict the relative steady-state binding (as a measure of avidity) of each peptide to its cognate MHC allele, based on canonical motifs. This computational approach predicts binding half-life of peptides within a given sequence for specific MHC alleles. The peptides were synthesized by Genemed Synthesis (San Francisco, CA).

Enzyme-linked immunospot assays. The enzyme-linked immunospot (ELISPOT) assay measuring mouse IFN- γ was used to assess specific T-cell responses to antigen-derived peptides. After immunization, inguinal lymph nodes and spleens were harvested from mice. HA-Multiscreen plates (Millipore, Burlington, MA) were coated with anti-mouse IFN- γ monoclonal antibody (AN18, Mabtech, Mariemont, OH). $CD8^+$ T cells were isolated using magnetic beads (MACS, Miltenyi Biotec, Auburn, CA) and then plated and stimulated with peptide-pulsed EL4 cells (mouse lymphoma cell line; 10^6 EL4 cells/mL incubated with 10 μ g peptide/mL). B16 melanoma cells, in which tyrosinase is naturally expressed, were also used as targets. $CD8^+$ cells were plated at 10^5 per well; EL4 cells were plated at 10^4 per well; and B16 cells were plated at 5×10^3 per well. Both EL4 and B16 targets were irradiated (100 Gy) before plating. After incubating targets with effectors at 37°C for 20 hours, plates were extensively washed with PBS/0.05% Tween, and 100 μ L biotinylated capture-antibody against mouse IFN- γ (monoclonal antibody R4-6A2-B; Mabtech) was added (2 μ g/mL). Plates were incubated an additional 2 hours at 37°C and washed with PBS/0.05% Tween. A total of 100 μ L avidin-biotin peroxidase complex (ABC Vectastain-Elite kit, Vector Laboratories, Burlingame, CA) were added, after two drops of each reagent were diluted in 10 mL PBS/0.05% Tween, and incubated for 1 hour at room temperature. The unbound complex was removed by successive washings with PBS/0.05% Tween and PBS alone. The substrate 3-amino-9-ethylcarbazole (Sigma-Aldrich) was added to the plates for 4 minutes (color development), and the reaction was stopped under running tap water. Spots were counted using an automated Zeiss ELISPOT Reader System with KS 4.3 software (36, 37).

Cytolytic assay. Two weeks after the last immunization with rVV constructs, splenocytes ($\sim 8 \times 10^6$) were harvested and restimulated *in vitro* with concanavalin A-treated (10%) and mitomycin C-treated B16 melanoma cells (8×10^4) as a source of antigen. The cultures were maintained for 5 days in RPMI/10% fetal bovine serum at 37°C under 5% CO_2 and then tested in a standard ^{51}Cr release assay. Cytotoxic activity of cultured splenocytes was tested in a short-term incubation assay by mixing 2×10^3 ^{51}Cr -labeled target cells with the effector cells at various ratios in 96-well microplates. After 5 hours at 37°C, supernatants were harvested, and radioactivity was counted in a microplate scintillation counter (Packard Instruments Co., Meriden, CT).

Tumor challenge. The B16F10 line was originally provided by Isaiah Fidler (M.D. Anderson Cancer Center, Houston, TX). B16F10LM3 is a pigmented mouse melanoma cell line of C57BL/6 origin that our laboratory has selected from the B16F10 line for aggressive *in vivo* growth and competency for lung metastases. For intradermal tumor challenge, C57BL/6 mice received 2.5×10^4 to 5×10^4 B16F10LM3 cells

(in 50 μ L RPMI) in the right flank 5 to 6 days after the last immunization and then were followed for the appearance of tumor. Tumors were noted as positive when visible or palpable at 2-mm diameter. For mice vaccinated with rVV, tumor challenge was done i.v. 5 days after the final immunization. These mice were injected via the tail vein with 2×10^5 B16F10LM3 melanoma cells in 200 μ L PBS. Mice were sacrificed at 12 to 16 days after tumor challenge. All lobes of both lungs were dissected, and surface lung metastases were counted under a dissecting microscope.

Data analysis. For the intradermal tumor challenges, results are reported as Kaplan-Meier survival curves, which were analyzed by log-rank using the SPSS statistical program (version 12.0). The ELISPOT results were analyzed as the mean and SE for five replicate wells using the GraphPad PRISM 4.00 and SigmaPlot 8.0 programs. For lung metastases, the mean number of lung metastases and SE were calculated per group.

Results

Xenogeneic immunization with tyrosinase DNA delays tumor growth. To evaluate the effect of xenogeneic DNA vaccination with tyrosinase *in vivo*, mice (15 per group) were immunized by gene gun weekly for 5 weeks with plasmids expressing either mouse or human tyrosinase DNA followed by challenge with B16F10LM3, derived from the spontaneously arising B16 melanoma. The Kaplan-Meier survival curves (Fig. 1A) show that mice immunized with mouse tyrosinase (syngeneic DNA) had no tumor-free survival benefit, whereas mice immunized with human tyrosinase (xenogeneic DNA) showed a weak tumor-free survival advantage compared with the untreated controls ($P = 0.05$). No autoimmune hypopigmentation was observed.

We characterized the immune response to mouse or human tyrosinase following DNA immunization. Sera from immunized and control mice were analyzed for the presence of anti-mouse tyrosinase antibodies using a colorimetric assay. If the serum contained precipitating antibodies against tyrosinase, which catalyzes the conversion of L-DOPA substrate into melanin, a pigment that is easily measured colorimetrically, then active enzyme would be detected in the precipitate. Although neither control mice nor mice immunized with syngeneic tyrosinase DNA ($n = 15$) produced detectable anti-mouse tyrosinase antibodies, 8 of the 15 mice immunized with human tyrosinase DNA developed an antibody response (Table 1). Sera scored as positive in the colorimetric assay were confirmed using an immunoprecipitation-Western blot assay (data not shown).

Because our previous study showed that immunization with xenogeneic DCT and gp100 results in the production of $CD8^+$ T-cell responses, we assessed T-cell responses to tyrosinase using ELISPOT assays. However, a lack of defined MHC class I-restricted epitopes for mouse tyrosinase led us to first identify peptides that were to be used in these assays. We used available algorithms to predict which mouse tyrosinase peptides had a high probability of binding to MHC class I molecules, based on comparison with canonical motifs. Thus, we identified candidate peptides 164, 255, 360, and 445 (Table 2).

Prior observations with the antigen TYRP-1/gp75 have shown that antibody responses can be enhanced by priming with xenogeneic DNA to overcome tolerance and then boosting with syngeneic DNA (8, 38). To understand whether the same strategy could lead to an enhanced immune response to tyrosinase, we immunized C57BL/6 mice (three per group) thrice at weekly intervals with mouse tyrosinase DNA (M) or

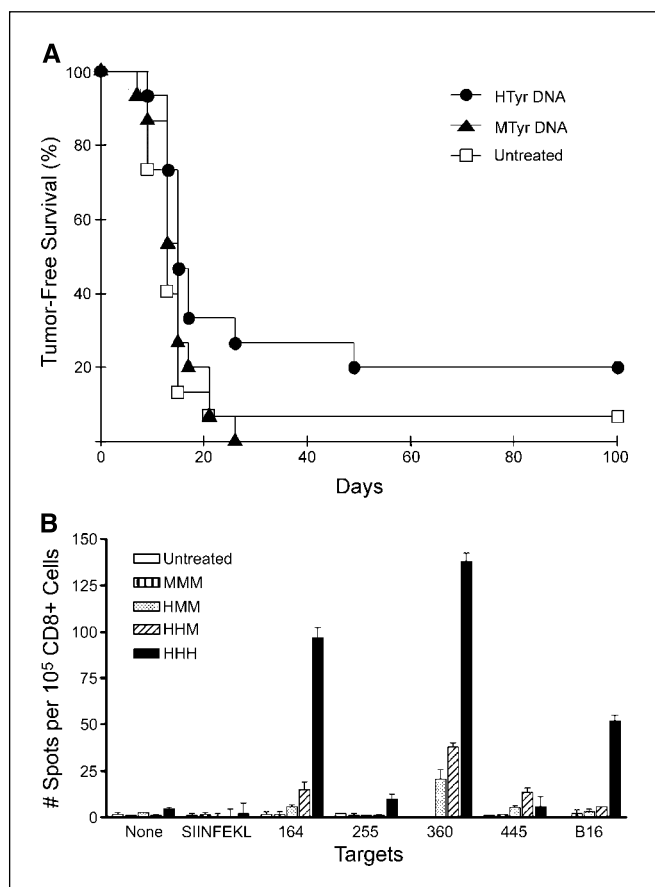


Fig. 1. Xenogeneic (human) tyrosinase plasmid DNA immunization delays tumor growth and induces a dose-dependent CD8⁺ T-cell response. *A*, tumor-free survival was evaluated in mice (15 per group) immunized three times at weekly intervals with either human or mouse tyrosinase DNA and challenged with B16 mouse melanoma cells. Mice immunized with human tyrosinase DNA had improvement in tumor-free survival compared with untreated mice ($P = 0.05$). This is the best of six repetitions, most of which show a trend toward improved survival. *B*, C57BL/6 mice (two mice per group) were immunized with human (HHH), mouse (MMM), or a sequence of human and mouse (HMM or HHM) tyrosinase DNA three times at weekly intervals or were left untreated. Only mice immunized with xenogeneic tyrosinase developed anti-mouse tyrosinase responses, and the intensity of the CD8⁺ response was higher with increasing number of vaccinations with xenogeneic plasmid DNA. The ovalbumin peptide SIINFEKL was used as an irrelevant control peptide to measure nonspecific CD8⁺ responses.

human tyrosinase DNA (H) in the following weekly sequences, by group: MMM, HMM, HHM, or HHH. Control mice were left untreated. For ELISPOT analyses, CD8⁺ T cells were purified from pooled inguinal lymph nodes and spleens and then incubated with EL4 stimulator cells loaded with different peptides (Fig. 1B). Another stimulator was syngeneic B16 melanoma, in which tyrosinase is endogenously expressed. Three weekly DNA immunizations with human tyrosinase DNA (HHH) induced CD8⁺ T cells that recognized syngeneic tyrosinase peptides as well as endogenous tyrosinase epitopes presented by B16 cells. Mice immunized only with syngeneic mouse tyrosinase DNA (MMM) had no detectable T-cell response, which is consistent with results obtained with other melanosomal antigens (9, 10). Only mice immunized with human tyrosinase developed a CD8⁺ T-cell response to mouse tyrosinase peptides. The T-cell response was dose dependent; that is, the number of IFN- γ ⁺ CD8 T cells (spots) counted in the

ELISPOT assay was higher with increasing numbers of human tyrosinase DNA injections.

Immunization with virus-like replicon particle encoding either mouse or human tyrosinase results in tumor immunity. Because of the potential advantages of VRP, we investigated the ability of VEEV-based VRP, derived from an attenuated variant of VEEV encoding mouse or human tyrosinase, to induce immunity to tyrosinase. Mice were immunized s.c. every 2 weeks for a total of three vaccinations with either mouse or human tyrosinase VRP (MVRP or HVRP, respectively) at a dose of 10^2 , 10^4 , or 10^6 infectious units per vaccine. Control mice were either unimmunized or immunized with 10^6 infectious units VRP encoding an irrelevant gene (*GFP-VRP*). Antibodies to mouse tyrosinase were assayed first as a means to identify an immunologically active dose. None of the mice receiving the 10^2 infectious units dose of either human or mouse tyrosinase VRP developed an antibody response (Table 1), whereas all 10 of 10 mice given the 10^4 or the 10^6 dose of mouse or human VRP developed antibody responses to mouse tyrosinase. Although this assay is not quantitative, we did serial dilutions of the serum (when sufficient amounts were available) to study any trends in the degree of antibody production for a given immunization strategy. Serum that had previously tested positive was serially diluted in half with PBS until the anti-tyrosinase antibodies no longer tested positive in the enzymatic assay described above. The dilution ranges for the analyzed samples (five per group) are reported in Table 1. The results shown suggest a trend for greater antibody production induced by either of the viral vectors relative to the DNA vector as well as a trend for a stronger antibody response by the mouse (syngeneic) VRP than the human VRP. No hypopigmentation of coat was observed. The 10^6 infectious units dose was used in all subsequent experiments to maximize immune responses.

CD8⁺ T-cell responses to VRP or DNA immunization were measured in ELISPOT assays (Fig. 2A). Mice immunized with three doses of human or mouse tyrosinase VRPs developed comparable or better CD8⁺ T-cell responses compared with

Table 1. DNA vaccines induce a partial antibody response only when using the xenogene, but VRP vaccines consistently induce an antibody response when given above a threshold dose

Vaccine (IU dose)	Responders/total	Dilution range for which samples remain positive
Not immunized	0/15	0
5 MTyr DNA	0/15	0
5 HTyr DNA	8/15	1:6-1:48
3 GFP-VRP (10^6)	0	0
3 MVRP (10^2)	0/10	0
3 MVRP (10^4)	10/10	Serum not available
3 MVRP (10^6)	10/10	1:96-1:384
3 HVRP (10^2)	0/10	0
3 HVRP (10^4)	10/10	Serum not available
3 HVRP (10^6)	10/10	1:48-1:96

Abbreviation: IU, infectious units.

Table 2. Mouse tyrosinase peptide selection for ELISPOT

MHC class I molecule	Position	Sequence	Predicted half-life (s)
K ^b	445	LGYDYSYL	174
D ^b	164	TPMFNDINI	220
D ^b	255*	RHPENPNLL	720
D ^b	360	SSMHNALHI	286

*All residues are conserved between mouse and human tyrosinase, except the two amino acids in boldface, which differ between the mouse and human sequences.

mice immunized with five doses of human tyrosinase DNA. However, higher T-cell responses were induced by five VRP inoculations, and, in particular, five VRP inoculations produced a substantially higher response than all of the other regimens. Therefore, immunization with syngeneic VRP induced the strongest CD8⁺ T-cell responses.

These observations raise the more general question whether the requirement for immunization with orthologues using plasmid DNA is overcome with viral immunization. Published studies have shown that whereas xenogeneic human TYRP-1 plasmid DNA is immunogenic, syngeneic TYRP-1 expressed by vaccinia virus is also immunogenic in melanoma models. In studies conducted here, C57BL/6 mice were injected with 10⁷ plaque-forming units of recombinant vaccinia vector expressing syngeneic mouse tyrosinase (MTyr rVV) twice, 1 week apart. Control mice were either immunized with null rVV or left untreated. Mice immunized with syngeneic tyrosinase induced more pronounced cytotoxic CD8⁺ T-cell responses against B16 melanoma than immunization with a human tyrosinase vaccinia construct (HTyr rVV; Fig. 2B). Two weeks after the last immunization, splenocytes were harvested, stimulated, and evaluated for cytotoxicity by standard ⁵¹Cr release assay, using B16 melanoma cells as targets. CTL responses were detected only in the mice immunized with MTyr rVV. No measurable CTL responses were identified in mice vaccinated with HTyr rVV or in the control groups. Thus, the immunogenicity of syngeneic tyrosinase expressed by a viral vector was a more general phenomenon shared by rVV and alphavirus VRP.

Immunization with virus-like replicon particle encoding mouse tyrosinase improves tumor-free survival. Because of the enhanced immune responses seen with higher and more frequent dosing of VRP, we investigated B16 tumor growth following immunization with high doses of either mouse (MVRP) or human (HVRP) tyrosinase VRP (five doses; 10⁶ particles per dose; Fig. 3A). Injection of five doses of syngeneic MVRP resulted in a significant improvement in tumor-free survival (15-18 mice per group), whereas five doses of HTyr-VRP did not induce detectable improvement. Thus, the results from the *in vitro* ELISPOT assays seemed correlated with the ability of VRP vaccines to reject B16 melanoma.

Similar results were observed when rVV constructs were used as the delivery vector. C57BL/6 mice were vaccinated with 10⁷ plaque-forming units of either HTyr rVV, MTyr rVV, or null rVV

and boosted 1 week later. These mice were challenged with B16 melanoma cells *i.v.*, and lung metastases were counted at 17 days (Fig. 3B). Mice immunized with vaccinia expressing syngeneic mouse tyrosinase (MTyr rVV) had greater tumor protection than those immunized with HTyr rVV.

Heterologous prime/boost DNA/virus-like replicon particle immunization enhances tumor-free survival. To establish if heterologous prime/boost regimen would increase immunity, we examined different combinations of plasmid DNA and VRP encoding tyrosinase. The results from a large number of permutations and combinations of DNA and VRP showed that the majority of mice in any group receiving at least two vaccinations with MVRP or HVRP produced antibodies to mouse tyrosinase (data not shown). Heterologous DNA prime/VRP boost regimens were also compared directly to repeated vaccinations with VRP. Mice were primed with either one vaccination with VRP or three injections of DNA and then boosted with two vaccinations with VRP. Control groups

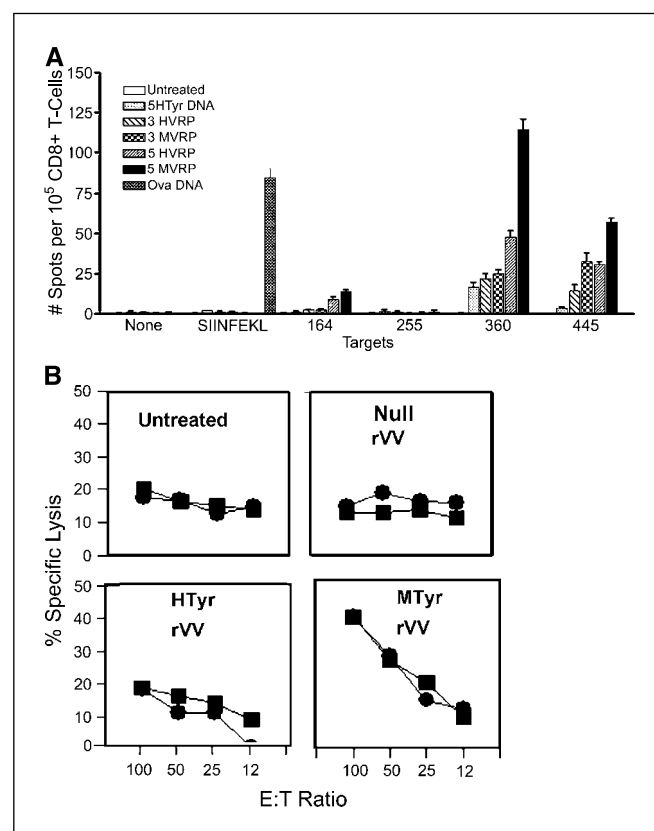


Fig. 2. Immunization with alphavirus particles encoding syngeneic (mouse) tyrosinase induces CD8⁺ T-cell responses. **A**, cells from inguinal lymph nodes and spleen were pooled from mice (three mice per group) that had been cutaneously immunized every two weeks with different regimens of VRP and were analyzed by ELISPOT assays against syngeneic mouse tyrosinase peptides. Columns, mean number of spots per five replicate wells; bars, SE. Mice immunized thrice with VRP encoding either mouse or human tyrosinase developed CD8⁺ T-cell responses comparable or better than those induced by human tyrosinase plasmid DNA. The response to five VRP vaccinations was higher than other vaccination regimens with five vaccine doses of mouse tyrosinase being the strongest of all the groups. CD8⁺ cells from mice immunized with an ovalbumin minigene DNA (Ova) stimulated with Ova SIINFEKL peptide-pulsed EL4 targets served as positive control. **B**, splenocytes were harvested from mice immunized *i.p.* twice in a weekly interval with vaccinia constructs expressing human or mouse tyrosinase and analyzed by ⁵¹Cr release assay against B16 melanoma. Points, % specific lysis for individual mice (two per group). SD of the triplicate determinations for each effector/target cell ratio was <10% in negative groups, and spontaneous release never exceeded 20%.

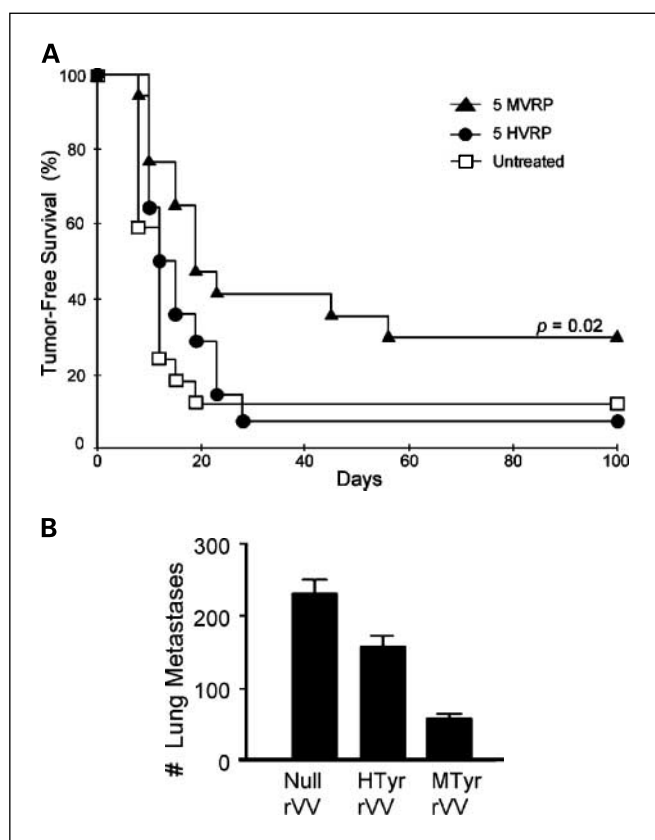


Fig. 3. Repeated immunizations with VRP or rVV vectors encoding syngeneic (mouse) tyrosinase provide tumor protection. *A*, Kaplan-Meier survival curves show tumor-free survival for mice immunized with five vaccinations of mouse or human tyrosinase VRP and challenged with a lethal dose of B16 melanoma cells. Compared with the control (untreated) group, tumor-free survival was significantly improved in the group receiving five vaccinations with MVRP (syngeneic antigen). $P = 0.02$ (log-rank analysis). *B*, immunization with rVV expressing syngeneic (mouse) tyrosinase provides resistance to lung metastases following i.v. challenge with a lethal dose of B16 melanoma, whereas rVV expressing xenogeneic (human) tyrosinase or control rVV provided no protection. Mice (10 per group) were immunized twice at a weekly interval and challenged with B16 5 days after the last immunization. Lung metastases were evaluated on day 17 following challenge. Representative of two experiments. Columns, mean number of surface lung metastases; bars, SE.

received five injections of human tyrosinase DNA alone or were left untreated. Mice were followed for 100 days after intradermal tumor challenge. When either a DNA or VRP prime was followed by two VRP boosts, a modest but significant effect on tumor-free survival (relative to untreated mice) was observed when at least one vaccination with VRP encoding mouse tyrosinase (syngeneic antigen) was given (Fig. 4). The 3MVRP group also showed statistically significant improvement in tumor-free survival relative to both the DNA alone and untreated groups, but the difference between 3MVRP and the heterologous DNA prime/virus boost did not reach statistical significance.

Discussion

This study evaluated different strategies for immunizing against the differentiation antigen tyrosinase. Using a computer algorithm and ELISPOT assays, we were able to identify and validate four new MHC class I-restricted epitopes for mouse tyrosinase. These peptides can be used to assess CD8⁺ T-cell

responses to mouse tyrosinase in C57BL/6 mice following immunotherapy.

When immunizing mice with plasmid DNA vaccines, only the xenogeneic orthologue was effective for antibody and CTL induction and showed a weak but reproducible improvement in tumor protection. These findings corroborate prior studies using other differentiation antigens, which showed that immunologic ignorance or tolerance can be overcome by immunizing with xenogeneic antigen (7–10). Nevertheless, the effects of xenogeneic DNA immunization in the current study were not as effective as the strategies incorporating Tyr-VRP into the antigen delivery scheme.

This study is the first to report the use of alphavirus-derived VRP vectors as a delivery system for cancer differentiation antigens. We examined the efficacy of these vectors by themselves, as well as their effects in a heterologous prime/boost strategy with plasmid DNA encoding the same antigen. The DNA-alone model gave only weak immune responses with xenogeneic tyrosinase DNA. In contrast, when the same genes, mouse and human tyrosinase, were delivered in a VRP vector, syngeneic mouse tyrosinase induced stronger antibody and T-cell responses that conferred modest and statistically significant tumor protection. In each instance, for VRP, the magnitude of the immune response to the syngeneic antigen was at least as good as the response to the xenogeneic construct, and with five immunizations, syngeneic tyrosinase VRP induced higher CD8⁺ T-cell responses than the xenogeneic vaccines. We had previously observed a similar phenomenon with immunization using vaccinia virus constructs, representing an entirely different class of virus (double-stranded DNA) for antigen delivery. Because the experiments that evaluated the vaccinia virus constructs were done several years ago in our laboratory and because conditions for handling this virus have changed, we were not able to directly compare VRP to rVV.

The superior effectiveness of immunization with the syngeneic mouse antigen compared with xenogeneic antigen seen in

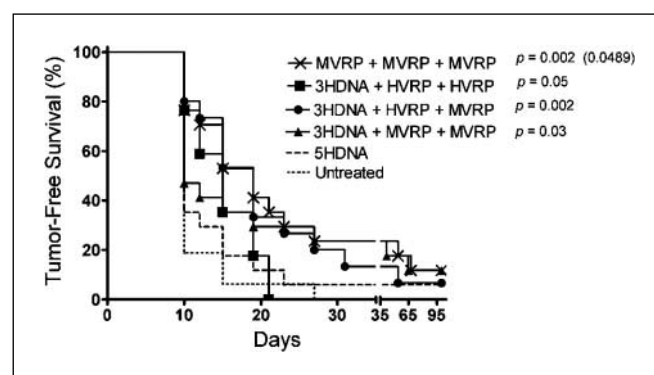


Fig. 4. Heterologous DNA prime/virus boost strategy provides tumor protection. Kaplan-Meier curves represent tumor-free survival for mice (15–17 per group) immunized with various combinations of human tyrosinase DNA (HDNA) and/or human or mouse tyrosinase VRP (HVRP and MVRP, respectively) and challenged with B16 melanoma cells. Mice receiving DNA only had five DNA vaccinations, whereas mice primed with DNA and boosted with VRP received three DNA vaccinations. Boosting with MVRP or MVRP and HVRP encoding tyrosinase provides tumor protection (compared with untreated animals), whether primed with xenogeneic DNA or syngeneic VRP. P s are the significance by log-rank analysis of the treatment group relative to untreated mice. For the 3MVRP group, there is an additional P shown in brackets that compares the 3MVRP group with the 5HDNA group.

our study has been shown in other systems that use virus-derived vectors. Leitner et al. (39) immunized mice with a Sindbis replicon-encoding TYRP-1/gp75 DNA vaccine and showed that tolerance could be broken with both mouse or human TYRP-1/gp75. A different study from this group showed that immunity and tumor protection could be induced by vaccinating with an rVV encoding mouse TYRP-1/gp75; however, there was no direct comparison between viruses encoding syngeneic and xenogeneic TYRP-1/gp75 (15). Finally, induction of T-cell and antitumor responses has been reported in animal models using VEEV VRPs encoding a syngeneic *neu* oncogene (26, 40). These studies did not directly compare syngeneic and xenogeneic orthologues and did not evaluate other vector delivery methods. These studies and ours together reveal the reproducible induction of responses by the syngeneic self-antigen encoded by viral replicons and viral nucleic acid vectors. Collectively, these results stand in sharp contrast to results using plasmid DNA vectors, in which immune tolerance or ignorance cannot be overcome by immunizing with syngeneic DNA (7–10, 41). For plasmid DNA vaccination, one mechanism by which tolerance is overcome involves enhanced binding of orthologous peptide to mouse MHC I molecules compared with the homologous syngeneic peptide (42, 43). It is also likely that regions of the xenogeneic antigen that are distinct from the syngeneic antigen may be recruiting other arms of the immune system (e.g., CD4⁺ help).

We do not have a clear explanation for why the syngeneic antigen is converted to an immunogenic antigen by delivery with VRP, but there are multiple plausible explanations. First, it is possible that more sustained antigen expression and the presence of helper epitopes provided by the viral particles augment the immune response. For RNA viruses, in particular double-stranded RNA viruses, TLR3-mediated induction of type I IFNs is triggered, which serves as a broad stimulus to the immune response (44). In addition, the high-error prone RNA replicases may generate many immunogenic mutants via creation of heteroclitic epitopes (45). Viral infection is also known to overtake the cellular machinery such that defective ribosomal products (DRiPs) are formed (e.g., prematurely truncated or misfolded polypeptides; ref. 46). DRiPs have been shown to represent a source of peptides for MHC I processing (46, 47), and truncated proteins have been shown to enhance epitope presentation (48). More specifically, our observations

have revealed that truncated mouse tyrosinase induces a CTL response in C57BL/6 mice.⁴ Furthermore, the high viral protein expression rate and cellular stress induced by viral infection can alter processing and enhance presentation of full-length wild-type antigens. Ostankovitch et al. (49) have shown that both misfolded wild-type tyrosinase and wild-type tyrosinase retained in the ER can each augment proteasome degradation and MHC I presentation of tyrosinase epitopes.

Our study also shows that in contrast to what has been observed in other viral systems (15, 20), administration of sequential vaccinations with VRP did not show visible effects of neutralizing immunity, in that development of immunity to the encoded antigen was not diminished, and actually increased, as more vaccinations with mouse tyrosinase VRP were administered. It has been shown previously that although immunization with replication-deficient VRP based on Semliki Forest virus did induce anti-Semliki Forest virus antibodies, these antibodies did not inhibit antigen-specific responses from occurring with additional booster doses of Semliki Forest virus-based vaccine (50).

Immunization with alphavirus-based VRP represents a strategy to induce T-cell and antibody responses to otherwise poorly immunogenic cancer antigens. Our results provide the foundation for the design of clinical trials using VRP expressing syngeneic antigens. Xenogeneic tyrosinase DNA vaccine is presently being evaluated in a phase I clinical trial in humans⁵ and has been shown to prolong survival in a canine melanoma trial (29). Although priming immunization with a plasmid DNA was predicted to enhance the immunogenicity of VRP, this phenomenon was not observed. In all experimental variations evaluated, mouse tyrosinase VRP induced the strongest T-cell response and the best tumor rejection. We conclude that VRP expressing human differentiation antigens would be the appropriate vehicles for clinical testing in patients with cancer.

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⁴ M. Engelhorn, J. Guevara-Patino, and A.N. Houghton, unpublished data.

⁵ J.D. Wolchok, unpublished data.

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