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Report

Venezuelan equine encephalitis replicon immunization overcomes intrinsic tolerance and elicits effective anti-tumor immunity to the ‘self’ tumor-associated antigen, *neu* in a rat mammary tumor model

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Key words: breast cancer, immunotherapy, *neu*, rat tumor model, replicon vector

Summary

Many tumor-associated antigens (TAAs) represent ‘self’ antigens and as such, are subject to the constraints of immunologic tolerance. There are significant barriers to eliciting anti-tumor immune responses of sufficient magnitude. We have taken advantage of a Venezuelan equine encephalitis-derived alphavirus replicon vector system with documented *in vivo* tropism for immune system dendritic cells. We have overcome the intrinsic tolerance to the ‘self’ TAA rat *neu* and elicited an effective anti-tumor immune response using this alphavirus replicon vector system and a designed target antigen in a rigorous rat mammary tumor model. We have demonstrated the capacity to generate 50% protection in tumor challenge experiments ($p = 0.004$) and we have confirmed the establishment of immunologic memory by both second tumor challenge and Winn Assay ($p = 0.009$). Minor antibody responses were identified and supported the establishment of T helper type 1 (Th1) anti-tumor immune responses by isotype. Animals surviving in excess of 300 days with established effective anti-tumor immunity showed no signs of autoimmune phenomena. Together these experiments support the establishment of T lymphocyte dependent, Th1-biased anti-tumor immune responses to a non-mutated ‘self’ TAA in an aggressive tumor model. Importantly, this tumor model is subject to the constraints of immunologic tolerance present in animals with normal developmental, temporal, and anatomical expression of a non-mutated TAA. These data support the continued development and potential clinical application of this alphaviral replicon vector system and the use of appropriately designed target antigen sequences for anti-tumor immunotherapy.

Introduction

One in eight American women will be diagnosed with breast cancer [1]. Despite significant treatment advances, a substantial percentage of women diagnosed with breast cancer will develop metastatic disease, often after many years, suggesting the presence of micrometastatic disease after initial treatment. Novel treatment methods directed at seeking out and eliminating this persistent micrometastatic disease might have substantial clinical benefit. The immune system is particularly well suited for this purpose. Breast

cancer patients have been documented to have immune responses to breast cancer tumor-associated antigens (TAAs) [2–8], but these immune responses are generally of low magnitude and are clearly insufficient to establish or maintain control of patients’ tumors. Many TAAs have been characterized as ‘altered self’ [9] or are mal-expressed ‘self’ molecules, which may account, in part, for the difficulty encountered in attempts to elicit robust antigen-specific, anti-tumor immune responses due to intrinsic tolerance to ‘self’. Recent promising studies in non-Hodgkins lymphoma and mela-

noma have demonstrated the capacity of immunotherapeutic strategies to elicit TAA-specific immune responses that are associated with clinical responses [10–13]. However, the clinical benefit of anti-tumor immunotherapy in other solid tumors such as breast cancer remains to be demonstrated.

Alphaviruses, such as Venezuelan equine encephalitis (VEE), are positive strand ribonucleic acid (RNA) viruses that have several characteristics which are potentially advantageous for the derivation of anti-tumor immunotherapeutic/vaccine vector systems [14,15], including demonstrated tropism for a subset of immune system dendritic cells (DCs) [16]. Several strategies using vector systems derived from alpha viruses have been described, including viral replicon particles (VRPs) [17–30]. VRPs are single-cycle vectors containing RNA replicons with an engineered multiple cloning site in place of the viral structural protein genes. Heterologous genes cloned into this site are expressed from the 26S subgenomic RNA promoter at very high levels. Replicon particles are produced by providing the missing structural protein genes *in trans* on two helper RNAs [31]. The application of VEE VRPs for immunization with various infectious disease antigens (one of which is also a TAA) has been reported [27, 32–41]. The potency of VEE VRPs in these studies suggests that VRPs might have similar efficacy in targeting a ‘self’ TAA.

We have used a rat mammary tumor model and selected the *neu* molecule, homologue of human HER2/*neu*, as a prototypical TAA to test our hypothesis that the application of VEE derived VRP immunotherapy will overcome intrinsic tolerance and elicit efficacious anti-tumor immunity. The selection of a rat model is complicated by the availability of fewer immunologic reagents, but the normal expression pattern of rat *neu*, in contrast to mice transgenic for rat *neu*, provides for a model; (1) that more closely matches the human clinical situation, (2) with normal intrinsic tolerance, and (3) the potential to observe elicited autoimmune phenomenon.

Materials and methods

Animals and cell lines. Six- to eight-week-old Fisher 344 female rats (NCI-FCRC, Frederick, MD) were obtained and housed in grouped cages under normal

vivarium conditions. Water and rodent chow were provided *ad libitum*. The rat mammary tumor cell line 13762 MAT B III (CRL-1666, ATCC, Manassas, VA) was obtained and cultured *in vitro* as recommended. Cells were harvested using Versene (Gibco Life technologies, Rockville, MD) and washed three times in phosphate-buffered saline prior to suspension in injection grade normal saline for inoculation into recipient animals. BHK cells (CCL-10, ATCC, Manassas, VA) were used for production and titration of VRPs. All work was performed under an approved and active animal experimental protocol. All experiments were performed with strict adherence to all institutional animal care and use guidelines. The expression level of rat *neu* and MHC class I molecules were routinely monitored by flow cytometry (FAC-Scan, B.D. Biosciences, San Diego, CA) using FITC labeled appropriate isotype controls, Ab-4 (Oncogene Science, Boston, MA) and OX-18 (B.D. Biosciences, San Diego, CA) respectively, Figure 1.

Target antigen sequence. Partial protein sequences from HER2/*neu* were used to probe the protein databases for regions of homology to known proteins. Regions with the least amount of homology with other normal proteins, including other members of the epidermal growth factor receptor (EGFR) family, were selected for inclusion in the target antigen sequence. Regions from the extra-cellular domain were not included to optimize the likelihood of intracellular expression, MHC class I processing/presentation,

Figure 1. 13762 MAT B II expression of MHC class I and rat *neu*: Representative, non-gated, data from routine fluorescent cytometry monitoring of the 13762 MAT B II tumor line. MHC class I, bold line – no shading; rat *neu*, bold line – gray shading; and isotype control thin line – no shading.

Figure 2. Comparative predicted amino acid sequences of human and rat c-erb B2. Standard single letter amino acid notations are used for the human sequence above and rat sequence below. Vertical bars = identical amino acids, colon = conservative substitutions, and periods = semi-conservative substitutions. Underlined regions represent those selected for inclusion in the target antigen sequence.

and T helper type 1 (Th1) immune responses. The exceptional degree of homology between *HER2/neu* and rat *neu* allowed the selection and construction of an entirely homologous target antigen sequence for rat *neu*, Figure 2. The selected sequences were obtained by rtPCR from mRNA isolated from the 13762 MAT B III tumor cell line. Total RNA was isolated using Trizol reagent and first strand cDNA synthesis was performed using Superscript II Reverse Transcriptase

(Gibco Life technologies, Rockville, MD) according to manufacturers instructions. Pfu thermostable polymerase (Stratagene, LaJolla, CA) was used for all PCR reactions. The following primers were used to amplify a small fragment that includes the transmembrane domain, + strand (bases 1855–1882) 5'-CTCCTACATGCCCATCTGGAAGTACCC-3' and – strand (bases 2093–2120) 5'-TAACTCAGTTTCCTGCAGCAGCTACG-3'. The following primers were

used to amplify a larger fragment derived from the cytoplasmic domain, + strand (modified from bases 3110–3142) 5'-GATTCTTCTCTCCGGAGCCCTACCCCAGGCAC-3' and – strand (bases 3920–3950) 5'-CAGCAAGGAAAGGTTCTCGGGGCAGGTTC-3'. The proximal fragment contains an intrinsic BspE I site and the terminal fragment positive strand primer was modified by changing the 11th base from a C to T creating a BspE I site (underlined) and inserting a G between bases 16 and 17 in order to maintain the reading frame. These two fragments were digested with BspE I (New England Biolabs, Beverly, MA) under standard conditions, purified by agarose gel electrophoresis, and ligated using Rapid Ligation Kit (Gene Choice, PGC Scientifics Corp. Frederick, MD) as per manufacturers instructions. The resultant ligation product was used for PCR amplification of the target antigen sequence using the following primers containing engineered Bgl I sites for directional cloning (underline), + strand 5'-CCCATCGCCACCATGGCCTCCTGTGTGGATCTGGATGAACGAGGC-3' and – strand 5'-ACGTGCCTTAAGGCTCATACAGGTACATCCAGGCCTAGGTACTC-3'. The resulting target antigen sequence, encoding a heterologous target antigen protein containing 275 amino acids, incorporates a 'start' methionine (M) and alanine (A) in the second position preceding the sequence from the smaller fragment. The fusion of the polypeptides encoded by the smaller and larger PCR fragments required a conserved substitution of arginine (R) for lysine (K) and the insertion of serine (S) at the fusion site in order to maintain the reading frame through the BspE I site in the resultant fusion protein, Figure 3. This amplified tumor antigen sequence was sub-cloned into a standard cloning vector for sequence confirmation and subsequent sub-

MASCVDLDERGCPAEQRASPVTFIATVVGVLFF
LILVVVVGILIKRRRQKIRSPPTGTGTAHRRHRS
SSTRSGGGELTLGLEPSEEGPPRSPLAPSEGAGS
DVFDGDLAMGVTKGLQSLSPHDLSPQLQRYSED
PTLPLPETDGYVAPLACSPQPEYVNQSEVQPQ
PPLTPGEPLPPVRPAGATLERPKTLSPGKNGVV
KDVFAFGGAVENPEYLVPREGTASPPHPSPAFS
PAFDNLYYWDQNSSEQGPPPSNFEGTPTAENP
EYLGLDVPV

Figure 3. Amino acid sequence of the designed tumor-associated target antigen. Standard single letter amino acid notations are used. Underlined amino acids note modifications/additions to the wild type sequences, see text for full description, italicized sequence represents the transmembrane domain.

cloning into the VEE VRP production constructs and for production of His tagged target antigen protein.

VEE VRP Production. The target antigen sequence, designed and constructed as above, and the influenza A hemagglutinin (H1) sequence were subcloned into the VEE replicon plasmid pVR200 that has been described previously [41]. This plasmid along with the two other plasmids encoding the structural gene sequences, the split helper plasmid system, were linearized by Not I digestion. *In vitro* transcription using T7 RNA polymerase was used to generate capped RNAs that were electroporated into BHK-21 cells for the production of the VRPs. VRPs were concentrated from culture supernatants via centrifugation through a 20% sucrose cushion prepared in Phosphate Buffered Saline (PBS). Infectious unit titers (IU/ml) were obtained by plating serial dilutions on BHK-21 monolayers with immunofluorescent evaluation of VEE non-structural gene products or heterologous protein expression. Extensive safety testing was performed prior to release of VEE VRPs for experimental evaluation to document the absence of replication competent virus. VRPs were resuspended in PBS with 1% normal rat serum and frozen at –80°C for shipment and diluted in this same buffer to an appropriate concentration for administration.

Immunization and phlebotomy. Animals were immunized with 200 µl of solution containing the appropriate concentration of VEE VRPs administered through a 27 g needle. All injections were performed with minimal restraining of conscious animals. The administration site was cleansed with 70% ethanol and allowed to dry prior to immunization. Subcutaneous (SC) immunizations were located approximately 0.5–1.0 cm cephalad and lateral to the base of the tail on the contralateral side to tumor inoculation. Intramuscular (IM) administrations were located in the quadriceps, rectus medius. Ten to 12 days after completion of immunization sequences, venous blood was obtained via standard saphenous vein phlebotomy. Serum was stored at –20°C prior to analysis.

His-tagged rNeu protein expression and purification. A histidine tag was added to the C-terminus of the rat *neu* target antigen coding sequence by PCR amplification using the following forward and reverse primers engineered to contain *EcoRV* and *AscI* sites, respectively (sites underlined), 5'-CGGATATCATGGCCTCCTGTGTGGATCTG-3' and

Figure 4. (A) Tumor challenge survival. Animals receiving VRP-rNeu intramuscular immunizations are depicted by the dashed line and open squares ($\square$), VRP-rNeu SC immunizations the circle dotted line and open circles ($\circ$). Control animals, receiving VRP-HA intramuscular immunizations, irrelevant antigen for vector and specificity control, are depicted by the square dotted line and open triangles ($\triangle$), receiving SC immunizations of 10^6 irradiated tumor cell by the solid line and open diamonds ($\diamond$), and animals that did not receive immunization by the thin line and closed squares ($\blacksquare$). Animals were euthanized when tumors exceeded a volume of $10,000 \text{ mm}^3$. (B) Individual rat tumor volumes. Each individual line represents a single animal in the cohort of VRP-rNeu intramuscular immunized animals. Time 0 equals tumor inoculation.

Figure 5. Tumor re-challenge survival. Survival of immunized animals receiving a second tumor challenge 140 days after the initial challenge. VRP-rNeu intramuscular route are depicted by the dashed line and open squares (□), SC immunization route by the dotted line and open circles (○), or 10^6 irradiated tumor cells by the solid line and open diamonds (◇).

Figure 6. Winn assay. Survival of naïve animals from time of inoculation of tumor and nylon wool, T-cell enriched immune splenocytes. Splenocytes isolated from animals receiving VRP-rNeu intramuscular immunizations are depicted by the dashed line and open squares (□), VRP-rNeu SC immunizations by the circle dotted line and open circles (○), VRP-HA, the irrelevant antigen, by the square dotted line and open triangles (△) Control, and non-immunized splenocytes by the thin line and closed squares (■). Animals were euthanized when tumors exceeded a volume of $10,000 \text{ mm}^3$.

tumor cells, in appropriate amounts to allow for 200 μ l inoculation volumes, just prior to administration to non-immunized naïve animals as described above for tumor challenges.

Results

Immunization with VRP vectors encoding the rat *neu* target antigen sequence resulted in 50% of animals being protected from tumor challenge, Figure 4. Immunization with VRP encoding the irrelevant antigen influenza hemagglutinin (HA) or sham immunization with vehicle alone resulted in no protection from tumor challenge. The survival benefit for VRP-*rNeu* immunized animals relative to control animals is statistically significant, $p = 0.004$ (two-tailed Fisher's Exact Test). The results depicted in Figure 4(a) are representative of three separate experiments that gave similar results. Interestingly, all animals receiving SC and a proportion of animals receiving IM VRP-*rNeu* immunizations developed tumors with 25–50% of these tumors permanently regressing, Figure 4(b). Overt protection from tumor challenge was observed only with the IM route of administration. There appeared to be a modest dose–response relationship over the range from 10^5 to 10^6 to 10^7 IU/immunization (data not shown).

Immunological memory was evaluated using both repeat tumor challenge and Winn assay experiments. Animals that had rejected their tumors were subjected to a second tumor challenge 140 days after the initial tumor challenge. A cohort of naïve animals was similarly challenged with tumor as a positive control and demonstrated the expected tumor development. All surviving animals previously immunized with VRP-*rNeu* were protected from a second tumor challenge while the control animals developed progressing tumors without regression, Figure 5. There were no surviving animals from the non-immunized or VRP-HA immunized cohorts. Immunologic memory elicited by the immunization procedure alone was assessed by the Winn assay. Nylon wool T cell enriched immune splenocytes purified from immunized rats were administered to naïve animals admixed with tumor at 100:1 and demonstrated significant protection, $p = 0.009$ (two-tailed Fisher's Exact Test), from tumor development relative to animals that received nylon wool T cell enriched non-immune splenocytes admixed with tumor at the same ratio, Figure 6. Nylon wool T cell enriched VRP-HA immune splenocytes protected two

Table 1. Post-immunization mean antibody titers

Immunization	Mean antibody titer	Range of antibody titers
10^7 VRP- <i>rNeu</i> IM	2793	1920–3840
10^7 VRP- <i>rNeu</i> SC	1975	640–3840
10^6 VRP- <i>rNeu</i> IM	2326	1280–5120
10^6 VRP- <i>rNeu</i> SC	988	120–2560
10^5 VRP- <i>rNeu</i> IM	2610	640–5120
10^5 VRP- <i>rNeu</i> SC	1568	480–3840
10^7 VRP-HA IM	135	<80–640
10^7 VRP-HA SC	145	<80–640
Irradiated 13762	<80	all <80
Non-immunized	<80	all <80

of six animals despite no previous demonstration of protection in challenge experiments. Flow cytometric evaluation of the nylon wool T cell enriched populations obtained from the various treatment cohorts demonstrated no significant differences in proportions of NK, CD3+, CD4+, CD8+ and MHC class II+ cells (data not shown).

We evaluated serum that was obtained from animals approximately 10 days after completing the immunization sequence for antibody responses to the rat *neu* target antigen by ELISA. The IM routes of administration resulted in higher mean antibody titers at all doses, Table 1. Control animals (sham, VRP-HA, and irradiated tumor immunized) did not elicit significant anti-rat *neu* target antigen immune responses. This data is representative of the antibody titers for all three experiments in which sera was collected. All positive samples were evaluated for isotype responses. Only IgG2a or IgG2b isotypes were identified in the anti-rat *neu* target antigen reactive components. No IgG1 anti-rat *neu* antibodies were detected (data not shown). There was no correlation with antibody titer and rejection of tumor challenge.

Discussion

This series of experiments demonstrates that VEE derived VRPs are an efficient vector system for overcoming the existing level of tolerance and eliciting anti-tumor immunity to a highly conserved TAA which is also a normally expressed 'self' protein. These results were obtained in a rat tumor model that has expression of the TAA in an entirely physiologic manner in contrast to the various rat *neu* transgenic

mouse models [43–57] and using a designed TAA target antigen selected to enhance both specificity and elicited Th1/ cytotoxic T lymphocyte (CTL) immune responses. The failure of immunization with VRPs expressing an irrelevant antigen, HA, to protect animals from tumor challenge strongly supports the generation of antigen-specific anti-tumor immune responses. The long-term survival of animals that were immunized and rejected the tumor challenges, without apparent toxicity, supports the absence of significant cross-reactive autoimmune responses. The establishment of immunological memory, limited humoral responses with exclusive Th1 associated isotypes, and the capacity of nylon wool-enriched immune splenocytes to protect naïve animals from tumor challenge in the Winn assay argue for VRP-elicited Th1 biased, T lymphocyte mediated anti-tumor immune responses. Taken together these results support the further investigation of this alphavirus-derived replicon vector system for anti-tumor immunotherapy including targeting over-expressed TAAs, even those that are normal ‘self’ proteins.

The minimal level of protection provided by VRP-HA immune splenocytes in the Winn assay was unexpected and is not readily explained. Given the absence of tumor challenge protection and antigen reactive humoral immune responses seen with VRP-HA immunization, we believe that the effector population for this unexpected finding may reside in the innate arm of the immune system. Although there were no differences in proportion of NK or MHC II+ cells in the enriched immune splenocyte populations from the various cohorts, NK activity was not assessed and differences in activity may account for this unexpected observation. There is no reason to believe that different target antigens, HA or rat *neu*, in the VRP vector would result in different levels of NK activity. Despite this unexpected result in the Winn Assay, there is a clear antigen-specific benefit across all tumor protection, re-challenge, and Winn assays.

The breast cancer TAA HER2/*neu* is a non-mutated, member of the EGFR family [58, 59], which is over-expressed in 25–30% of human breast cancers and numerous other tumor types [60–64]. This molecule contains a number of MHC binding polypeptide T lymphocyte epitopes [65–77] and immune responses to HER2/*neu* have been described in breast cancer patients [74, 77–79]. The targeting of this molecule by the therapeutic antibody, Herceptin®, has shown clinical efficacy in a proportion of the patients that over-express HER2/*neu* [80] supporting this protein

as a viable target antigen. The observation of unexpected cardiotoxicity in some patients treated with Herceptin® [80] accentuates the potential difficulties encountered when targeting a ‘self’ TAA.

We used a designed target antigen derived from selected portions of rat *neu*, and by extension HER2/*neu*, because we sought to both elicit immune responses biased towards Th1 and CTL effectors and to maximize specificity. Other groups have evaluated either the whole protein, extra-cellular domain, intra-cellular domain, or selected MHC binding peptides as target antigens [43–47, 67, 81–92]. The presence of large regions of highly conserved sequences in both the intra- and extra-cellular domains suggested that a designed target antigen sequence, such as ours, might avoid cross-reactive autoimmune responses while potentially allowing for processing and presentation of multiple antigenic epitopes, in the context of a broader range of MHC molecules, than with the use of MHC binding peptides. The targeting of two distinct and separate regions of HER2/*neu* by Herceptin® and this VRP based immunotherapy would suggest that these two treatments would not be antagonistic and indeed might be synergistic.

Our choice of a syngeneic rat mammary tumor model was driven by the desire to have as stringent a model as possible for evaluating antigen-specific anti-tumor immunotherapeutic strategies. The presence of a physiologically expressed, highly conserved homologue of a human TAA with its intrinsic level of tolerance more than compensated for the drawback of the restricted number of well characterized, rat specific, immunologic reagents relative to mouse systems. To date, similar studies to those described above have not been able to be performed in non-transgenic mouse models. A full-length murine cDNA homologue for human HER2/*neu* has not been demonstrated, although a number of groups have used knock-out techniques to study presumptive *erbB2* function in mice [93, 94]. In contrast, the highly conserved rat homologue rat *neu*, both wild type and mutated/activated (tumorigenic) sequences, has been well characterized. Transgenic mouse models using both wild type and activated rat *neu* have been developed and have been useful in studies of anti-tumor immunotherapies including those targeting *neu* [43–57]. It is widely acknowledged that expression of transgene encoded products can result in immunologic tolerance [47] however, the expression pattern is not always consistent with normal physiology. Additionally, the mammary tumor cell line we selected expresses relatively low levels

of MHC class I on the cell surface, recapitulating findings in human breast cancers [95], and modestly over-expresses rat *neu* both of which add rigor to the model. Thus, we believe that rat *neu* and rat mammary tumor models are more clinically relevant for evaluating both the capacity to elicit anti-tumor, antigen-specific immune responses and any untoward autoimmune phenomena.

Several characteristics of VEE derived VRPs appear to contribute to the efficiency of these vector systems, including (1) the very high level of heterologous gene expression, (2) their specific cell and tissue tropisms, and (3) the vector-associated cytopathic effect. The life cycle of alpha viruses and alphaviral-derived vectors occur entirely within the cytoplasm that obviates transport of genetic material across the nuclear membrane as a concern and removes the potential for chromosomal integration or adventitious splicing of engineered coding sequences. The genomic positive strand RNA gives rise to amplified subgenomic RNAs that are then directly translated for the synthesis of the encoded heterologous protein resulting in very high levels of viral encoded protein synthesis, up to 25% of total cellular protein [41, 96], providing abundant albeit transient expression of heterologous proteins. The expression of the heterologous protein is intracellular and thus, available to the endogenous antigen processing and presentation pathway. The host tissue receptivity for VEE and derived replicons includes lymphoid and neuronal cells, and specific targeting to DC has been described [15, 16]. The cytopathic effect, believed to result primarily in apoptotic cell death [30, 97–114], can result in uptake of heterologous protein contained in the cellular debris via the extrinsic antigen-processing pathway for presentation on MHC class II and in the case of DCs also ‘cross-priming’ into the MHC class I pathway. Furthermore, the associated cytopathic effect may yield pro-inflammatory signals and be implicated in enhanced expression and loading of host heat shock proteins with peptides from expressed heterologous polypeptides.

Alphavirus replicon vectors, derived from VEE virus, have recently been used for targeting xenoantigens in two other models of anti-tumor immunotherapy. The full length prostate specific membrane antigen (PSMA) coding sequence has been used as the heterologous protein in one system [115, 116]. Mice immunized with VRPs designed to express PSMA developed both antibody and cellular immune responses reflective of a Th1 bias as characterized by cytokine production and murine antibody isotype. Velders et al.

evaluated human papilloma virus (HPV) E7 as a target antigen in a VRP immunization strategy [27] using a mouse model consisting of C57BL/6 mice and the syngeneic C3 tumor cell line expressing HPV-16 E7. This VRP immunization regimen provided 100% protection against the tumor challenge and was associated with increased E7 specific CTL responses, E7 peptide tetramer staining of CD8+ T cells, and IFN γ ELISPOT responses. Evaluation of the E7-VRP immunization strategy in a 1-week palpable tumor nodule treatment model resulted in regression of tumor in 67% of the animals at 60 days. This vector system has also been extensively evaluated in prophylactic immunization strategies for various other infectious diseases which have yielded important mechanistic and toxicity data [32–41].

The data contained within this report, along with that reported by the groups targeting HPV E7 and PSMA, suggest that the VEE VRP vector system is a very potent anti-tumor immunotherapeutic vector system. The absence of toxicity in immunized animals capable of rejecting a tumor challenge, in our report, suggests that non-mutated ‘self’ proteins can be targeted by the strategic selection of regions to be targeted. Using this vector system and the designed target antigen, the pre-existing level of tolerance can be overcome and effective anti-tumor immunity can be elicited to an over-expressed ‘self’ TAA without apparent development of clinically relevant cross-reactive autoimmunity. Studies are underway to more extensively characterize the VRP elicited anti-tumor immune response and this data will facilitate design of immunologic monitoring strategies for potential clinical trials. The capacity to mount an effective immune response to a tumor that moderately over-expresses the non-mutated TAA *neu* suggests that this strategy may be more broadly applicable than existing antibody therapies that require 3+ immunohistochemical over-expression or gene amplification. This strategy, including the designed target antigen, could be readily translated into human clinical trials due to the exceptional homology between rat *neu* and HER2/*neu*, and could be a valuable addition to therapeutic armamentarium for patients with breast cancer.

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References

1. Jemal A, Thomas A, Murray T, Thun M: Cancer statistics, 2002. *CA Cancer J Clin* 52: 23–47, 2002
2. Croce MV, Price MR, Segal-Eiras A: Expression of monoclonal-antibody-defined antigens in fractions isolated from human breast carcinomas and patients' serum. *Cancer Immunol Immunother* 40: 132–137, 1995
3. Angelopoulou K, Yu H, Bharaj B, Gai M, Diamandis EP: p53 gene mutation, tumor p53 protein overexpression, and serum p53 autoantibody generation in patients with breast cancer. *Clin Biochem* 33: 53–62, 2000
4. Disis ML, Calenoff E, McLaughlin G, Murphy AE, Chen W, Groner B, Jeschke M, Lydon N, McGlynn E, Livingston RB et al.: Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. *Cancer Res* 54: 16–20, 1994
5. Disis ML, Pupa SM, Gralow JR, Dittadi R, Menard S, Cheever MA: High-titer HER-2/neu protein-specific antibody can be detected in patients with early-stage breast cancer. *J Clin Oncol* 15: 3363–3367, 1997
6. Goydos JS, Elder E, Whiteside TL, Finn OJ, Lotze MT: A phase I trial of a synthetic mucin peptide vaccine. Induction of specific immune reactivity in patients with adenocarcinoma. *J Surg Res* 63: 298–304, 1996
7. Sandmaier BM, Oparin DV, Holmberg LA, Reddish MA, MacLean GD, Longenecker BM: Evidence of a cellular immune response against sialyl-Tn in breast and ovarian cancer patients after high-dose chemotherapy, stem cell rescue, and immunization with Theratope STn-KLH cancer vaccine. *J Immunother* 22: 54–66, 1999
8. Tilkin AF, Lubin R, Soussi T, Lazar V, Janin N, Mathieu MC, Lefrere I, Carlu C, Roy M, Kayibanda M et al.: Primary proliferative T cell response to wild-type p53 protein in patients with breast cancer. *Eur J Immunol* 25: 1765–1769, 1995
9. Houghton AN: Cancer antigens: immune recognition of self and altered self. *J Exp Med* 180: 1–4, 1994
10. Hsu FJ, Caspar CB, Czerwinski D, Kwak LW, Liles TM, Syrengelas A, Taidi-Laskowski B, Levy R: Tumor-specific idiotype vaccines in the treatment of patients with B-cell lymphoma – long-term results of a clinical trial. *Blood* 89: 3129–3135, 1997
11. Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, Restifo NP, Sznol M, Schwarz SL, Spiess PJ, Wunderlich JR, Seipp CA, Einhorn JH, Rogers-Freezer L, White DE: Impact of cytokine administration on the generation of antitumor reactivity in patients with metastatic melanoma receiving a peptide vaccine. *J Immunol* 163: 1690–1695, 1999
12. Bendandi M, Gocke CD, Kobrin CB, Benko FA, Sternas LA, Pennington R, Watson TM, Reynolds CW, Gause BL, Duffey PL, Jaffe ES, Creekmore SP, Longo DL, Kwak LW: Complete molecular remissions induced by patient-specific vaccination plus granulocyte-monocyte colony-stimulating factor against lymphoma. *Nat Med* 5: 1171–1177, 1999
13. Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ, Topalian SL, Sherry R, Restifo NP, Hubicki AM, Robinson MR, Raffeld M, Duray P, Seipp CA, Rogers-Freezer L, Morton KE, Mavroukakis SA, White DE, Rosenberg SA: Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 298: 850–854, 2002
14. Powers AM, Brault AC, Shirako Y, Strauss EG, Kang W, Strauss JH, Weaver SC: Evolutionary relationships and systematics of the alphaviruses. *J Virol* 75: 10118–10131, 2001
15. Strauss JH, Strauss EG: The alphaviruses: gene expression, replication, and evolution. *Microbiol Rev* 58: 491–562, 1994
16. MacDonald GH, Johnston RE: Role of dendritic cell targeting in Venezuelan equine encephalitis virus pathogenesis. *J Virol* 74: 914–922, 2000
17. Hsu KF, Hung CF, Cheng WF, He L, Slater LA, Ling M, Wu TC: Enhancement of suicidal DNA vaccine potency by linking Mycobacterium tuberculosis heat shock protein 70 to an antigen. *Gene Ther* 8: 376–383, 2001
18. Andersson C, Vasconcelos NM, Sievertzon M, Haddad D, Liljeqvist S, Berglund P, Liljestrom P, Ahlberg N, Stahl S, Berzins K: Comparative immunization study using RNA and DNA constructs encoding a part of the Plasmodium falciparum antigen Pf332. *Scand J Immunol* 54: 117–124, 2001
19. DiCiommo DP, Bremner R: Rapid, high level protein production using DNA-based Semliki Forest virus vectors. *J Biol Chem* 273: 18060–18066, 1998
20. Leitner WW, Ying H, Driver DA, Dubensky TW, Restifo NP: Enhancement of tumor-specific immune response with plasmid DNA replicon vectors. *Cancer Res* 60: 51–55, 2000
21. Kohno A, Emi N, Kasai M, Tanimoto M, Saito H: Semliki Forest virus-based DNA expression vector: transient protein production followed by cell death. *Gene Ther* 5: 415–418, 1998
22. Johanning FW, Conry RM, LoBuglio AF, Wright M, Sumerel LA, Pike MJ, Curiel DT: A Sindbis virus mRNA polynucleotide vector achieves prolonged and high level heterologous gene expression in vivo. *Nucleic Acids Res* 23: 1495–1501, 1995
23. Dubensky Jr TW, Driver DA, Polo JM, Belli BA, Latham EM, Ibanez CE, Chada S, Brumm D, Banks TA, Mento SJ, Jolly DJ, Chang SM: Sindbis virus DNA-based expression vectors: utility for *in vitro* and *in vivo* gene transfer. *J Virol* 70: 508–519, 1996
24. Schlesinger S: Alphavirus vectors: development and potential therapeutic applications. *Expert Opin Biol Ther* 1: 177–191, 2001
25. Morris-Downes MM, Phenix KV, Smyth J, Sheahan BJ, Liljeqvist S, Mooney DA, Liljestrom P, Todd D, Atkins GJ: Semliki Forest virus-based vaccines: persistence, distribution and pathological analysis in two animal systems. *Vaccine* 19: 1978–1988, 2001
26. Lundstrom K, Schweutzer C, Richards JG, Ehrenguber MU, Jenck F, Mulhardt C: Semliki Forest virus vectors for *in vitro* and *in vivo* applications. *Gene Ther Mol Biol* 4: 23–31, 1999
27. Velders MP, McElhiney S, Cassetti MC, Eiben GL, Higgins T, Kovacs GR, Elmishad AG, Kast WM, Smith LR: Eradication of established tumors by vaccination with Venezuelan equine encephalitis virus replicon particles delivering human papillomavirus 16 E7 RNA. *Cancer Res* 61: 7861–7867, 2001
28. Lachman LB, Rao XM, Kremer RH, Ozpolat B, Kiriakova G, Price JE: DNA vaccination against neu reduces breast cancer incidence and metastasis in mice. *Cancer Gene Ther* 8: 259–268, 2001

29. Cheng WF, Hung CF, Hsu KF, Chai CY, He L, Polo JM, Slater LA, Ling M, Wu TC: Cancer immunotherapy using Sindbis virus replicon particles encoding a VP22-antigen fusion. *Hum Gene Ther* 13: 553–568, 2002
30. Yamanaka R, Zullo SA, Tanaka R, Blaese M, Xanthopoulos KG: Enhancement of antitumor immune response in glioma models in mice by genetically modified dendritic cells pulsed with Semliki forest virus-mediated complementary DNA. *J Neurosurg* 94: 474–481, 2001
31. Frolov I, Frolova E, Schlesinger S: Sindbis virus replicons and Sindbis virus: assembly of chimeras and of particles deficient in virus RNA. *J Virol* 71: 2819–2829, 1997
32. Geisbert TW, Pushko P, Anderson K, Smith J, Davis KJ, Jahrling PB: Evaluation in nonhuman primates of vaccines against Ebola virus. *Emerg Infect Dis* 8: 503–507, 2002
33. Lee JS, Dyas BK, Nystrom SS, Lind CM, Smith JF, Ulrich RG: Immune protection against staphylococcal enterotoxin-induced toxic shock by vaccination with a Venezuelan equine encephalitis virus replicon. *J Infect Dis* 185: 1192–1196, 2002
34. Balasuriya UB, Heidner HW, Davis NL, Wagner HM, Hullinger PJ, Hedges JF, Williams JC, Johnston RE, David Wilson W, Liu IK, James MacLachlan N: Alphavirus replicon particles expressing the two major envelope proteins of equine arteritis virus induce high level protection against challenge with virulent virus in vaccinated horses. *Vaccine* 20: 1609–1617, 2002
35. Harrington PR, Yount B, Johnston RE, Davis N, Moe C, Baric RS: Systemic, mucosal, and heterotypic immune induction in mice inoculated with Venezuelan equine encephalitis replicons expressing Norwalk virus-like particles. *J Virol* 76: 730–742, 2002
36. Wilson JA, Hart MK: Protection from Ebola virus mediated by cytotoxic T lymphocytes specific for the viral nucleoprotein. *J Virol* 75: 2660–2664, 2001
37. Schultz-Cherry S, Dybing JK, Davis NL, Williamson C, Suarez DL, Johnston R, Perdue ML: Influenza virus (A/HK/156/97) hemagglutinin expressed by an alphavirus replicon system protects chickens against lethal infection with Hong Kong-origin H5N1 viruses. *Virology* 278: 55–59, 2000
38. Pushko P, Bray M, Ludwig GV, Parker M, Schmaljohn A, Sanchez A, Jahrling PB, Smith JF: Recombinant RNA replicons derived from attenuated Venezuelan equine encephalitis virus protect guinea pigs and mice from Ebola hemorrhagic fever virus. *Vaccine* 19: 142–153, 2000
39. Davis NL, Caley IJ, Brown KW, Betts MR, Irlbeck DM, McGrath KM, Connell MJ, Montefiori DC, Frelinger JA, Swanstrom R, Johnson PR, Johnston RE: Vaccination of macaques against pathogenic simian immunodeficiency virus with Venezuelan equine encephalitis virus replicon particles. *J Virol* 74: 371–378, 2000
40. Hevey M, Negley D, Pushko P, Smith J, Schmaljohn A: Marburg virus vaccines based upon alphavirus replicons protect guinea pigs and nonhuman primates. *Virology* 251: 28–37, 1998
41. Pushko P, Parker M, Ludwig GV, Davis NL, Johnston RE, Smith JF: Replicon-helper systems from attenuated Venezuelan equine encephalitis virus: expression of heterologous genes *in vitro* and immunization against heterologous pathogens *in vivo*. *Virology* 239: 389–401, 1997
42. Attia MA, DeOme KB, Weiss DW: Immunology of spontaneous mammary carcinomas in mice II. Resistance to a rapidly and a slowly developing tumor. *Cancer Res* 25: 451–457, 1965
43. Foy TM, Bannink J, Sutherland RA, McNeill PD, Moulton GG, Smith J, Cheever MA, Grabstein K: Vaccination with Her-2/neu DNA or protein subunits protects against growth of a Her-2/neu-expressing murine tumor. *Vaccine* 19: 2598–2606, 2001
44. Pilon SA, Piechocki MP, Wei WZ: Vaccination with cytoplasmic ErbB-2 DNA protects mice from mammary tumor growth without anti-ErbB-2 antibody. *J Immunol* 167: 3201–3206, 2001
45. Piechocki MP, Pilon SA, Wei WZ: Complementary antitumor immunity induced by plasmid DNA encoding secreted and cytoplasmic human ErbB-2. *J Immunol* 167: 3367–3374, 2001
46. Mukai K, Yasutomi Y, Watanabe M, Kenjo A, Aota T, Wang L, Nishikawa H, Ishihara M, Fujita T, Kuribayashi K, Shiku H: HER2 peptide-specific CD8(+) T cells are proportionally detectable long after multiple DNA vaccinations. *Gene Ther* 9: 879–888, 2002
47. Reilly RT, Gottlieb MB, Ercolini AM, Machiels JP, Kane CE, Okoye FI, Muller WJ, Dixon KH, Jaffee EM: HER-2/neu is a tumor rejection target in tolerized HER-2/neu transgenic mice. *Cancer Res* 60: 3569–3576, 2000
48. Weinstein EJ, Kitsberg DI, Leder P: A mouse model for breast cancer induced by amplification and overexpression of the neu promoter and transgene. *Mol Med* 6: 4–16, 2000
49. Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ: Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc Natl Acad Sci USA* 89: 10578–10582, 1992
50. Bouchard L, Lamarre L, Tremblay PJ, Jolicoeur P: Stochastic appearance of mammary tumors in transgenic mice carrying the MMTV/c-neu oncogene. *Cell* 57: 931–936, 1989
51. Muller WJ, Sinn E, Pattengale PK, Wallace R, Leder P: Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell* 54: 105–115, 1988
52. Nanni P, Nicoletti G, De Giovanni C, Landuzzi L, Di Carlo E, Cavallo F, Pupa SM, Rossi I, Colombo MP, Ricci C, Astolfi A, Musiani P, Forni G, Lollini PL: Combined allogeneic tumor cell vaccination and systemic interleukin 12 prevents mammary carcinogenesis in HER-2/neu transgenic mice. *J Exp Med* 194: 1195–1205, 2001
53. Pupa SM, Invernizzi AM, Forti S, Di Carlo E, Musiani P, Nanni P, Lollini PL, Meazza R, Ferrini S, Menard S: Prevention of spontaneous neu-expressing mammary tumor development in mice transgenic for rat proto-neu by DNA vaccination. *Gene Ther* 8: 75–79, 2001
54. Machiels JP, Reilly RT, Emens LA, Ercolini AM, Lei RY, Weintraub D, Okoye FI, Jaffee EM: Cyclophosphamide, doxorubicin, and paclitaxel enhance the antitumor immune response of granulocyte/macrophage-colony stimulating factor-secreting whole-cell vaccines in HER-2/neu tolerized mice. *Cancer Res* 61: 3689–3697, 2001
55. Rovero S, Boggio K, Carlo ED, Amici A, Quaglini E, Porcedda P, Musiani P, Forni G: Insertion of the DNA for the 163–171 peptide of IL1 β enables a DNA vaccine encoding p185(neu) to inhibit mammary carcinogenesis in Her-2/neu transgenic BALB/c mice. *Gene Ther* 8: 447–452, 2001

56. Di Carlo E, Rovero S, Boggio K, Quaglino E, Amici A, Smorlesi A, Forni G, Musiani P: Inhibition of mammary carcinogenesis by systemic interleukin 12 or p185neu DNA vaccination in Her-2/neu transgenic BALB/c mice. *Clin Cancer Res* 7: 830s–837s, 2001
57. Reilly RT, Machiels JP, Emens LA, Ercolini AM, Okoye FI, Lei RY, Weintraub D, Jaffee EM: The collaboration of both humoral and cellular HER-2/neu-targeted immune responses is required for the complete eradication of HER-2/neu-expressing tumors. *Cancer Res* 61: 880–883, 2001
58. Yamamoto T, Ikawa S, Akiyama T, Semba K, Nomura N, Miyajima N, Saito T, Toyoshima K: Similarity of protein encoded by the human c-erbB-2 gene to epidermal growth factor receptor. *Nature* 319: 230–234, 1986
59. Bargmann CI, Hung MC, Weinberg RA: The neu oncogene encodes an epidermal growth factor receptor-related protein. *Nature* 319: 226–230, 1986
60. Agus DB, Bunn Jr PA, Franklin W, Garcia M, Ozols RF: HER-2/neu as a therapeutic target in non-small cell lung cancer, prostate cancer, and ovarian cancer. *Semin Oncol* 27: 53–63; discussion 92–100, 2000
61. Olayioye MA, Neve RM, Lane HA, Hynes NE: The ErbB signaling network: receptor heterodimerization in development and cancer. *Embo J* 19: 3159–3167, 2000
62. Hung MC, Lau YK: Basic science of HER-2/neu: a review. *Semin Oncol* 26: 51–59, 1999
63. Lofts FJ, Gullick WJ: c-erbB2 amplification and overexpression in human tumors. *Cancer Treat Res* 61: 161–179, 1992
64. Press MF, Jones LA, Godolphin W, Edwards CL, Slamon DJ: HER-2/neu oncogene amplification and expression in breast and ovarian cancers. *Prog Clin Biol Res* 354A: 209–221, 1990
65. Kawashima I, Tsai V, Southwood S, Takesako K, Sette A, Celis E: Identification of HLA-A3-restricted cytotoxic T lymphocyte epitopes from carcinoembryonic antigen and HER-2/neu by primary *in vitro* immunization with peptide-pulsed dendritic cells. *Cancer Res* 59: 431–435, 1999
66. Castilleja A, Carter D, Efferson CL, Ward NE, Kawano K, Fisk B, Kudelka AP, Gershenson DM, Murray JL, O'Brian CA, Ioannides CG: Induction of tumor-reactive CTL by C-side chain variants of the CTL epitope HER-2/neu protooncogene (369–377) selected by molecular modeling of the peptide: HLA-A2 complex. *J Immunol* 169: 3545–3554, 2002
67. Disis ML, Schiffman K, Gooley TA, McNeel DG, Rinn K, Knutson KL: Delayed-type hypersensitivity response is a predictor of peripheral blood T-cell immunity after HER-2/neu peptide immunization. *Clin Cancer Res* 6: 1347–1350, 2000
68. Disis ML, Gooley TA, Rinn K, Davis D, Piepkorn M, Cheever MA, Knutson KL, Schiffman K: Generation of T-cell immunity to the HER-2/neu protein after active immunization with HER-2/neu peptide-based vaccines. *J Clin Oncol* 20: 2624–2632, 2002
69. Keogh E, Fikes J, Southwood S, Celis E, Chesnut R, Sette A: Identification of new epitopes from four different tumor-associated antigens: recognition of naturally processed epitopes correlates with HLA-A*0201-binding affinity. *J Immunol* 167: 787–796, 2001
70. Kobayashi H, Kennedy R, Lu J, Davila E, Celis E: MHC-binding peptides as immunotherapeutics for cancer. *Immunol Invest* 29: 105–110, 2000
71. Lee TV, Johnston DA, Thomakos N, Honda T, Efferson CL, Ioannides CG: Helper peptide G89 (HER-2: 777–789) and G89-activated cells regulate the survival of effectors induced by the CTL epitope E75 (HER-2, 369–377). Correlation with the IFN-gamma: IL-10 balance. *Anticancer Res* 22: 1481–1490, 2002
72. Peiper M, Goedegebuure PS, Linehan DC, Ganguly E, Douville CC, Eberlein TJ: The HER2/neu-derived peptide p654–662 is a tumor-associated antigen in human pancreatic cancer recognized by cytotoxic T lymphocytes. *Eur J Immunol* 27: 1115–1123, 1997
73. Peoples GE, Smith RC, Linehan DC, Yoshino I, Goedegebuure PS, Eberlein TJ: Shared T cell epitopes in epithelial tumors. *Cell Immunol* 164: 279–286, 1995
74. Perez SA, Sotiropoulou PA, Sotiriadou NN, Mamalaki A, Gritzapis AD, Echner H, Voelter W, Pawelec G, Papamichail M, Baxevanis CN: HER-2/neu-derived peptide 884–899 is expressed by human breast, colorectal and pancreatic adenocarcinomas and is recognized by *in vitro*-induced specific CD4(+) T cell clones. *Cancer Immunol Immunother* 50: 615–624, 2002
75. Rongcun Y, Salazar-Onfray F, Charo J, Malmberg KJ, Evrin K, Maes H, Kono K, Hising C, Petersson M, Larsson O, Lan L, Appella E, Sette A, Celis E, Kiessling R: Identification of new HER2/neu-derived peptide epitopes that can elicit specific CTL against autologous and allogeneic carcinomas and melanomas. *J Immunol* 163: 1037–1044, 1999
76. Scardino A, Gross DA, Alves P, Schultze JL, Graff-Dubois S, Faure O, Tourdot S, Chouaib S, Nadler LM, Lemonnier FA, Vonderheide RH, Cardoso AA, Kosmatopoulos K: HER-2/neu and hTERT cryptic epitopes as novel targets for broad spectrum tumor immunotherapy. *J Immunol* 168: 5900–5906, 2002
77. Sotiriadou R, Perez SA, Gritzapis AD, Sotiropoulou PA, Echner H, Heinzel S, Mamalaki A, Pawelec G, Voelter W, Baxevanis CN, Papamichail M: Peptide HER2(776–788) represents a naturally processed broad MHC class II-restricted T cell epitope. *Br J Cancer* 85: 1527–1534, 2001
78. Disis ML, Pupa SM, Gralow JR, Dittadi R, Menard S, Cheever MA: High-titer HER-2/neu protein-specific antibody can be detected in patients with early-stage breast cancer. *J Clin Oncol* 15: 3363–3367, 1997
79. Disis ML, Calenoff E, McLaughlin G, Murphy AE, Chen W, Groner B, Jeschke M, Lydon N, McGlynn E, Livingston RB et al.: Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. *Cancer Res* 54: 16–20, 1994
80. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, Norton L: Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 344: 783–792, 2001
81. Shiku H, Wang L, Ikuta Y, Okugawa T, Schmitt M, Gu X, Akiyoshi K, Sunamoto J, Nakamura H: Development of a cancer vaccine: peptides, proteins, and DNA. *Cancer Chemother Pharmacol* 46(Suppl): S77–S82, 2000
82. Wei WZ, Shi WP, Galy A, Lichlyter D, Hernandez S, Groner B, Heilbrun L, Jones RF: Protection against mammary tumor growth by vaccination with full-length, modified human ErbB-2 DNA. *Int J Cancer* 81: 748–754, 1999

83. Rovero S, Amici A, Carlo ED, Bei R, Nanni P, Quaglino E, Porcedda P, Boggio K, Smorlesi A, Lollini PL, Landuzzi L, Colombo MP, Giovarelli M, Musiani P, Forni G: DNA vaccination against rat her-2/Neu p185 more effectively inhibits carcinogenesis than transplantable carcinomas in transgenic BALB/c mice. *J Immunol* 165: 5133–5142, 2000
84. Pietersz GA, Apostolopoulos V, McKenzie IF: Generation of cellular immune responses to antigenic tumor peptides. *Cell Mol Life Sci* 57: 290–310, 2000
85. Jager E, Jager D, Knuth A: Strategies for the development of vaccines to treat breast cancer. *Recent Results Cancer Res* 152: 94–102, 1998
86. Esserman LJ, Lopez T, Montes R, Bald LN, Fendly BM, Campbell MJ: Vaccination with the extracellular domain of p185neu prevents mammary tumor development in neu transgenic mice. *Cancer Immunol Immunother* 47: 337–342, 1999
87. Disis ML, Cheever MA: HER-2/neu protein: a target for antigen-specific immunotherapy of human cancer. *Adv Cancer Res* 71: 343–371, 1997
88. Dakappagari NK, Douglas DB, Triozzi PL, Stevens VC, Kaumaya PT: Prevention of mammary tumors with a chimeric HER-2 B-cell epitope peptide vaccine. *Cancer Res* 60: 3782–3789, 2000
89. Amici A, Smorlesi A, Noce G, Santoni G, Cappelletti P, Capparuccia L, Coppari R, Lucciarini R, Petrelli C, Provinciali M: DNA vaccination with full-length or truncated neu induces protective immunity against the development of spontaneous mammary tumors in HER-2/neu transgenic mice. *Gene Ther* 7: 703–706, 2000
90. Amici A, Venanzi FM, Concetti A: Genetic immunization against neu/erbB2 transgenic breast cancer. *Cancer Immunol Immunother* 47: 183–190, 1998
91. Chen SA, Tsai MH, Wu FT, Hsiang A, Chen YL, Lei HY, Tzai TS, Leung HW, Jin YT, Hsieh CL, Hwang LH, Lai MD: Induction of antitumor immunity with combination of HER2/neu DNA vaccine and interleukin 2 gene-modified tumor vaccine. *Clin Cancer Res* 6: 4381–4388, 2000
92. Mittelman A, Lucchese A, Sinha AA, Kanduc D: Monoclonal and polyclonal humoral immune response to EC HER-2/NEU peptides with low similarity to the host's proteome. *Int J Cancer* 98: 741–747, 2002
93. Ozcelik C, Erdmann B, Pilz B, Wettschureck N, Britsch S, Hubner N, Chien KR, Birchmeier C, Garratt AN: Conditional mutation of the ErbB2 (HER2) receptor in cardiomyocytes leads to dilated cardiomyopathy. *Proc Natl Acad Sci USA* 99: 8880–8885, 2002
94. Crone SA, Zhao YY, Fan L, Gu Y, Minamisawa S, Liu Y, Peterson KL, Chen J, Kahn R, Condorelli G, Ross Jr J, Chien KR, Lee KF: ErbB2 is essential in the prevention of dilated cardiomyopathy. *Nat Med* 8: 459–465, 2002
95. Tait BD: HLA class I expression on human cancer cells. Implications for effective immunotherapy. *Hum Immunol* 61: 158–165, 2000
96. Liljestrom P, Garoff H: A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. *Biotechnology (NY)* 9: 1356–1361, 1991
97. Allsopp TE, Scallan MF, Williams A, Fazakerley JK: Virus infection induces neuronal apoptosis: a comparison with trophic factor withdrawal. *Cell Death Differ* 5: 50–59, 1998
98. Fazakerley JK, Allsopp TE: Programmed cell death in virus infections of the nervous system. *Curr Top Microbiol Immunol* 253: 95–119, 2001
99. Glasgow GM, McGee MM, Sheahan BJ, Atkins GJ: Death mechanisms in cultured cells infected by Semliki Forest virus. *J Gen Virol* 78: 1559–1563, 1997
100. Grandgirard D, Studer E, Monney L, Belser T, Fellay I, Borner C, Michel MR: Alphaviruses induce apoptosis in Bcl-2-overexpressing cells: evidence for a caspase-mediated, proteolytic inactivation of Bcl-2. *Embo J* 17: 1268–1278, 1998
101. Griffin DE: The Gordon Wilson lecture: unique interactions between viruses, neurons and the immune system. *Trans Am Clin Climatol Assoc* 107: 89–98, 1995
102. Hardy PA, Mazzini MJ, Schweitzer C, Lundstrom K, Glode LM: Recombinant Semliki forest virus infects and kills human prostate cancer cell lines and prostatic duct epithelial cells *ex vivo*. *Int J Mol Med* 5: 241–245, 2000
103. Jackson AC, Rossiter JP: Apoptotic cell death is an important cause of neuronal injury in experimental Venezuelan equine encephalitis virus infection of mice. *Acta Neuropathol (Berl)* 93: 349–353, 1997
104. Jan JT, Griffin DE: Induction of apoptosis by Sindbis virus occurs at cell entry and does not require virus replication. *J Virol* 73: 10296–10302, 1999
105. Jan JT, Chatterjee S, Griffin DE: Sindbis virus entry into cells triggers apoptosis by activating sphingomyelinase, leading to the release of ceramide. *J Virol* 74: 6425–6432, 2000
106. Karpf AR, Brown DT: Comparison of Sindbis virus-induced pathology in mosquito and vertebrate cell cultures. *Virology* 240: 193–201, 1998
107. Lundstrom K: Alphavirus vectors for gene therapy applications. *Curr Gene Ther* 1: 19–29, 2001
108. Mastrangelo AJ, Zou S, Hardwick JM, Betenbaugh MJ: Antiapoptosis chemicals prolong productive lifetimes of mammalian cells upon Sindbis virus vector infection. *Biotechnol Bioeng* 65: 298–305, 1999
109. Murphy AM, Morris-Downes MM, Sheahan BJ, Atkins GJ: Inhibition of human lung carcinoma cell growth by apoptosis induction using Semliki Forest virus recombinant particles. *Gene Ther* 7: 1477–1482, 2000
110. Murphy AM, Sheahan BJ, Atkins GJ: Induction of apoptosis in BCL-2-expressing rat prostate cancer cells using the Semliki Forest virus vector. *Int J Cancer* 94: 572–578, 2001
111. Nargi-Aizenman JL, Griffin DE: Sindbis virus-induced neuronal death is both necrotic and apoptotic and is ameliorated by *N*-methyl-D-aspartate receptor antagonists. *J Virol* 75: 7114–7121, 2001
112. Nava VE, Rosen A, Veluona MA, Clem RJ, Levine B, Hardwick JM: Sindbis virus induces apoptosis through a caspase-dependent, CrmA-sensitive pathway. *J Virol* 72: 452–459, 1998
113. Rosen A, Casciola-Rosen L, Ahearn J: Novel packages of viral and self-antigens are generated during apoptosis. *J Exp Med* 181: 1557–1561, 1995
114. Zrachia A, Dobroslav M, Blass M, Kazimirsky G, Kronfeld I, Blumberg PM, Kobiler D, Lustig S, Brodie C: Infection of glioma cells with Sindbis virus induces selective activation and tyrosine phosphorylation of protein kinase C delta. Implications for Sindbis virus-induced apoptosis. *J Biol Chem* 277: 23693–23701, 2002

115. Gardner JP, Donovan G, Morrissey D, Caley I, Durso RJ, Cohen M, Arrigale RR, Zhan C, Israel RJ, WC O: A novel alphavirus replicon vaccine encoding PSMA for immunotherapy of prostate cancer. In: 93rd Annual Meeting, American Association for Cancer Research, San Francisco, CA, 2002, pp 3017
116. Donovan GP, Gardner JP, Morrissey DM, Schulke N, Zhan C, Durso RJ, Arrigale RR, Varlamova O, Scalzo TM, Chodera AJ, Heston WD, WC O: Clinical development of immunotherapies targeting prostate specific membrane an-

tigen (PSMA). In: Thirty-eighth Annual Meeting American Society of Clinical Oncology, Orlando, FL, 2002, pp 25

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