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Breast cancer vaccines delivered by dendritic cell-targeted lentivectors induce potent antitumor immune responses and protect mice from mammary tumor growth

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Abstract

Breast cancer immunotherapy is a potent treatment option, with antibody therapies such as trastuzumab increasing 2-year survival rates by 50%. However, active immunotherapy through vaccination has generally been clinically ineffective. One potential means of improving vaccine therapy is by delivering breast cancer antigens to dendritic cells (DCs) for enhanced antigen presentation. To accomplish this in vivo, we pseudotyped lentiviral vector (LV) vaccines with a modified Sindbis Virus glycoprotein so that they could deliver genes encoding the breast cancer antigen alpha-lactalbumin (Lalba) or erb-b2 receptor tyrosine kinase 2 (ERBB2 or HER2) directly to resident DCs. We hypothesized that utilizing these DC-targeting lentiviral vectors as a breast cancer vaccine could lead to an improved immune response against self-antigens found in breast cancer tumors. Indeed, single injections of the vaccine vectors were able to amplify antigen-specific CD8 T cells 4–6 fold over naïve mice, similar to the best published vaccine regimens. Immunization of these mice completely inhibited tumor growth in a foreign antigen environment (LV-ERBB2 in wildtype mice), and it reduced the rate of tumor growth in a self-antigen environment (LV-Lalba in wildtype or LV-ERBB2 in MMTV-huHER2 transgenic). These results

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Ethical standards

All procedures were performed according to the guidelines established in the *Guide for the Care and Use of Laboratory Animals*[24], and the protocols were approved by the USC IACUC (2011-11623, 2011-11683). The manuscript does not contain clinical studies or patient data.

Conflict of interest

The authors declare that they have no conflict of interest.

show that a single injection with targeted lentiviral vectors can be an effective immunotherapy for breast cancer. Furthermore, they could be combined with other immunotherapeutic regimens to improve outcomes for patients with breast cancer.

Graphical abstract

Keywords

Cancer vaccine; lentiviral vector; breast cancer immunotherapy; ERBB2/Her2; alpha-lactalbumin

1. Introduction

Breast cancer is the most commonly diagnosed cancer in women, and causes 40,000 deaths per year in the United States [1]. One treatment option for these patients is administration of trastuzumab [2], an antibody that targets the ErbB2 protein (also known as Her2), which is a protein tyrosine kinase of the epidermal growth factor receptor family. Treatment with trastuzumab has reduced death rates by 50% in patients with ErbB2+ breast cancer, and has improved 5-year survival rates to the equivalent of those observed for patients with ErbB2– breast cancer [3]. The success of this passive immunotherapy has inspired multiple approaches towards developing an active immunotherapy treatment, in which patients' immune systems are stimulated to develop their own immune response to the tumor.

Typically, active immunotherapies have involved vaccination with one or more breast cancer-specific antigens. Antigens can be delivered in many different ways, including peptides, recombinant viral vectors, whole tumor cells or lysates, plasmid DNA, or directly stimulated DCs [4]. To date, these attempts have generally shown limited clinical benefit [5]. One common exception to this pattern is the success of vaccines that utilize DCs to present the target antigen. DCs are the most powerful antigen-presenting cell, and directly including them in any vaccination regimen is likely to create a stronger immune response [6].

Lentiviral vectors (LV) are a versatile tool for gene delivery. Their advantages include efficient transduction of non-dividing cells, low natural anti-vector immunity, and a low potential for genotoxicity due to insertional mutagenesis. More importantly, they can be pseudotyped with many different glycoproteins, which facilitates the direct targeting of these vectors to a specific cell type [7–9]. Previous work in our laboratory has shown that LV pseudotyped with a modified Sindbis glycoprotein can directly target DC-SIGN on the surface of DCs [10]. This targeting was able to elicit a stronger immune response than a non-targeted vector [11]. Here, we sought to utilize LV to deliver breast cancer antigens to DCs in order to stimulate the host's immune response against a tumor.

A critical element in the development of an effective cancer vaccine is the selection of target antigen. Criteria that define a good target include: (1) presence in the tumor, (2) absence in healthy tissues, (3) immunogenicity, (4) oncogenic potential, (5) evidence for therapeutic efficacy, (6) known epitopes, and (7) existing models for studying vaccine-specific effects [12]. Previous vaccines in breast cancer therapy have targeted Lalba, MUC1, Mammaglobin-A, ErbB2, and NY-BR-1, among others [13–17]. The most advanced have proceeded to Phase II clinical trials [18–20]. In this work, we chose to investigate ErbB2 and Lalba as two potential targets.

As described above, ErbB2 has been a common target of passive immunotherapy. This protein is a membrane-bound receptor tyrosine kinase and is overexpressed in 20 – 30% of human breast cancers [3]. However, it is expressed at low-to-medium levels in only a few healthy tissues. Lalba, on the other hand, is expressed in no healthy tissues except during lactation, while it is expressed at high levels in a subset of human breast cancers, especially triple negative breast cancer [21]. ErbB2's immunogenicity in mice has been described, and its epitopes are known, while epitopes for Lalba are unknown.

In this study, we have identified Lalba and ERBB2 as two promising candidates for therapeutic vaccination. We engineered each of these two genes into a DC-targeted LV and utilized these vectors for immunization. Mice that were immunized with these LV could generate a T cell immune response to the target antigen. A single immunization was sufficient to prevent or slow the development of tumors in both transplanted and spontaneous mouse models of breast cancer.

2. Materials and Methods

2.1 Plasmids and mice

Lentiviral vectors were created using the FUW LV backbone, which was originally engineered to generate high-titer vectors that can transduce a variety of cell types [22]. LV-Lalba was created by PCR-amplifying the entire mouse Lalba coding sequence (Openbiosystems cDNA, accession #BC069916) and cloning it into FUW using the BamHI and EcoRI restriction sites. LV-ERBB2_ECD was created by PCR-amplifying the extracellular region (amino acids 1–652) of the ERBB2 coding sequence (Genecopoeia #HOC20199, accession #BC156755) and cloning the product into FUW at the HpaI and AscI restriction sites. LV-ERBB2-FL-ki was created by first PCR-amplifying the full-length ERBB2 coding sequence and cloning it into FUW at the HpaI and AscI restriction sites, followed by site-directed mutagenesis of K753A (Quikchange II, Agilent Technologies). LV-GFP was previously published as FUGW [10].

Balb/cJ mice were purchased from Jackson laboratories. MMTV-huHER2 transgenic mice [23] were obtained from Genentech (strain FVB/Tg.MMTV.f.huHER2 #5, South San Francisco, CA). All mice were maintained in the animal facilities at the University of Southern California (USC) under controlled temperature and a 12 h light/dark cycle, with free access to water and standard laboratory chow. All procedures were performed according to the guidelines established in the *Guide for the Care and Use of Laboratory Animals* [24], and the protocols were approved by the USC IACUC (2011-11623, 2011-11683).

2.2 Production of lentiviral vectors

LV were produced as previously described [25]. Briefly, 293T cells were transiently transfected with a standard calcium phosphate precipitation method. Tissue culture plates (15 cm) were transfected with 37.5 ug of transfer plasmid (LV-Lalba or LV-ERBB2_ECD) along with 18.75 ug of packaging plasmids REV, RRE, and SVGmu. Supernatants were collected at 48 and 72 hours post-transfection, and they were concentrated by ultracentrifugation at $80,000 \times g$ for 90 minutes. Pellets were resuspended in HBSS and titrated on 293T.DC-SIGN cells.

2.3 Construction of 4T1.Lalba and 4T1.ERBB2 cell lines

The 4T1 cell line was purchased from ATCC (#CRL-2539). Antigen positive cell lines (4T1.Lalba or 4T1.ERBB2) were generated by LV transduction of 4T1 cells [26] with either LV-Lalba or LV-ERBB2-FL-ki at a MOI of 10, followed by limiting dilution cloning. Clones were tested for antigen expression by RT-qPCR and/or flow cytometry staining. Primers for qPCR were [forward: TGAGGAAGGTGAAGGTGCTTGGAT, reverse: AGCCATAGGGCATAAGCTGTGTCA] for ERBB2 and [forward: TGGCTATCAAGGCATCTCTTTG, reverse: TCGGGGAAGTCACTACTTTTACA] for Lalba. Staining of ErbB2 was measured using an in-house fluorescein-conjugated peptide targeting Her2 (a generous gift of S. Fiocco), incubated at 6 μM for 1 hour at 37 °C.

2.4 Tumor antigen expression analysis

Oncomine™ (Compendia Bioscience™, part of Life Technologies™, Ann Arbor, MI) was used for analysis and visualization. Outlier analysis at the 75th – 95th percentile was performed for 25 independent data sets, and the gene rank for overexpression at the given percentile was determined.

2.5 Immunization and tumor challenge

LV immunization was performed as previously described [27]. Briefly, 1×10^7 TU of concentrated LV stock was injected subcutaneously into the footpad of 8-week-old mice. For intracellular cytokine staining, spleens were harvested at 14 days post-immunization. For tumor challenge, immunizations were administered both prophylactically (14 days prior to tumor challenge) and therapeutically (6 days after challenge).

Tumor challenge was performed by implanting 5×10^5 4T1.ERBB2 or 4T1.Lalba cells orthotopically in mouse mammary glands. Tumor size was measured every 2–3 days with fine-toothed calipers. Tumor volume was calculated as $length \times width \times height / 2$. Mice were euthanized when tumors reached 1500 mm³ or became ulcerated.

2.6 Intracellular cytokine staining

Splenocytes (1×10^6) were cultured with an ErbB2 peptide TYLPTNASL [28] (Genscript) at 10 $\mu g/mL$, for 6 hours in the following solution: RPMI media, 10% FBS, 10 U/mL penicillin, 100 ug/mL streptomycin, 2 mM L-glutamine, 0.67 uL/mL GolgiPlug™ (BD Biosciences #555029). Cells were washed and stained with antibodies to mouse CD4 and CD8 (Biolegend #130312, #100706). Then they were permeabilized and stained with

antibodies to mouse IFN γ (BD Biosciences #554412). Flow cytometry was performed on a MACSQuant Analyzer (Miltenyi Biotec). Analysis of expression was performed using FlowJo software.

2.7 Quantitative RT-PCR

Tumors were excised and dissociated in 0.2% collagenase. RNA was extracted with Trizol (Life technologies) according to the manufacturer's instructions. Two-step qRT-PCR was performed with the High Capacity cDNA Reverse Transcription kit and the Power SYBR® Green PCR Master Mix (Applied Biosystems). Human ERBB2 expression was normalized to mouse GAPDH expression using the formula, $2^{-(ERBB2\text{ Ct} - GAPDH\text{ Ct})}$. Primers for ERBB2 were AACCAAGAGGTGACAGCAGAG and TATTGGCACTGGTAACTGCCC. Primers for GAPDH were GCCTTCCGTGTTCTACC and CCTCAGTGTAGCCCAAGATG.

2.8 Statistical Analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software). Two sample comparisons were assessed with the Student's *t* test. Survival analyses were performed using the log-rank test. Transplanted tumor growth curves were compared with the two-way ANOVA test.

2.9 IFN- γ Enzyme-linked immunospot assay (ELISpot)

ELISpot was conducted as previously described [27]. Briefly, splenocytes from LV-Lalba- or LV-GFP-immunized mice were plated at 2×10^5 cells/well in 150 μ l complete medium + 10 μ g/ml cell lysate from 4T1.Lalba cells. After a 24-hour incubation at 37 °C, IFN- γ spots were developed as recommended by the manufacturer (BD Biosciences) and quantified by a Zeiss ELISPOT reader.

Results and Discussion

3.1 Identification and design of lentiviral vectors targeting breast cancer antigens

Our first goal in designing breast cancer-specific LV was to select antigens that would be the most promising candidates for a vaccine-mediated immunotherapy. We combined a review of the literature with a meta-analysis using OncoPrint software (Life Technologies) to select two antigens most suited to our goals. Both these antigens are highly expressed in subsets of human cancers (Fig. 1A). ErbB2 has been extensively studied, and multiple forms of vaccines have been previously published. Lalba is less well characterized, but one strong advantage for this protein is that it is not expressed in healthy tissues outside of lactation, and thus is an ideal candidate for post-menopausal prophylactic vaccination [14].

Each of these two genes was cloned into the FUW LV backbone (Fig. 1C) [22]. The entire mouse Lalba coding sequence was inserted into the LV. In the case of ErbB2, only the extracellular domain of ErbB2 was inserted into the vector, as the dominant epitopes previously identified in mice and humans fall within this region [28,29]. LV particles were produced by transfection into 293T cells according to standard protocols, followed by

ultracentrifugation. These particles were pseudotyped with codon-optimized SVGmu [10] to enable their targeting to dendritic cells.

3.2 LV immunization can generate a strong immune response to ErbB2 in a foreign- and self-antigen environment

Having produced high-titer LV, we first sought to measure whether immunization with these vectors could generate an antigen-specific immune response. We performed a single immunization of Balb/cJ mice with 1×10^7 TU of LV-ERBB2 and harvested splenocytes two weeks after immunization. By coculturing bulk splenocytes with a peptide of a known ErbB2 H-2 K^d epitope [28], we were able to identify the percentage of CD8⁺ T cells that were specific for ErbB2 (Fig. 2A and 2B). Mice immunized with LV-ERBB2 generated a marked amount of ErbB2-specific CD8⁺ T cells as compared with mice immunized with a LV-GFP control LV (1.68% vs. 0.25%, $p < 0.001$, t test). This compares favorably to previously published DNA, vector and DC vaccines, which have yielded 3–7-fold increases in tumor-specific CD8⁺ T cells with more complicated immunization regimens [28,30–32].

Such a clear difference was not unexpected, given that the antigen being delivered by the LV is a human antigen, not a mouse antigen. In order to more closely model a “self” tumor antigen setting, we performed the same experiment in mice that were transgenic for human ErbB2. We used hybrid mice from a cross between MMTV-huHER2 transgenic and wild-type BALB/cJ mice. In these offspring, constant expression of human ErbB2 ensures that this antigen will be recognized as a self-antigen, while CD8 T cells are able to recognize the H-2 K^d epitope of our ErbB2 peptide. Again, we found that mice immunized with the LV-ERBB2 vaccine generated more ErbB2-specific CD8 T cells than LV-GFP-immunized mice (Fig. 2, 0.66 vs. 0.16%, $p < 0.01$, t test). Thus, a single immunization produced a significant T cell immune response in a self-antigen environment.

Without a known epitope for Lalba in mice, it was more difficult to directly assay specific immune responses induced by LV immunization. Using an ELISpot assay with T cells cocultured with cell lysate from Lalba-overexpressing 4T1 cells, we were able to identify a modest increase in the number of IFN- γ T cells in mice immunized with LV-Lalba vs. those immunized with the control (Fig. S1, 27.8 vs. 5.3 SFC, $p < 0.01$, t test).

3.3 LV immunization can inhibit the growth of transplanted tumors

Having determined that a single injection of LV could generate a strong T cell immune response, we hypothesized that such a response would be sufficient to prevent or slow the growth of antigen-positive tumors. We tested this hypothesis using the mouse mammary tumor cell line, 4T1, a well-established model of human breast cancer [33]. We transduced parental 4T1 cells with LV encoding either full-length Lalba or a kinase-deficient version of human ERBB2 [28], followed by clonal isolation via limited dilution. These cell lines demonstrated high levels of antigen expression, as assessed by flow cytometry and RT-PCR (Fig. S2). Notably, the 4T1.ERBB2 cell line demonstrated slower growth kinetics with a higher degree of variation than the 4T1 and 4T1.Lalba cell lines (Fig. S3).

Tumor challenge of immunized subjects was performed in Balb/cJ mice, with vaccines administered either prophylactically or therapeutically. For mice challenged with

4T1.ERBB2, LV-ERBB2 immunization was effective in both a prophylactic and therapeutic setting (Fig. 3A and 3B). None of the mice prophylactically immunized with LV-ERBB2 developed tumors, whereas all control mice developed palpable tumors ($p < 0.01$, ANOVA). Therapeutic immunization was also effective, as LV-ERBB2 immunization extended tumor doubling time by 1.6-fold (9.3 vs 14.8 days, $p < 0.05$, ANOVA), and a smaller mean tumor size was detectable by day 42 after inoculation ($p < 0.01$, t test). We note that several of the control mice displayed reduced tumor growth, including one that controlled tumor growth completely. These mice may be able to control the tumor because these tumors express human ErbB2, a foreign antigen, and as shown in Fig. 2, naïve Balb/cJ mice can develop an immune response to the human antigen.

For mice challenged with 4T1.Lalba, LV-Lalba immunization was effective when administered prophylactically (Fig. 3C and 3D). With both immunizations, tumors grew in all mice; however, the rate of growth in LV-Lalba mice was reduced, with a smaller mean tumor size detectable at day 23 after inoculation ($p < 0.01$, t test). These mice survived 35% longer than the control group (35 vs. 26 days, $p < 0.02$, log-rank test), demonstrating that the antigen-specific immunization was able to delay tumor growth.

3.4 LV immunization can prevent the development of spontaneous ErbB2+ tumors

The above transplantation experiments show that LV immunization can prevent or slow the growth of tumors in a transient model. To better model tumor development in humans, we chose to use MMTV-huHER2 transgenic mice as a model for spontaneous tumor growth. As originally reported by Finkle et al. [23], these mice express high levels of human ErbB2 in mammary glands and other tissues. A majority of females (76%) develop spontaneous mammary gland adenocarcinomas, starting at 23 weeks of age, but these carcinomas can be prevented by passive immunotherapy with anti-ErbB2 antibodies. Here, we sought to test the hypothesis that targeted LV immunization with LV-ERBB2 could prevent tumor development and progression.

To test this hypothesis, we immunized MMTV-huHER2 transgenic mice with either LV-ERBB2 or LV-GFP prior to tumor onset, at the age of 8 – 10 weeks (Fig. 4A). The mice were observed for 80 weeks, and spontaneous tumor development was observed in 75% of the control group ($n=4$). Only 29% of the LV-ERBB2 group developed tumors by 55 weeks ($n=7$), when one tumor-free LV-ERBB2 mouse had to be euthanized due to swelling of the Harderian gland. Vaccination significantly delayed early tumor-induced death (Fig. 4B, $p < 0.05$, Gehan-Breslow-Wilcoxon test), although the improvement in median survival was less well supported ($p < 0.07$, log-rank test), as no mice in either group developed tumors after week 52.

Of the mice that did develop tumors, most exhibited rapid tumor growth. However, one of the LV-ERBB2 tumors grew at a very slow rate (Fig. 4C). This tumor demonstrated reduced ErbB2 expression (Fig. 4D), indicating that this tumor may have escaped from an antigen-specific immune response.

Conclusions

In this study, we have created a lentiviral vector vaccine that can be used to target breast cancer-specific antigens. We demonstrated that these vaccines can produce a T cell immune response after a single injection in mice. Furthermore, these injections are sufficient to prevent or slow the growth of tumors in multiple models of breast cancer. These vaccines represent a possible active immunotherapy approach to the treatment of breast cancer.

One advantage of DC-targeted lentiviral vectors as immunization reagents is that they are able to achieve high levels of protection with only a single injection. In order to elicit strong immune responses, DNA vaccines must generally be injected multiple times over the course of an experiment [32,35–38]. In contrast, all our experiments assayed the immune response to a single intra-footpad injection of the lentiviral vaccine. Previous work in our laboratory has shown that boosting immunizations can elicit even stronger immune responses, with minimal anti-vector immunogenicity [27]. Thus, these LV vaccines have the potential to elicit a much stronger immune response than a DNA vaccine. A strong response is critical when designing an anticancer immunotherapy because tumors often contain immunosuppressive microenvironments [39].

Our spontaneous challenge results adhere closely to those of a DNA and cell-based vaccine that were tested previously in the same strain of mice [34]. According to that work, DNA vaccination led to 65% tumor-free survival at 80 weeks and cell-based vaccination led to approximately 40% tumor-free survival, compared to our results, which yielded 69% tumor-free survival at 80 weeks. The major advantage of the LV approach is that it can achieve equal levels of protection with a single injection, whereas the cell-based immunization required twice-weekly injections for the lifetime of the mice, and the DNA vaccine required 16 injections over 80 weeks. This advantage is counterbalanced by questions surrounding the safety of LV vaccines.

Because lentiviral vectors are derived from HIV-1, a human pathogen, they have rarely been used directly in humans. However, lentiviral vectors have been engineered with numerous safety controls, and in the limited set of trials that have reported results utilizing lentiviral vectors for gene delivery, neither oncogenesis nor development of replication-competent lentivirus has been observed [40]. Recently, results from a Phase I clinical trial (NCT02122861) of an NY-ESO-1 lentiviral vector vaccine showed strong T cell responses and no serious adverse events [41]. This study and others like it will provide the necessary foundation for determining whether these powerful and flexible lentiviral vectors can be used in future cancer vaccines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard abbreviations

LV	Lentiviral vector
MOI	Multiplicity of infection
COPA	Cancer outlier profile analysis
SFC	Spot-forming cells

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Highlights

- A dendritic cell-targeted lentivector vaccine delivers breast cancer antigens
- Immunization with the ERBB2 antigen generates a strong CD8 T cell response
- A single immunization protects against mammary tumors in a self-antigen environment

A

B

C

Fig. 1. ErbB2 and Lalba are potential antigens for lentiviral vaccine immunotherapy. (A) Meta-analysis of ERBB2 and LALBA mRNA expression in multiple tumor samples. Cancer Outlier Profile Analysis (COPA) [42] across 25 human breast cancer datasets identified ERBB2 and LALBA as highly overexpressed in a subset of tumor samples, with median gene ranks of 158 and 287, respectively. Brighter red indicates a more significant outlier score for a given dataset of tumor samples. Below, microarray mRNA expression levels from several representative datasets are shown [43–45], demonstrating that ERBB2 or LALBA is overexpressed in a subset of patients with breast cancer. (B) Diagram showing the protein structure of the ERBB2 and LALBA genes. Signal peptides (SP) and transmembrane domains (TM) are indicated. (C) Diagram of the LV constructs used in the experiments (not to scale). The extracellular domain of ErbB2 and the entire coding sequence of mouse Lalba were cloned into a LV construct under the control of the ubiquitin promoter (Ubi). Oncomine™ (Compendia Bioscience™, part of Life Technologies™, Ann Arbor, MI) was used for analysis and visualization.

Fig. 2.

Lentiviral vector vaccines generate a strong immune response to human ErbB2 in both foreign- and self-antigen environments. Intracellular cytokine staining was performed on splenocytes after stimulation with an ErbB2 peptide. The number of IFN- γ -expressing CD8⁺ cells as a percentage of total CD8⁺ cells was measured. (A) Representative flow cytometry plots show the percentage of IFN- γ ⁺ cells after gating for CD8⁺CD4⁻ cells. The top panel shows Balb/cJ mice that were immunized with either the LV-ERBB2 or LV-GFP vaccine. The bottom panel shows MMTV-huHer2-Balb/cJ cross-bred mice immunized with the same conditions. (B) The ICCS data from all mice studied under the 4 conditions described. In both Balb/cJ and huHer2-Balb/cJ cross-bred mice, LV-ERBB2 immunization generated a higher antigen-specific T cell response than LV-GFP (*t* test, $p < 0.001$, $p < 0.01$ respectively).

Fig. 3.

Lentiviral vector vaccines protect Balb/cJ mice against transplanted tumor challenge. Balb/cJ mice were challenged with 4T1.ERBB2 (A and B) or 4T1.Lalba (C and D) tumor cells, and tumor growth was measured over time. LV-ERBB2 immunization was performed prophylactically (A, 14 days before challenge) or therapeutically (B, 6 days after challenge). Tumor size in individual mice is plotted with solid light gray or red lines, while mean tumor size is plotted with dashed lines. A reduction in tumor growth was observed in prophylactic ($p < 0.01$) and therapeutic settings ($p < 0.05$, two-way ANOVA). (C and D) LV-Lalba immunization was performed prophylactically (14 days before challenge). Replicates and means are plotted in (C) as above. A survival curve is plotted in (D), using 1000 mm³ as the survival cutoff. A reduction in tumor growth was observed beginning at day 23 ($p < 0.01$, t test), and median survival of immunized mice increased from 26 to 35 days ($p < 0.02$, log-rank test).

Fig. 4.

LV vaccines protect transgenic huHer2 mice against spontaneous tumor growth. (A) Transgenic MMTV-huHer2 mice were immunized with LV-ERBB2 or LV-GFP at 8 weeks of age. Tumor development in these mice was monitored for 80 weeks. (B) Survival is shown as the time to develop 750 mm³ tumors. LV-ERBB2 immunization led to long-term survival of > 80 weeks in the majority of mice, while LV-GFP led to early death in 75% of the mice (Gehan-Breslow-Wilcoxon, $p < 0.05$). (C) The rate of tumor growth was measured after first palpation, with all 3 observed tumors growing rapidly in the LV-GFP group, while 1 out of 2 tumors in the LV-ERBB2 immunized group developed much more slowly. (D) Human ERBB2 mRNA expression was measured from tumor extracts. Expression levels in the fast growing tumors (LV-GFP and LV-ERBB2 Fast) were higher than those in LV-ERBB2 Slow

(t test, $p < 0.02$), while expression in LV-ERBB2 Slow was higher than background levels (vs. E0771, t test, $p < 0.04$).

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