

A Novel Human Her-2/neu Chimeric Molecule Expressed by *Listeria monocytogenes* Can Elicit Potent HLA-A2 Restricted CD8-positive T cell Responses and Impact the Growth and Spread of Her-2/neu-positive Breast Tumors

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Abstract Purpose: The aim of this study was to efficiently design a novel vaccine for human Her-2/neu-positive (hHer-2/neu) breast cancer using the live, attenuated bacterial vector *Listeria monocytogenes*.

Experimental Design: Three recombinant *L. monocytogenes* – based vaccines were generated that could express and secrete extracellular and intracellular fragments of the hHer-2/neu protein. In addition, we generated a fourth construct fusing selected portions of each individual fragment that contained most of the human leukocyte antigen (HLA) epitopes as a combination vaccine (*L. monocytogenes* – hHer-2/neu chimera).

Results: Each individual vaccine was able to either fully regress or slow tumor growth in a mouse model for Her-2/neu-positive tumors. All three vaccines could elicit immune responses directed toward human leukocyte antigen-A2 epitopes of hHer-2/neu. The *L. monocytogenes* – hHer-2/neu chimera was able to mimic responses generated by the three separate vaccines and prevent spontaneous outgrowth of tumors in an autochthonous model for Her-2/neu-positive breast cancer, induce tumor regression in transplantable models, and prevent seeding of experimental lung metastases in a murine model for metastatic breast cancer.

Conclusion: This novel *L. monocytogenes* – hHer-2/neu chimera vaccine proves to be just as effective as the individual vaccines but combines the strength of all three in a single vaccination. These encouraging results support future clinical trials using this chimera vaccine and may be applicable to other cancer types expressing the Her-2/neu molecule such as colorectal and pancreatic cancer.

The Her-2/neu receptor is considered to be an oncogene, because its overexpression (1, 2), point mutations (3), or deletions (4) have been associated with several types of cancers. Neoplasms that are linked to dysregulation of Her-2/neu include cancers of the breast, lung, colon, stomach, and pancreas (5–7). Her-2/neu overexpression by human tumors

is an independent predictor of poor prognosis (2, 8, 9) and is associated with aggressive disease and resistance to high-dose chemotherapies such as tamoxifen, cytoxan, methotrexate, and 5-fluorouracil (9). Her-2/neu is a member of the epithelial growth factor receptor family with tyrosine kinase activity (10). It is a large (185 KD) protein comprising three domains: an extracellular, a transmembrane, and an intracellular domain.

Listeria monocytogenes is an intracellular facultative bacterium that has been investigated as a vaccine vector for cancer for over a decade (11). Once it enters the host, *Listeria* is rapidly taken up by immune cells such as dendritic cells and macrophages through phagocytosis. Due to the pore-forming action of a listerial cytotoxin called Listeriolysin-O (LLO; ref. 12), a fraction of the bacteria can escape from the phagosomes and multiply in the host cytosol. Because of this particular dual life cycle, which provides the bacterium with access to both phagosomal and intracellular compartments, antigens delivered by *L. monocytogenes* – based vaccines are presented on both major histocompatibility complex class I and II molecules. As a result, cellular immune responses elicited against these antigens include both CD4-positive and CD8-positive T cells (13). Because LLO is cytotoxic, a defense mechanism has evolved between the bacterium and the host cell, resulting in the rapid uptake and degradation of cytosolic LLO by proteasomes.

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Translational Relevance

Her-2/neu overexpression or mutations are associated with several types of human cancer, including breast, ovarian, pancreatic, gastric, and colon. The use of *Listeria monocytogenes* as a powerful vaccine vector has recently gained considerable interest and is currently being tested in clinical trials. In this study we extended our earlier results showing that Her-2/neu could be targeted using *L. monocytogenes* – based immunotherapy. We generated a vaccine-therapeutic that contained most of the human Her2/neu human leukocyte antigen epitopes and could cause regression in several murine tumor models and protect against breast cancer metastasis in mice. Our article shows the power of combination *L. monocytogenes* – based vaccines and identifies new human targets for this vector. Given the improvement in our preclinical understanding of these constructs and the ability of these vaccine-therapeutics to generate human leukocyte antigen -A2 restricted CTLs, we anticipate the future use of these vaccines in human trials for Her-2/neu-positive breast cancer.

Specific signal sequences, called PEST sequences, have been identified in LLO and seem to be responsible for rapid turnover of the protein (14–16). Thus, theoretically, proteins fused to LLO should also be shunted for rapid degradation and consequently, improved surface presentation. This was suggested by studies indicating that vaccines harboring antigens fused to LLO are far more effective than nonfused antigens (17, 18). We took advantage of this knowledge to develop three *L. monocytogenes* – based vaccines, which can deliver segments of the human Her-2/neu antigen.

Previously we showed that immunization with recombinant *L. monocytogenes* expressing and secreting fusions of a truncated nonhemolytic form of LLO and five segments of the rat Her-2/neu gene could cause regression of established rat Her-2/neu-positive tumors in mice. This response was mediated by CD8-positive T cells (18). Furthermore, we extended these studies using Her-2/neu transgenic mice, showing that *L. monocytogenes* – Her-2/neu vaccines were able to break immune tolerance and cause a significant delay in the growth of spontaneous breast tumors in these mice (19).

The encouraging results from the rat Her2/neu vaccines prompted us to develop this technology toward human use. In the present work we report on the construction of three recombinant *L. monocytogenes* strains harboring either one of the two extracellular segments (EC1 and EC2) or an intracellular segment (IC1) of the human Her-2/neu (hHer-2/neu) gene. These regions were chosen because they include most of the known human leukocyte antigen (HLA) epitopes (20). In addition, we generated a construct composed of the immunodominant sequences from the three previous vaccines to create a *L. monocytogenes* – hHer-2/neu chimera vaccine. This combined immunotherapeutic provides the advantage of targeting several immunodominant regions of the hHer-2/neu molecule in a single vaccination.

For all of the vaccines, we show that *L. monocytogenes* was able to express and secrete each of the LLO-hHer-2/neu fusion

proteins, and cause active tumor regression *in vivo*. Our *L. monocytogenes* – hHer-2/neu chimera vaccine was able to induce FVB/N H2^q and HLA-A2 – restricted responses to all three regions of hHer-2/neu originally cloned. In addition, vaccination with *L. monocytogenes* – hHer-2/neu chimera was able to prevent lung seeding of the aggressive metastatic breast cancer line 4T1 suggesting that this vaccine may also have applications for preventing breast cancer spread.

Materials and Methods

Materials. Oligos were synthesized by Invitrogen, and DNA sequencing was done by Genewiz Inc. Flow cytometry reagents were purchased from Becton Dickinson (BD) Biosciences. Cell culture media and supplements were from Gibco/Invitrogen. Other reagents, unless indicated, were from Sigma. Her-2/neu HLA-A2 peptides were synthesized by EZbiolabs. All bioluminescent work was conducted under guidance by the Small Animal Imaging Facility at the University of Pennsylvania in Philadelphia.

Mice and cell lines. C57BL/6 and FVB/N mice were purchased from Charles River laboratories. Breeding pairs of HLA-A2 transgenic mice were obtained from Dr. Linda Sherman, the Scripps Research Institute, La Jolla, California. They were bred and maintained at the animal facilities of the University of Pennsylvania School of Medicine. The FVB/N Her-2/neu transgenic mice (21) were housed and bred at the animal core facility at the University of Pennsylvania. The FVB/N rat Her-2/neu transgenic mouse overexpresses the rat Her-2/neu molecule (96% homologous to mouse Her-2/neu). Experiments on mice were done in accordance with regulations by the Institutional Animal Care and Use Committee of the University of Pennsylvania. The NT-2 tumor cell line was derived from a spontaneous mammary tumor in a rat Her-2/neu transgenic FVB/N mouse (21) and was grown as described previously (18). DHFR-G8 (3T3/neu) cells were obtained from the American Type Culture Collection and were grown according to its recommendations. The 4T1-Luciferase line (4T1-Luc) was a generous gift from Dr. Ellen Pure at the Wistar Institute, Philadelphia, and was grown in complete medium as described for NT-2.

Listeria constructs. Her-2/neu-pGEM7Z was kindly provided by Dr. Greene at the University of Pennsylvania and contained the full-length human Her-2/neu (hHer-2) gene cloned into the pGEM7Z plasmid (Promega). This plasmid was used as a template to amplify three segments of hHer-2/neu, namely, EC1, EC2, and IC1, by PCR using pfx DNA polymerase (Invitrogen) and the oligos indicated in Supplemental Table S1. The Her-2/neu chimera construct was generated by direct fusion by the SOEing PCR method and each separate hHer-2/neu segment as templates. Primers are shown in Supplemental Table S1. Each segment was cloned into the *Listeria* plasmid pGG55 (22) at *Xho*I and *Spe*I sites, in frame with a truncated, nonhemolytic fragment of LLO (Supplemental Fig. S1A and Fig. 4A). The sequences of each insert, LLO, and *prfA* genes were confirmed by DNA sequencing analysis. Each plasmid was electroporated into electro-competent *prfA*-negative *Listeria* strain XFL-7 (22) and positive clones were selected on brain heart infusion agar plates containing chloramphenicol (34 µg/mL) and streptomycin (250 µg/mL). In some experiments similar *Listeria* strains expressing rat Her-2/neu (*L. monocytogenes* – rHer-2) fragments were used for comparative purposes. These have been previously described (18).

Expression and secretion of Her-2/neu fragments by Listeria. Expression and secretion of fusion proteins from *L. monocytogenes* were tested as described previously (22). Each construct was passaged twice in mice as described (23); also see the supplemental data section for a complete description of the methods used.

Tumor regression studies. Because the rat and human Her-2/neu proteins are highly homologous, we used the rat Her-2/FVB/N mouse model to test for the therapeutic antitumor effects of each of the human *L. monocytogenes* – Her-2/neu constructs. Groups of 8 female mice (8-10 weeks old) were inoculated s.c. on the right flank with 1×10^6 NT-2 tumor cells resuspended in 200 μ L PBS. Once the tumors reached a palpable size of 4 to 5 mm (on day 7), the mice were immunized i.p. with one of the *L. monocytogenes* – Her-2/neu vaccines as follows: *L. monocytogenes* – hHer-2/EC1 (1×10^8 CFU), *L. monocytogenes* – hHer-2/EC2 (2.5×10^7 CFU), *L. monocytogenes* – hHer-2/IC1 (1×10^8 CFU), *L. monocytogenes* – rHer-2/EC1 (1×10^7 CFU), *L. monocytogenes* – rHer-2/EC2 (5×10^7 CFU), *L. monocytogenes* – rHer-2/IC1 (1×10^7 CFU), or *L. monocytogenes* – hHer-2/neu chimera (5×10^8 CFU) resuspended in 200 μ L PBS. These doses were the maximum tolerated dose for each construct as determined by an *in vivo* toxicity assay. The prime dose was followed by two boosts at 7-d intervals. Tumors were monitored weekly and tumor masses were measured with calipers in two perpendicular diameters. Mice were sacrificed if tumors reached a mean diameter of 2 cm. In the control groups, mice received either PBS or an equivalent dose of an irrelevant *L. monocytogenes* vaccine (*L. monocytogenes* – LLO-NY-ESO-1₁₀₁₋₁₅₆).

IFN- γ ELISpot assay. Secretion of IFN- γ by mouse splenocytes in response to peptide stimulation was tested by enzyme-linked immu-

nospot (ELISpot) assay (24), complete methods of which can be found in Supplemental Data. Cells were incubated in the presence of 10 μ g/mL Her-2/neu HLA-A2 peptides: Her-2/EC1: HLYQGCVV (48-56; ref. 25), EC2: KIFGSLAFL (369-378; ref. 26), IC1: RLLQETELV (689-697; ref. 27), and 5 U/mL of interleukin-2, and incubated overnight at 37°C in the presence of 5% CO₂. Development of IFN- γ spots was done by the method described previously (28). The wells were washed five times between each incubation step. Spots were counted by an ELISpot reader (C.T.L., v2006).

Intracellular staining and analysis by flow cytometry. Wild-type C57BL/6 or HLA-A2 transgenic mice were immunized i.p. once with *L. monocytogenes* – hHer-2/EC1, EC2, IC1, or chimera, or were left naive. Spleens were harvested 7 d later and isolated splenocytes were prepared. Cells were resuspended at 10^7 cells/mL and incubated in complete RPMI medium for 5 h at 37°C plus 5% CO₂ in the presence of 1 μ mol/L of each corresponding peptide, Brefeldin-A (Golgi transport inhibitor), and 5 U/mL of mouse interleukin-2. They were then washed twice with complete RPMI medium and incubated with 100 μ L of 2.4G2 hybridoma supernatant for Fc γ receptor blocking (American Type Culture Collection) for 1 h or overnight at 4°C. Cells were stained for surface molecules, using anti-CD8 α (FITC), anti-CD11b (perCP-Cy5.5), anti-CD62L (APC) antibodies. They were then permeabilized and fixed using the Golgi-Stop or Golgi-plug

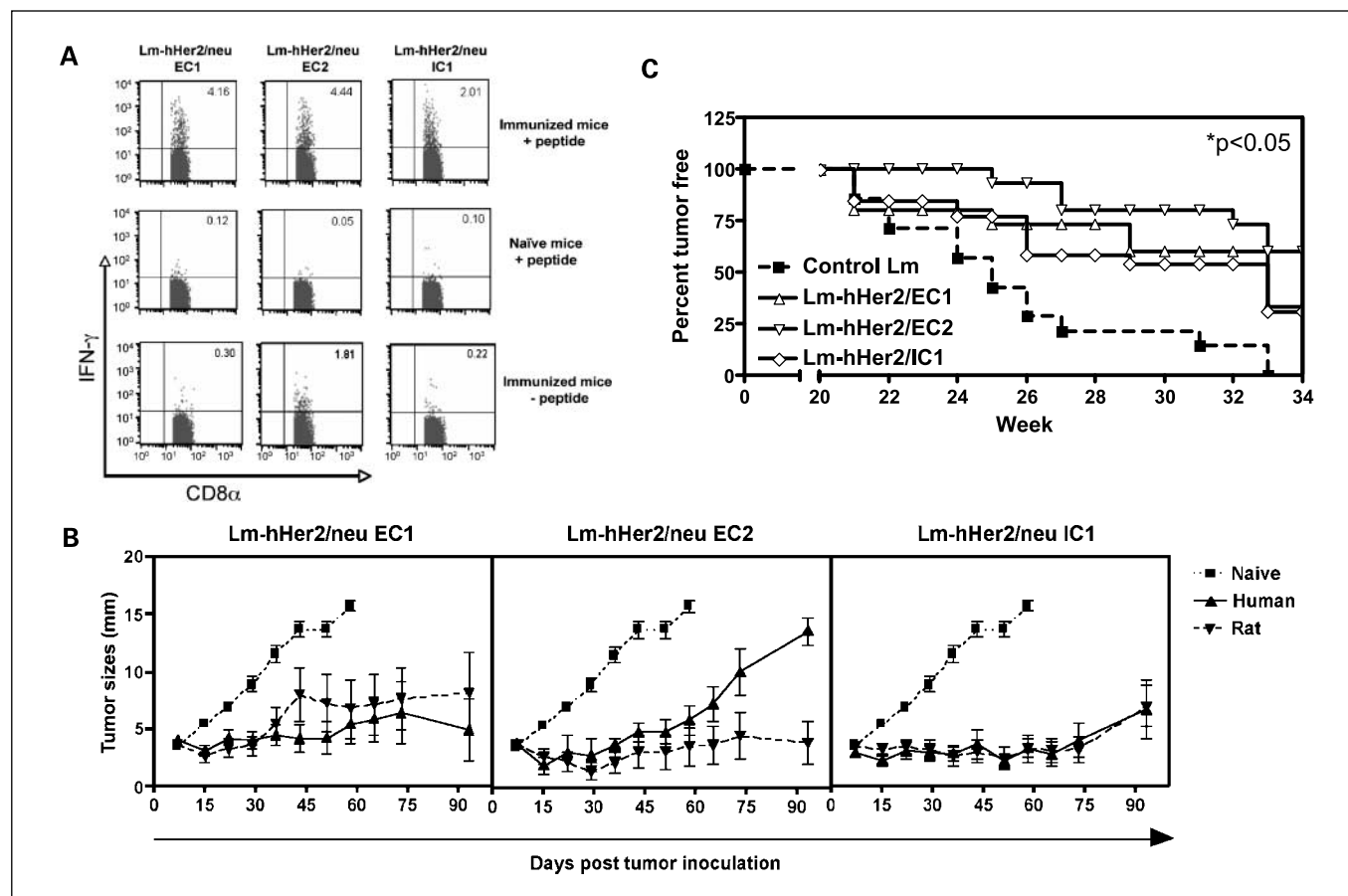


Fig. 1. Construction and validation of individual human Her-2/neu vaccines. **A**, IFN- γ secretion by splenocytes from HLA-A2 transgenic mice immunized with *L. monocytogenes* – Her-2/neu constructs. Mice were immunized with each of the *L. monocytogenes* – Her-2/neu constructs. Splenocytes were isolated and incubated overnight in the presence of each of the corresponding HLA-A2 peptides for each segment or without peptide in the presence of golgi plug. After a 6-h stimulation *in vitro*, cells were prepared for intracellular staining and flow cytometry. **B**, FVB/N mice were inoculated s.c. with NT-2 tumor cells and then immunized after 7 d with different *L. monocytogenes* – hHer-2/neu constructs or were left naive. Tumor sizes were monitored weekly. Tumor sizes are expressed as the average of two perpendicular diameters of the tumor. **C**, FVB/N rat Her-2/neu transgenic mice were injected six times with each individual vaccine or a control *L. monocytogenes*. Vaccination started at 6 wk of age and continued every 3 wk until week 21. Tumor burden was monitored on a weekly basis. Significant differences were observed between the hEC1 and hIC1 groups. Graph shows mean \pm SE, * $P < 0.05$.

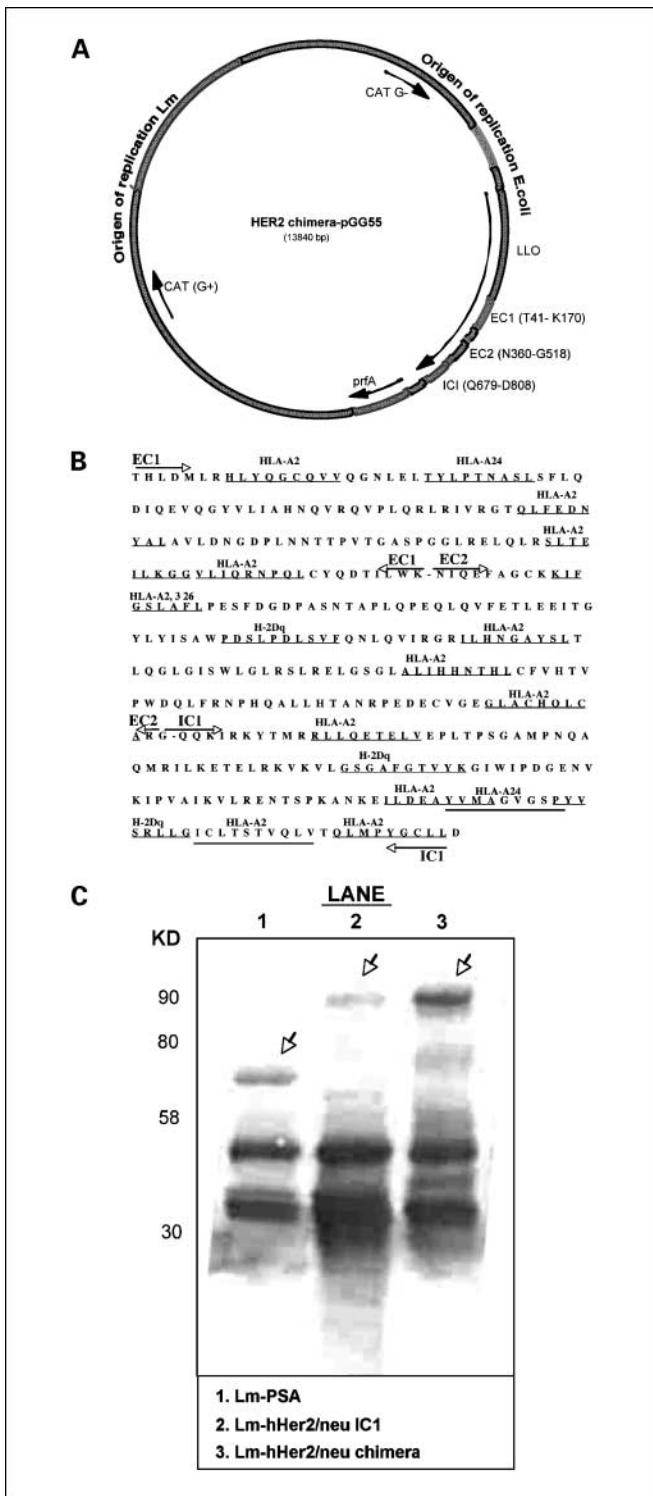


Fig. 2. Construction of the *L. monocytogenes* – hHer-2/neu chimera vaccine. **A**, three gene fragments of Her-2/neu harboring clusters of HLA epitopes from the extracellular and intracellular domains of the protein were fused by SOEing PCR method. This chimeric segment was then cloned as an open reading frame with a truncated, nonhemolytic fragment of LLO into pGG55, a *L. monocytogenes* expression plasmid. **B**, the amino acid sequence of the hHer-2/neu chimera, with marked HLA epitopes and corresponding positions of each fragment (as compared with the Her-2/neu wild-type protein). **C**, expression and secretion of LLO-hHer-2/neu chimera by *L. monocytogenes* was shown by Western blot analysis of the proteins using trichloroacetic acid – precipitated cell culture supernatants. LLO fusion proteins were detected using a polyclonal anti-LLO antibody. LLO-prostate specific antigen and LLOHer-2/IC1 were used as controls.

permeabilization Kit (BD Biosciences), and then stained for IFN- γ using antimouse IFN- γ antibody. Typically, 500,000 events were acquired using a flow cytometer FACSCalibur machine (BD Biosciences). All data were analyzed using Cellquest software (BD Biosciences). IFN- γ positive cells were expressed as a percentage of gated CD8-positive CD62L^{low} cells.

INF- γ ELISA. Splenocytes from immunized HLA-A2-mice (2-3 per group) were cultured in 24-well plates at 5×10^6 cells/well *in vitro* in the presence of 5 μ mol/L of the peptides listed above, in 2 mL of complete RPMI medium. Samples from supernatants were obtained on day 3 and tested for the presence of IFN- γ using mouse IFN- γ ELISA kit (BD Biosciences) according to the manufacturer's recommendations.

Cytotoxicity assay. Groups of three mice (FVB/N) were immunized three times with 1-wk intervals with *L. monocytogenes* – hHer-2/neu chimera (1×10^8 CFU), *L. monocytogenes* – hHer-2/neu ICI or *L. monocytogenes* – NY-ESO1 as a negative control (control *L. monocytogenes*), or were left naïve. Splenocytes were harvested and stimulated *in vitro* for 5 d with mitomycin C-treated NT-2 cells. A standard cytotoxicity assay was done using europium-labeled 3T3/neu (DHFR-G8) cells as targets according to the method previously described (28).

Metastasis studies and bioluminescent imaging. Mice were given a total of three vaccinations prior to i.v. injection with 50,000 4T1 cells expressing the integrated luciferase reporter gene (4T1-Luc). The 4T1-Luc cells were a generous gift from the Ellen Pure Laboratory. The corresponding substrate, D-Luciferin, was injected i.p. at 5 to 10 mg/mouse in 200 μ L of PBS. The mice were placed in the dark chamber of a Xenogen IVIS imaging system (X-100; Xenogen Corporation), under anesthesia following i.p. injection of ketamine (80 mg/kg)/xylazine (12 mg/kg; Sigma). Photographic and luminescence images were captured with a charge-coupled device camera, and the luminescence intensity was quantitated using Living Image software (version 2.11) from Xenogen according to the manufacturer's instructions. Longitudinal imaging was done on a weekly basis until at least 4 wk post tumor inoculation. All mice were imaged for the same exposure and length of time. Images show normalized graphics.

Statistical analysis. Nonparametric Mann-Whitney and Kruskal Wallis tests were applied to compare tumor sizes among different treatment groups. The log-rank χ^2 test was used for survival data. The Mann-Whitney test was used to compare the means in the ELISpot experiments. $P < 0.05$ was considered statistically significant in these analyses. All statistical analyses were done with either Prism software, version 4.0a (2006), or SPSS software, version 15.0 (2006). For all FVB/N rat Her-2/neu transgenic studies we used 8 to 15 mice per group; for all wild-type FVB/N studies we used at least 8 mice per group unless otherwise stated. All studies were repeated at least once except for the long-term tumor study in Her-2/neu transgenic mouse model, where the groups included 15 mice. This experiment was done once.

Results

Construction of recombinant Listeria strains secreting LLO-hHer-2/neu fusions. Three recombinant *L. monocytogenes* strains were designed to express and secrete the EC1, EC2, or ICI segments of the hHer-2/neu antigen. These segments were chosen because they harbored clusters of known HLA-A2 and other HLA epitopes such as HLA-A24 and HLA-A3 (Supplemental Fig. S1A and B). The secretion by *L. monocytogenes* is restricted by the size and hydrophobicity of proteins, and therefore each segment was designed carefully to cover as many epitopes as possible, avoiding large sizes and hydrophobic regions. Each segment was cloned into a *L. monocytogenes* plasmid (pGG55) in frame with a truncated, nonhemolytic segment of LLO, which lacks the cholesterol-binding domain. The expression and secretion of these fusion proteins by *L. monocytogenes* was tested in culture supernatants, using an

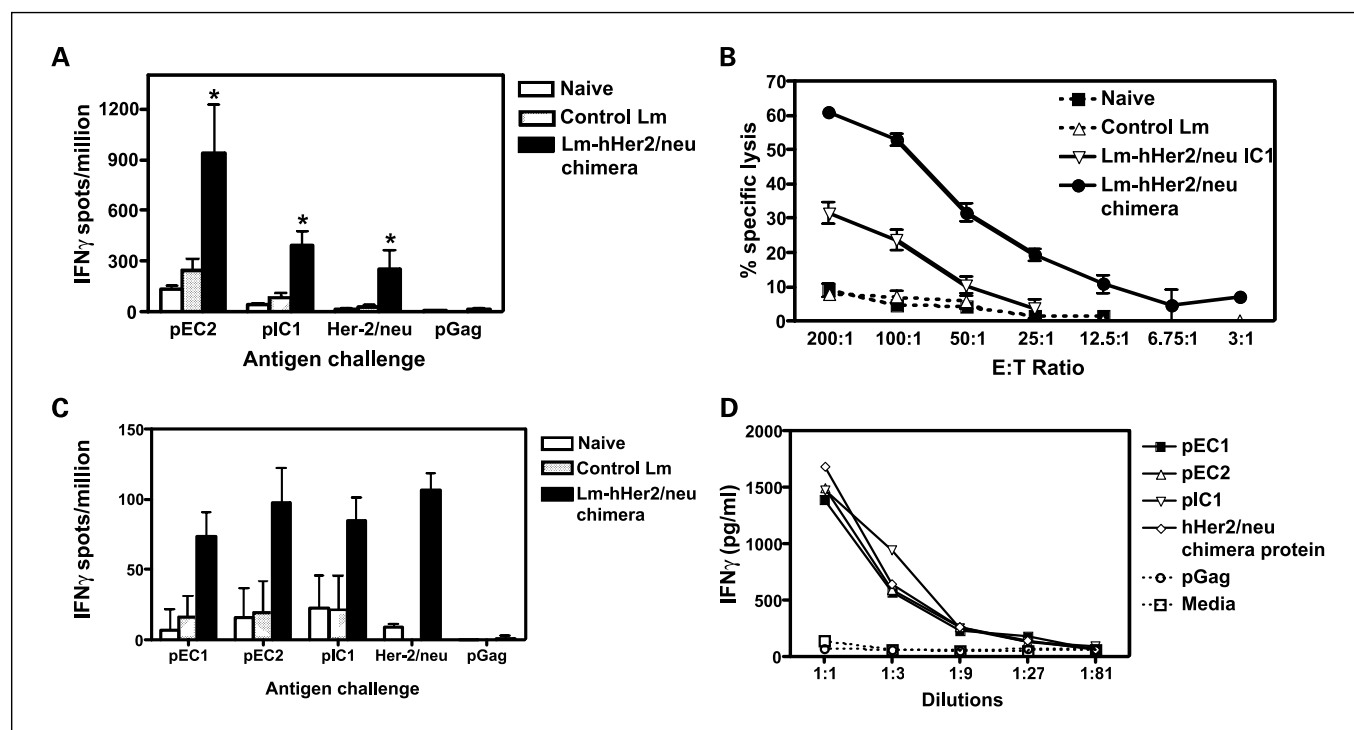


Fig. 3. *L. monocytogenes* – hHer-2/neu chimera immunogenicity. **A**, immunogenicity of the *L. monocytogenes* – hHer-2/neu chimera to induce specific immune responses to mapped epitopes in the FVB/N mouse shown. IFN- γ secretion in response to H2^d mapped epitopes shown using ELISpot. **B**, cytotoxicity assay used to determine potential CTLs in the spleens of immunized FVB/N mice. Mice immunized with *L. monocytogenes* – hHer-2/neu chimera were able to kill target Her-2/neu-positive cells more efficiently than naive or an irrelevant vaccine control. The chimera also showed cytotoxicity greater than that elicited by the individual human IC1 vaccine. **C**, immunogenicity of the *L. monocytogenes* – hHer-2/neu chimera to induce specific immune responses to mapped human epitopes after immunization in the C57Bl/6 HLA-A2 transgenic mouse. **D**, ELISpot and 3-d culture supernatants from an ELISA (bottom) show responses to all three regions of the hHer-2/neu protein. Naive and control *L. monocytogenes* were used as background, an HIV epitope pGag used as a third party peptide. Recombinant hHer-2/neu protein was used to encompass all possible epitopes. Specific responses seen for both models above background controls. * $P < 0.05$; all graphs show mean \pm SE.

anti-LLO polyclonal antibody. LLO fusion proteins of the expected sizes were detected from each of the supernatants. Thus, LLO-Her-2/EC1, EC2, and IC1 were shown to be 82, 71, and 94 KD, respectively (Supplemental Fig. S1C). Each strain was passaged two times in mice to obtain a stabilized level of virulence. The expression of LLO-Her-2/neu segments was still retained by the bacteria recovered from the spleens 3 days post-injection after each passage, showing the *in vivo* stability and plasmid retention capacity of the different strains (data not shown).

Individual *L. monocytogenes*–LLO-Her-2/neu fragment vaccines elicit immunogenic responses in mice. To investigate the immunogenic properties of *L. monocytogenes*–Her-2/neu vaccines, transgenic HLA-A2 or wild-type C57Bl/6 mice were first immunized with each individual *L. monocytogenes* – hHer-2/neu construct. Isolated splenocytes were prepared and tested for IFN- γ secretion. The induction of IFN- γ by CD8-positive T cells was tested by intracellular staining. Cells were stained for surface CD8 and CD62L molecules and intracellular IFN- γ . As expected, CD8-positive T cells isolated from HLA-A2 transgenic mice immunized with *L. monocytogenes* – hHer-2/neu constructs produced greater amounts of IFN- γ in response to their specific HLA-A2 peptide (Fig. 1A). This response ranged between 2% of CD8-positive T cells for *L. monocytogenes* – hHer-2/neu/IC1 and 4.4% for that of *L. monocytogenes*–hHer-2/neu/EC2. Splenocytes from naive or immunized mice incubated in the absence of peptides displayed low IFN- γ responses.

***L. monocytogenes*–hHer-2/neu fragment vaccines caused regression of established NT-2 tumors in mice.** High homology between the rat and hHer-2/neu proteins allowed us to use the NT-2 tumor model to determine the effect of vaccination with the *L. monocytogenes* – hHer-2/neu recombinants on tumor growth. FVB/N mice were inoculated s.c. with NT-2 tumor cells. Seven days later, when the tumors became palpable, the animals received an i.p. immunization with *L. monocytogenes* – hHer-2/EC1, *L. monocytogenes*–hHer-2/EC2 or *L. monocytogenes* – hHer-2/IC1. A naive control group was included in each experiment. For comparative purposes *L. monocytogenes* strains expressing both the rat and hHer-2/neu sequences were used. Immunizations were repeated two more times at 1-week intervals. Tumors were monitored weekly for 90 days. Each of the hHer-2/neu constructs as well as rat constructs were shown to slow or completely abolish established tumors in this mouse strain (Fig. 1B). There were no statistically significant differences between the antitumor effects of the three human vaccines or between the human and rat Her-2/neu-*L. monocytogenes* vaccines in this model, except for the later time points measured for hEC2. All immunized groups were significantly different when compared with the naive group. This trend continued when we looked at spontaneous outgrowth of Her-2/neu-positive tumors in the autochthonous model (Fig. 1C). All three vaccines were able to significantly delay the development of breast tumors beyond the control *L. monocytogenes* vaccination.

Construction of recombinant *L. monocytogenes* strains secreting a LLO-Her-2/neu chimera protein. Although each of the individual *L. monocytogenes* – hHer-2/neu fragment vaccines was shown to have antitumor effects, development of three vaccines for one clinical application, such as breast cancer, presents several obstacles and is impractical. In addition, the use of fragment vaccines has been associated with selection of tumors harboring mutations in the epitopes corresponding to the immunizing fragment (19). Thus, for clinical development and testing, one single vaccine harboring all of these fragments would be a clear advantage. Toward that end, we generated a *L. monocytogenes* strain expressing a chimeric hHer-2/neu protein (*L. monocytogenes* – hHer-2/neu chimera) that would contain all three regions in a single construct (Fig. 2A and B). The immunodominant regions of all three Her-2/neu fragments were included in a single chimeric protein as a fusion to the truncated, nonhemolytic LLO protein. The construct encoded several immunodominant epitopes found in the FVB/N H2^d model and also contained a number of mapped HLA epitopes (Fig. 2B). The *L. monocytogenes* – hHer-2/neu chimera construct was able to secrete the LLO-hHer-2/neu chimera fusion protein as shown by Western blots (Fig. 2C). Furthermore, immunization with the *L. monocytogenes* – hHer-2/neu chimera generated strong immune responses in both FVB/N H-2^d (Fig. 3A and B) and HLA-A2–restricted CD8-positive T cells *ex vivo* (Fig. 3C and D). This was shown by secretion of IFN- γ by splenocytes of immunized mice in response to the corresponding peptides (Fig. 3A and C). In addition, the *L. monocytogenes* – hHer-2/neu chimera was also shown to generate T cells with cytotoxic activity against a rHer-2/neu-expressing tumor cell line 3T3/neu when tested by an *in vitro* CTL assay (Fig. 3B). The ELISA in Fig. 3D shows the ability of the *L. monocytogenes* – hHer-2/neu chimera construct to induce IFN- γ secretion by splenocytes in response to all three regions of the human Her-2/neu molecule.

The *L. monocytogenes* – Her-2/neu chimera can significantly impact the growth and development of Her-2/neu-positive breast tumors *in vivo*. The antitumor properties of the *L. monocytogenes* – hHer-2/neu chimera vaccine was then tested using

two accepted murine models for breast cancer (21): a foreign-antigen model using the rat Her-2/neu-expressing cell line NT-2, implanted into a syngeneic FVB/N host; and a self-antigen model in the rat Her-2/neu transgenic mouse strain. These models provided an opportunity to test the ability of the vaccine to cause regression or prevention of Her-2/neu-positive tumors *in vivo*. For the foreign-antigen model, FVB/N mice were implanted with 1×10^6 NT-2 cells s.c.; when the tumors reached a palpable size of 4 to 5 mm on day 4 postinoculation, vaccination was started. Mice were vaccinated three times with *L. monocytogenes* – hHer-2/neu chimera, or a control *L. monocytogenes* vaccine. *L. monocytogenes* – hHer-2/neu chimera vaccination was able to slow down (5 of 7 mice; 71%) or cause complete regression (2 of 7 mice; 28%) of NT-2 tumors in this mouse strain (Fig. 4A; * $P < 0.05$).

The transplantable murine model using the NT-2 cell line is a fast-growing tumor model with little tolerance toward the Her-2/neu antigen. A more challenging tumor model, where tolerance toward the Her-2/neu antigen might play a significant role in attenuating the immunotherapeutic efficacy of a vaccine, is the rat Her-2/neu transgenic mouse model (21). In this model, mice develop spontaneous, slow-growing mammary tumors between 4 and 8 months of age. We therefore used this mouse strain to test whether the *L. monocytogenes* – hHer-2/neu chimera vaccine was able to overcome tolerance toward the Her-2/neu self-antigen. Because this is a prophylactic model and these spontaneous tumors do not start appearing until about week 20, we were able to repeat immunizations more times, as compared with the fast-growing model. Thus, these mice were immunized six times with *L. monocytogenes* – hHer-2/neu chimera and observed twice a week for the emergence and growth of spontaneous mammary tumors for up to 52 weeks. Spontaneous tumor formation is detected by palpation of the upper and lower mouse mammary glands, which can identify tumors as small as 1 to 2 mm in diameter. Even in this challenging model, immunization with *L. monocytogenes* – hHer-2/neu chimera vaccine was able to delay the onset of autochthonous tumors, showing that this vaccine is able to overcome tolerance toward Her-2/neu

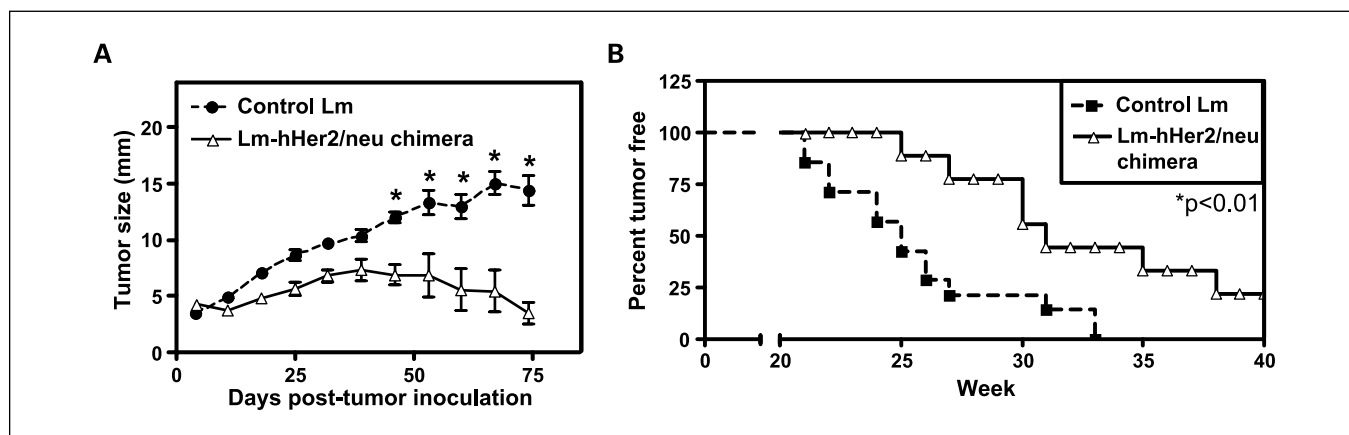


Fig. 4. Tumor regression and prevention studies for *L. monocytogenes* – hHer-2/neu chimera vaccine. **A**, FVB/N mice were inoculated s.c. with NT-2 tumor cells and then immunized the following week with either the *L. monocytogenes* – hHer-2/neu chimera vaccine or a control *L. monocytogenes* vaccine. Tumor sizes were monitored weekly. Tumor sizes are expressed as the average of two perpendicular diameters of the tumor. Graphs show mean \pm SE; * $P < 0.05$; $n = 8$ /group. **B**, FVB/N rat Her-2/neu transgenic mice were injected six times with each individual vaccine or a control *L. monocytogenes* vaccination started at 6 wk of age and continued every 3 wk until week 21. Tumor burden was monitored on a weekly basis. Significant differences were observed for our chimera vaccination. * $P < 0.01$; $n = 14$ /group.

(Fig. 4B; $*P < 0.01$). Mice in the control group started to show signs of spontaneous mammary tumors by week 21 and by week 33 all mice had detectable tumors. The *L. monocytogenes* – hHer-2/neu chimera vaccine caused a significant delay in the onset of tumor development. By week 37, 33% of immunized mice were still tumor-free, whereas none of the mice in the control group were tumor-free (Fig. 4B).

***L. monocytogenes* – hHer-2/neu chimera impacts on tumor growth of metastatic breast cancer.** A commonly used model for testing immunotherapeutics on metastasis is the lung-seeding model that makes use of experimental metastasis introduced via tail-vein injection. We used a syngeneic mouse model, the mammary carcinoma cell line 4T1 that stably expresses the firefly luciferase gene (4T1-Luc) under the control of a lentivirus promoter. This model thus allows us to use bioluminescent technology to track metastases and lung seeding *in vivo* postimmunization. The 4T1 mouse mammary carcinoma line was originally derived from a spontaneous breast tumor isolated from a Balb/c mouse. To work as a model for Her-2/neu, we first showed that 4T1-Luc does in fact express mouse Her-2/neu. This was shown by the weak but detectable expression in Western blots as compared with the higher expression of rat Her-2/neu by the NT-2 cell line (Fig. 5A). In order to test our vaccine in this model, Balb/c mice were immunized with the *L. monocytogenes* – hHer-2/neu chimera or a control *L. monocytogenes* vaccine weekly for a total of four weeks. Mice were then injected i.v. with 50,000 4T1-Luc cells. On a weekly basis, mice were anesthetized and injected with a luciferase substrate (D-Luciferin) and imaged longitudinally using noninvasive bioluminescence imaging. Lung seeding was apparent by day 11 and control-treated mice rapidly became colonized with 4T1-Luc cells by day 25, whereas none of the chimera-treated mice showed any signs of lung seeding until at least day 32, at which point the control-treated mice had become ill and were sacrificed (Fig. 5B). On day 32, only 33% of the chimera-treated mice showed any lung tumors, which was confirmed via visual examination of the lung tissue *ex vivo* (data not shown). Thus, mice immunized with the control *L. monocytogenes* rapidly became diseased by lung tumors, but the chimeric *L. monocytogenes* vaccination significantly delayed tumor burden, time to progression (day 11 for control, day 32 for chimera), and eventual systemic disease (as evidenced by morbidity).

Discussion

Several immunotherapeutic approaches targeting the Her-2/neu receptor have been reported in the literature, including both passive and active immunizations (29). A humanized anti-Her-2/neu antibody, Herceptin (trastuzumab), was approved by the Food and Drug Administration in 1998, confirming the potential of this antigen to be used as an immunotherapeutic target against Her-2/neu-positive tumors. Herceptin binds to the extracellular domain of Her-2/neu and deactivates it by various mechanisms (for a review see ref. 30). Because this is a passive immunization, weekly doses are needed continuously for tumor control. Unlike a passive approach, active immunization with a therapeutic cancer vaccine results in a gradual and lasting response, in that it could abolish the tumor and induce a long-term memory

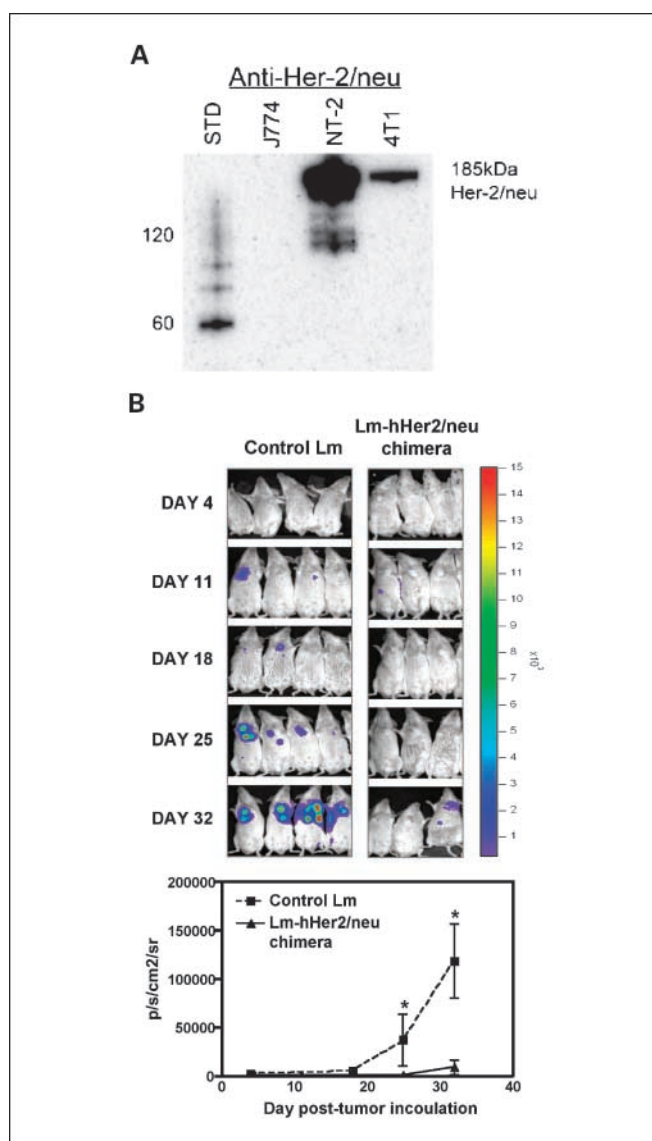


Fig. 5. *L. monocytogenes* – hHer-2/neu chimera vaccine prevents lung metastases. **A**, listed cell lines (J774, NT-2, 4T1-Luc) were grown in complete cell culture medium and lysed using 2× Nu-PAGE lysis buffer and ran on a SDS-PAGE then transferred, and a Western blot probed with an anti-Her-2/neu antibody that cross-reacts across several species including mouse and rat. The J774 macrophage cell line as a negative control does not express Her-2/neu, the NT-2 line overexpresses rat Her-2/neu under a transgene, and 4T1-Luc expresses low, physiologic levels of mouse Her-2/neu. **B**, Balb/c mice were immunized with *L. monocytogenes* – hHer-2/neu chimera or a control *L. monocytogenes* vaccine for 4 wk sequentially. Fifty thousand 4T1-Luc cells were injected i.v. and mice were imaged every week following D-Luciferin injection. Average bioluminescence imaging radiance was measured by gating each mouse individually, gate size being the same for every animal measured. Living Image software from Xenogen calculated the average number of photons collected per second of exposure per cm² of gated surface area. Graph shows mean ± SE; $*P < 0.05$, $n = 3-4$ mice/group, experiment repeated once.

response that could protect the patient from future disease recurrence. Specifically, *L. monocytogenes* – based vaccines have shown advantages over other vaccine approaches, because in addition to antigen-specific responses, they also engage a number of innate immune responses such as stimulation of cytokine release including IFN- γ and interleukin-6, reduce intratumoral T regulatory cells (28, 31), and are able to break

tolerance toward self-antigens (19, 32). Here, we report the development of three therapeutic vaccines targeting the hHer-2/neu antigen and a fourth vaccine targeting all three regions in a single vaccination.

We used a live attenuated strain of *L. monocytogenes* as an intracellular vaccine vector to deliver these segments of the hHer-2/neu protein: two segments of the extracellular and one from the intracellular domains. The full-length Her-2/neu is too large for successful secretion from *L. monocytogenes*, so we chose shorter fragments, which were predicted to be well tolerated and easily secreted by *L. monocytogenes*, in addition to harboring several known HLA-A2, HLA-A3, HLA-A24, and other HLA epitopes (for a review on hHer-2 HLA epitopes see ref. 33). Interestingly, because the rat and hHer-2/neu proteins are highly homologous, most of the H-2^q epitopes previously mapped for FVB/N mice (19, 34–37) were conserved in the human segments (highlighted in Fig. 2). One exception was found in the EC1 segment where there was one amino acid difference in the peptide 300-309 (human/rat PYNYLSTE/DV; Supplemental Fig. S1B). Despite this difference, *L. monocytogenes*-hHer-2/EC1 was also able to cause tumor regression in this model; however, we also have evidence that there are multiple H-2^q restricted epitopes present in this fragment (19, 36, 37). Other mismatches were found in the EC2 segment that might explain the difference observed between the rat and human EC2 immunized groups. The human EC2 domain contains three amino acids within key epitopes that differ from the rat counterpart (rat “PDSLRLDLSVF” → human “PDSLPLDLSVF”; rat “ALIRHNAHL” → human “ALIHNTHL”). Despite these differences, we were still able to use the rat model to show the efficacy of *L. monocytogenes*-hHer-2/neu vaccines. Interestingly, when equivalent rat Her-2/neu fragment vaccines were tested, the intracellular segment (IC1), which includes the kinase domain of the hHer-2/neu receptor, was shown to be the most efficacious and immunogenic fragment (36).

The three segments of hHer-2/neu were fused in frame to a fragment of LLO (22), which harbors signals for secretion out of *L. monocytogenes* and possibly for rapid protein degradation in proteosomes (14). The size and hydrophobicity of LLO fusion proteins are limiting factors for secretion from *L. monocytogenes*; thus each segment was designed taking these factors into consideration. As a result, none of these segments included the transmembrane domain of hHer-2/neu, which is the most hydrophobic region of the hHer-2/neu protein and where the immunodominant HLA-A2 epitope (665-673, VVLGVVFGI) is located (20). Theoretically, this might present an advantage in our vaccine design, because tolerance to self-antigens is often directed toward the immunodominant epitopes and not to the subdominant epitopes (37–39). Nevertheless, this theory still needs to be further investigated.

The encouraging results observed with *L. monocytogenes*–hHer-2/neu fragment vaccines prompted us to take this vaccine for further development toward clinical testing. Nonetheless, advancing three vaccines toward development would require triple resources, more regulatory concerns, and various technical obstacles for proper manufacturing/dosing, which seemed to be extremely impractical. For instance, if a mixture of the three vaccines was to be used, then the dose would have to be lowered to reduce the total *L. monocytogenes* vector toxicity

(additive LD₅₀ values). Decreasing each vaccine as to match the 0.1 LD₅₀ value for total bacteria per dose has been shown to severely compromise the efficacy of our *L. monocytogenes*–based vaccines.³ Furthermore, previously we showed that tumor escape can occur when fragment vaccines are used (19), specifically due to point mutations in the CD8-specific epitopes harbored in that fragment. Covering a wider range of epitopes would then be advantageous, as it would reduce the chance of tumor escape. In order to circumvent these issues, we decided to generate a fourth vaccine that would cover most of the known HLA epitopes located on the three fragments by direct fusion of the selected regions of hHer-2/neu EC1, EC2, and IC1 fragments. This proved to be a successful approach. *L. monocytogenes*-hHer-2/neu chimera was able to induce regression of transplanted tumors (Fig. 4A) and overcome tolerance in a spontaneous model of breast cancer (Fig. 4B). As breast cancer progresses, tumor cells eventually gain access to lymph nodes and/or the circulation that allows for spreading to distal organs. Metastatic breast cancer usually spreads to the bone, but can also spread to the lung, liver, and brain. Recently it was found that nearly 70% of brain metastases in advanced-stage breast cancer are Her-2/neu-positive (40) and that the action of the anti-Her-2/neu monoclonal antibody, trastuzumab, may be due to its direct effect on the Her-2/neu-positive breast cancer stem cell population (41). Based on these findings we hypothesized that our *L. monocytogenes*–Her-2/neu chimeric vaccine may also make an impact on the spread of metastatic breast cancer. Indeed, immunization with the chimera construct was able to prevent the lung seeding in a poor Her-2/neu-expressing syngeneic model for advanced-stage metastatic breast cancer (Fig. 5). The power of this construct is the ability to deliver most of the known hHer-2/neu epitopes in a single vaccine.

In conclusion, each of the individual *L. monocytogenes*–hHer-2/neu vaccines and our combined *L. monocytogenes*–hHer-2/neu chimera vaccine were able to overcome self-tolerance to an endogenous tumor protein, induce tumor regression in a foreign-antigen model, and prolong the development of spontaneous breast carcinoma. In addition, our chimeric vaccine may provide protection from the seeding and/or growth of lung metastases. The strength of this later point warrants further investigation as true, natural metastasis involves several evasion mechanisms that experimental metastases may lack.

Because of the encouraging results of this construct we are continuing to develop this vaccine toward a product for human use by generating a vector that is antibiotic-independent, more attenuated/safer, and more suitable for clinical use. Our results from the first-in-man clinical trial with a recombinant *L. monocytogenes*–expressing HPV16E7 showed that this vector can be used safely in late-stage cancer patients, causing limited and manageable toxicity (42). For obvious reasons, a more attenuated and antibiotic-independent bacterial strain with equivalent efficacy is preferred for human use. In addition, we are seeking out strategies to enhance protein expression by this vaccine to increase efficacy and the resulting immune responses. It is possible that these vaccines may also be

³ Unpublished observations.

beneficial for other malignancies such as ovarian, prostate, pancreatic, and colorectal cancer as these also express Her-2/neu (43).

Disclosure of Potential Conflicts of Interest

Advaxis, Inc. is a for-profit vaccine and therapeutic company that has licensed or has an option to license all patents from the University of Pennsylvania that concern the use of *L. monocytogenes* or listerial products as vaccines. The following authors

have a financial interest in Advaxis, Inc., and thus may have a conflict of interest: Drs. Paulo C. Maciag, Anu Wallecha, Sandra Rivera, Vafa Shahabi, and Yvonne Paterson. Drs. Zhen-Kun Pan and Matthew M. Seavey do not hold any conflict of interest.

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