Fusion to Listeriolysin O and Delivery by *Listeria monocytogenes* Enhances the Immunogenicity of HER-2/neu and Reveals Subdominant Epitopes in the FVB/N Mouse[1]

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Five overlapping fragments of rat HER-2/neu have been expressed in recombinant *Listeria monocytogenes*. Each fragment of HER-2/neu is secreted as a fusion protein with a truncated, nonhemolytic form of listeriolysin O (LLO). Lm-LLO-EC1, Lm-LLO-EC2, and Lm-LLO-EC3 overlap the extracellular domain of HER-2/neu, whereas Lm-LLO-IC1 and Lm-LLO-IC2 span the intracellular domain. All five strains controlled the growth of established NT-2 tumors, a rat HER-2/neu-expressing tumor line derived from a spontaneously arising mammary tumor in a FVB/N HER-2/neu-transgenic mouse. The antitumor effect of each of these vaccine constructs was abrogated by the in vivo depletion of CD8+ T cells, although only one known epitope has been defined previously and is present in Lm-LLO-EC2. Anti-HER-2/neu CTL responses were generated by each of the rLm vaccine constructs. With the use of a panel of 3T3 cell lines expressing overlapping fragments of HER-2/neu, regions of HER-2/neu with potential CD8+ T cell epitopes have been defined. DNA vaccines expressing either a fragment or full-length HER-2/neu were constructed in LLO-fused and non-LLO-fused forms. CTL analysis of the DNA vaccines revealed a broadening in the regions of HER-2/neu recognizable as targets when the target Ag is fused to LLO. These studies show the efficacy of *L. monocytogenes*-based HER-2/neu vaccines in a murine model of breast cancer and also that the immunogenicity of self-Ags can be increased by fusion to LLO and delivery by *L. monocytogenes* revealing subdominant epitopes. *The Journal of Immunology,* **2005, 175: 3663–3673.**

A member of the epidermal growth factor receptor family of tyrosine kinases, HER-2/neu is a 185-kDa glycoprotein. It consists of an extracellular domain, a transmembrane domain, and an intracellular domain, which is known to be involved in cellular signaling (1–4). It is overexpressed in 25–40% of all breast cancers and is also overexpressed in many cancers of the ovaries, lung, pancreas, and gastrointestinal tract (5–7). The overexpression of HER-2/neu is associated with uncontrolled cell growth and signaling, both of which contribute to the development of tumors (2, 8). Patients with cancers that overexpress HER-2/neu exhibit tolerance even with detectable humoral (9), CD8+ T cell (10), and CD4+ T cell (11) responses directed against HER-2/neu.

Listeria monocytogenes is an intracellular pathogen that primarily infects APCs and has adapted for life in the cytoplasm of these cells (12, 13). Host cells, such as macrophages, actively phagocytose *L. monocytogenes*, and the majority of the bacteria are degraded in the phagolysosome (14). Some of the bacteria escape into the host cytosol by perforating the phagosomal membrane through the action of a hemolysin, listeriolysin O (LLO)[4] (13, 15). Once in the cytosol, *L. monocytogenes* can polymerize the host actin and pass directly from cell to cell further evading the host immune system and resulting in a negligible Ab response to *L. monocytogenes* (13).

L. monocytogenes is an attractive vaccine vector because proteins produced by this bacterium can be presented as short peptides via both the MHC class I and class II pathways generating both CD8+ and CD4+ T cell responses to these Ags (16). Direct killing of tumor cells occurs through the function of CD8+ T cells, but this killing may be enhanced through the activation of CD4+ T cells. MHC class II molecules present peptides derived from listerial proteins in the phagolysosome to activate CD4+ T cells (16). Conversely, proteins that are produced by *L. monocytogenes* in the host cytosol are presented to CD8+ T cells by MHC class I molecules (17, 18). Taking advantage of the ability of *L. monocytogenes* to target Ags to both of these pathways should lead to a strong cellular immune response against the Ags it produces. Our lab has previously shown this in response to the human papillomavirus (HPV) E7 protein and the influenza nucleoprotein (NP) (17, 19–21). Both of these systems used recombinant *L. monocytogenes* containing a plasmid with a truncated, nonhemolytic, LLO fused to the N terminus of either E7 (Lm-LLO-E7) or NP (Lm-LLO-NP) (17, 19). In both these cases, complete tumor regression can be seen upon vaccination of mice-bearing tumors with the target Ag (19, 20). In particular, Lm-LLO-E7 induces the complete regression of >75% of established E7-expressing murine tumors. This vaccine is also capable of generating an E7-specific CD8+ T cell response that can be used to effectively kill target tumor cells in a CTL assay, and the antitumor response is abrogated in vivo upon depletion of CD8+ T cells (19).

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[4] Abbreviations used in this paper: LLO, listeriolysin O; HPV, human papillomavirus; NP, nucleoprotein; PEST, proline, glutamic acid, serine, and threonine.

Based on these results, we have adapted the Lm-LLO-Ag system for the self-Ag HER-2/neu. Several other vaccines using the HER-2/neu tumor Ag in the mouse have been studied previously. In wild-type FVB/N mice, whole cell vaccination has been effective in preventing tumor growth upon tumor challenge but is not effective as a treatment for established tumors (22). Recombinant vaccinia virus transduced with HER-2/neu has also been shown to be fairly effective in a tumor prevention model (23). HSP-110-HER-2/neu chaperone complex vaccines containing the intracellular domain of HER-2/neu have also been described previously (24). It has been shown previously that depletion of either $CD4^+$ or $CD8^+$ T cells in tumor-bearing mice results in decreased tumor regression vs undepleted mice (25). Because of the ability of *L. monocytogenes* to generate both an Ag-specific $CD4^+$ or $CD8^+$ T cell response, it appears to be a promising vaccine vector for HER-2/neu. In addition, the use of a bacterium such as *L. monocytogenes* to deliver fragments of HER-2/neu fused to LLO may make self-Ags, such as HER-2/neu, immunogenic enough to induce an anti-HER-2/neu tumor response in mice with HER-2/neu-positive tumors.

In this study, we describe a series of *L. monocytogenes*-based vaccines for HER-2/neu. Each of these vaccines is capable of significantly impacting on tumor growth and is able to induce an anti-HER-2/neu CTL response, despite the fact that there is only one known $CD8^+$ T cell epitope, which falls into a region covered by only one vaccine (Lm-LLO-EC2) (26). These results suggest that there are subdominant HER-2/neu epitopes that are revealed through this vaccination strategy that are immunogenic enough to induce $CD8^+$ T cells that can control tumor growth. This study further attempts to determine whether these subdominant epitopes are revealed through 1) the use of *L. monocytogenes* as a vaccine vector, 2) fusion of the Ag to LLO, 3) splitting HER-2/neu into fragments, or 4) a combination of these possibilities.

Materials and Methods

L. monocytogenes *and vaccine construction*

The *L. monocytogenes* strains used for these studies are Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, and Lm-LLO-IC2, all of which contain a fragment of rat HER-2/*neu* fused to the listerial *hly* gene in an episomal expression system that has been described previously by our lab (19, 21). Fragments of HER-2/*neu* have been cloned into the plasmid pGG-55, and the amino acids in each fragment are 20–326 in Lm-LLO-EC1, 303–501 in Lm-LLO-EC2, 479–655 in Lm-LLO-EC3, 690–1081 in Lm-LLO-IC1, and 1020–1260 in Lm-LLO-IC2 (see Fig. 1*A*). Each fragment was amplified by PCR from the pNINA plasmid, which contains the full-length rat HER-2/*neu* gene using the following primers: EC1 (bp 74–994) 5′-CACGCGGATGAAATCGATAAGCTCGAGCCCCCCGGAATCGCGG GCAC (*Xho*I site underlined) and 3′-CCGGACTAGTGACCTCTTGGTTA TTCGGGGGGACACACC (*Spe*I site underlined); EC2 (bp 923-1519) 5′-CC GGGTCGACTGCCCCTACAACTACCTGTCTACG (*Sal*I site underlined) and 3′-CCGGACTAGTTTACTTGTCATCGTCGTCCTTGTAGTCCCCACT GTGGAGCAGGGCCTG (*Spe*I site underlined, FLAG tag sequence in italics); EC3 (bp 1451–1981) 5′-CCGGGTCGACTGCTTTGTACACACTGTA CCTTGG (*Sal*I site underlined) and 3′-CCGGACTAGTTTACTTGTCA TCGTCGTCCTTGTAGTCCGGGCTGGCTCTCTGCTCTGC (*Spe*I site underlined, FLAG tag in italics); IC1 (bp 2084–3259) 5′-CCGG CTCGAGTATACGATGCGTAGGCTGCTGCAGG (*Xho*I site underlined) and 3′-CCGGACTAGTAGCCAGTGGAGATCTGGGGGGGCCC (*Spe*I site underlined); and IC2 (bp 3073–3796) 5′-CCGGCTCGAGGGTGACCTGG TAGACGCTGAAG (*Xho*I site underlined) and 3′-CCGGACTAGTTACAG GTACATCCAGGCCTAGG (*Spe*I site underlined). Each of these fragments was amplified by PCR and cloned into the pCR 2.1 expression system (Invitrogen Life Technologies). Each fragment was then excised from the pCR2.1 plasmid with the delineated enzymes. For all the constructs, the *E7* gene was excised from the pGG-55 plasmid (19) with *Xho*I and *Spe*I. Each HER-2/neu fragment was then ligated into the E7 site (ends cut with *Sal*I are compatible with *Xho*I cuts). The XFL-7 strain (19) of *L. monocytogenes* was then electroporated with each plasmid. Bacteria were grown in brain heart infusion medium (BD) with 50 μg/ml chloramphenicol and then frozen in 1-ml aliquots

at $-80°C$. The LD_{50} of each vaccine was determined as previously described by vaccinating mice with various doses of each vaccine construct (19). The LD_{50} of each vaccine construct is as follows: 1×10^8 CFU for Lm-LLO-EC1, 5×10^8 CFU for Lm-LLO-EC2, 1×10^9 CFU for Lm-LLO-EC3, 1×10^8 CFU for Lm-LLO-IC1, and 1×10^8 CFU for Lm-LLO-IC2.

Lm-LLO-E7 as used as a control for non-Ag-specific effects throughout this study. This strain is isogenic with the Lm-LLO-HER-2/neu strains, except for the Ag expressed and has been described previously (19).

The DNA vaccines were all inserted into the pcDNA 3.1 plasmid (Invitrogen Life Technologies). Full-length HER-2/*neu* and the *EC1* fragment were amplified by PCR for both the LLO-fused and non-LLO-fused versions of the DNA vaccine. The primers used were as follows: full-length HER-2/*neu* no fusion 5′-CCGGGCTAGCATGGTCATCATGGAGCTG GCCGG (*Nhe*I site underlined) and 3′-CCGGGATATCTTACTTGT CATCGTCGTCCTTGTAGTCTCATACAGGTACATCCAGGCC (*Eco*RV site underlined, FLAG tag in italics, stop codon in bold); full-length HER-2/*neu* fused to LLO 5′-CCGGGTCGACATGGTCATCATGGAGCTG GCCGG (*Sal*I site underlined) and the same 3′ primer as for the full-length HER-2/*neu* with no fusion; *EC1* no LLO fusion 5′ primer same as full-length HER-2/*neu* no fusion 5′ primer and 3′-CCGGGATATCTTACTTGTCATCG TCGTCCTTGTAGTCTCAGACCTCTTGGTTATTCGGGGG (*Eco*RV site underlined, FLAG tag in italics, stop codon in bold); *EC1* with LLO fusion 5′ primer same as the 5′ primer used for the full-length HER-2/*neu* with LLO fusion and 3′ primer same as the 3′ primer for *EC1* no LLO fusion. All the fragments were cloned into the multicloning site of pcDNA3.1 downstream from the CMV promoter either containing LLO or not. *Escherichia coli* were transformed with each plasmid and the bacteria were grown in Luria-Bertani medium (BD) with 50 μg/ml ampicillin.

Western blotting

The HER-2/neu *L. monocytogenes* vaccines were grown overnight in Luria-Bertani medium with 50 μg/ml chloramphenicol at 37°C. Supernatants were TCA precipitated and resuspended in $1\times$ LDS sample buffer (Invitrogen Life Technologies). Fifteen microliters of each sample were loaded on a 4–12% Bis-Tris SDS-PAGE gel (Invitrogen Life Technologies). The gel was then transferred to a Immobilon-P polyvinylidene fluoride membrane (Millipore) and blotted with a polyclonal rabbit serum raised to the first 30 residues of the LLO protein (anti-proline, glutamic acid, serine, and threonine (PEST)). The secondary Ab was an HRP-conjugated anti-rabbit Ab (Amersham Biosciences).

Mice

Six- to 8-wk-old female FVB/N mice were purchased from Charles River Laboratories.

Cell lines

The FVB/N syngeneic NT-2 tumor cell line was developed from a spontaneously occurring mammary tumor in a FVB/N HER-2/neu-transgenic mouse (27). NT-2 tumor cells constitutively express low levels of rat HER-2/neu and are tumorigenic in wild-type syngeneic mice. NT-2 cells were grown in RPMI 1640 medium supplemented with 20% FCS, 10.2 mM HEPES, 2 mM L-glutamine, 100 μM nonessential amino acids, 1 mM sodium pyruvate, 50 U/ml penicillin G, 50 μg/ml streptomycin, 20 μg/ml insulin, and 2 μg/ml gentamicin at 37°C with 5% CO_2. NIH 3T3 cells are a mouse fibroblast line and the 3T3-neu lines were made from these wild-type cells as described previously (26). Briefly, wild-type 3T3 cells were transduced with overlapping fragments of the rat HER-2/neu gene, creating nine 3T3 HER-2/neu fragment lines, and one 3T3 line expressing the full-length rat HER-2/neu. The 3T3 HER-2/neu fragment lines encompass the following overlapping amino acid regions: 3T3-neu-1 spans 1–165; 3T3-neu-2 spans 148–291; 3T3-neu-3 spans 274–426; 3T3-neu-4 spans 410–553; 3T3-neu-5 spans 531–687; 3T3-neu-6 spans 665–820; 3T3-neu-7 spans 797–952; 3T3-neu-8 spans 929-1085; and 3T3-neu-9 spans 1063–1260. The NIH 3T3 and all the derived cells were cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 μM nonessential amino acids, 1 mM sodium pyruvate, 50 U/ml penicillin G, and 50 μg/ml streptomycin. Culture medium for the 3T3-neu cell lines was supplemented with 1 mg/ml G418. Cells were grown at 37°C with 5% CO_2.

Tumor regression

Six- to 8-wk-old FVB/N mice were injected s.c. on the right flank with 2×10^6 NT-2 tumor cells in 200 μl of PBS. One week posttumor inoculation, the tumors reached a palpable size at 4–5 mm. Each treatment group consisted of eight mice, which were vaccinated with 0.1 LD_{50} i.p. Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, Lm-LLO-IC2, Lm-LLO-E7, or PBS on days 7, 14, and 21 after tumor inoculation.

Measurement of tumor growth

Tumors were measured every 2 days with calipers spanning the shortest and longest surface diameters. Plots show the mean of these two measurements as the tumor size in millimeters vs time. Mice were sacrificed when mean tumor diameter reached 20 mm, and tumor measurements for each time point are shown only for the surviving mice.

$CD8^+$ cell depletion

$CD8^+$ T cells were depleted in NT-2 tumor-bearing mice with 0.5 mg of 2.43 (21) on days 6, 7, 8, 11, 14, 17, 20, and 23 posttumor injection. The 2.43 (anti-$CD8^+$ T cell) Ab was affinity purified from ascites on a protein G-Sepharose column (Amersham Biosciences). This Ab has been shown by many investigators to specifically deplete only $CD8^+$ T cells. In contrast, a similarly purified, isotype-matched rat Ab, GL117.41, which produces an anti-$E.$ $coli$ β-galactosidase, had no effect on tumor growth in similar transplantable tumor models (19, 28). $CD8^+$ T cell populations were reduced by >95% as measured by flow cytometric analysis on day 24 (data not shown). Mice were vaccinated and tumors measured as described above.

Flow cytometric analysis

FVB/N mice, 6–8 wk old, were immunized with either PBS, 0.1 LD_{50} Lm-LLO-EC2, or 0.1 LD_{50} Lm-LLO-E7 and then boosted 21 days later. Three-color flow cytometry for CD8 (53-6.7, FITC conjugated), CD62 ligand (Mel-14, APC conjugated) (BD Biosciences), and HER-2/neu H-2^q tetramer (PE conjugated) was performed using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences). The tetramer was loaded with a H-2^q-specific PDSLRDLSVF peptide. Tetramers were provided by the National Institute of Allergy and Infectious Diseases Tetramer Core Facility of Emory University (Atlanta, GA), under the direction of Dr. J. Altman, and the National Institutes of Health AIDS Research and Reference Reagent Program. Splenocytes were harvested 5 days after the boost and were stained at room temperature with the tetramer for 1 h at a 1/200 dilution. Cells were then stained on ice with anti-CD8 and anti-CD62L Abs for 30 min. The splenocytes were then analyzed as described above comparing $CD8^+,CD62L^{low},tetramer^+$ cells generated by either PBS, Lm-LLO-EC2, or Lm-LLO-E7 vaccination. Analysis was done using FlowJo software (Tree Star).

^{51}Cr release assay

Six- to 8-wk-old FVB/N mice were immunized i.p with 0.1 LD_{50} Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, Lm-LLO-IC2, Lm-LLO-E7, or PBS or with 50 μg i.m. of pcDNA neu, pcDNA LLO-neu, pcDNA EC1, or pcDNA LLO-EC1. Mice were then sacrificed 9 days after immunization, and spleens were harvested. Splenocytes were then cultured with irradiated NT-2 (20,000 rad) tumor cells at a 100:1 ratio of splenocytes to tumor cells with 20 U/ml IL-2 (Roche). Following 4 days of culture, the splenocytes were used as effector cells in a standard ^{51}Cr release assay. Briefly, target cells (3T3-wt, 3T3-neu, 3T3-neu-1, 3T3-neu-2, 3T3-neu-3, 3T3-neu-4, 3T3-neu-5, 3T3-neu-6, 3T3-neu-7, 3T3-neu-8, and 3T3-neu-9 lines) were labeled with chromium and were then cultured with splenocytes at the E:T ratios of 200:1, 100:1, 50:1, and 25:1 in triplicate for 4 h. In addition, 3T3-wt cells were pulsed for a minimum of 1 h before labeling with ^{51}Cr with 1 μM of the H-2^q-specific PDSLRDLSVF peptide. Following the incubation, 100 μl of supernatant were assayed for ^{51}Cr release. The percent-specific lysis was determined as ((experimental counts per minute − spontaneous counts per minute)/(total counts per minute − spontaneous counts per minute)) × 100.

DNA vaccination

Female FVB/N mice that were 6–8 wk old were given 7 × 10^5 NT-2 tumor cells s.c. on the right flank. Mice were then vaccinated i.m. with either 50 μg of GM-CSF, 50 μg of pcDNA neu + GM-CSF, 50 μg of pcDNA LLO-neu + GM-CSF, 50 μg of pcDNA EC1 + GM-CSF, or 50 μg of pcDNA LLO-EC1 + GM-CSF or i.p. with PBS or 0.1 LD_{50} Lm-LLO-EC1. The vaccines were given on days 3, 10, and 18 and tumors were measured every 3 days. Mice were sacrificed when their tumors reached 20 mm in size.

Statistics

The Student t test was used for statistical analyses, and significant differences ($p < 0.05$) are noted.

Results

Generation of L. monocytogenes strains that secrete fragments of HER-2/neu

Five recombinant $L.$ $monocytogenes$ strains that express and secrete overlapping fragments of the rat HER-2/neu gene have been designed and constructed (Fig. 1A). In the design of these vaccine constructs, the signal sequence and transmembrane domains of HER-2/neu have been deleted due to the hydrophobicity of these regions and the inability of $L.$ $monocytogenes$ to secrete extremely hydrophobic domains. All the fragments have been cloned into the pGG-55 expression system described previously (19). The Lm-LLO-HER-2/neu constructs have been designed based on the effectiveness of an artificial tumor Ag, NP, and the HPV tumor Ag, E7, in this system (19–21). The secretion of each of the HER-2/neu fragment vaccines has been confirmed by Western blot of secreted Listerial proteins (Fig. 1B). Secretion of the fusion protein LLO-E7 by Lm-LLO-E7 as a control is shown at 67 kDa. An 83-kDa recombinant protein is secreted by Lm-LLO-EC1, 70 kDa by Lm-LLO-EC2, 68 kDa by Lm-LLO-EC3, 92.5 kDa by Lm-LLO-IC1, and 74 kDa by Lm-LLO-IC2. Each of these strains is highly attenuated as compared with the wild-type 10403S, but the virulence of each strain is comparable to Lm-LLO-E7, which is ~1 × 10^9 CFU.

Vaccination with each Lm-based HER-2/neu construct leads to stasis in tumor growth

Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, and Lm-LLO-IC2 were each compared with PBS controls and Lm-LLO-E7-vaccinated mice for their ability to impact on the growth
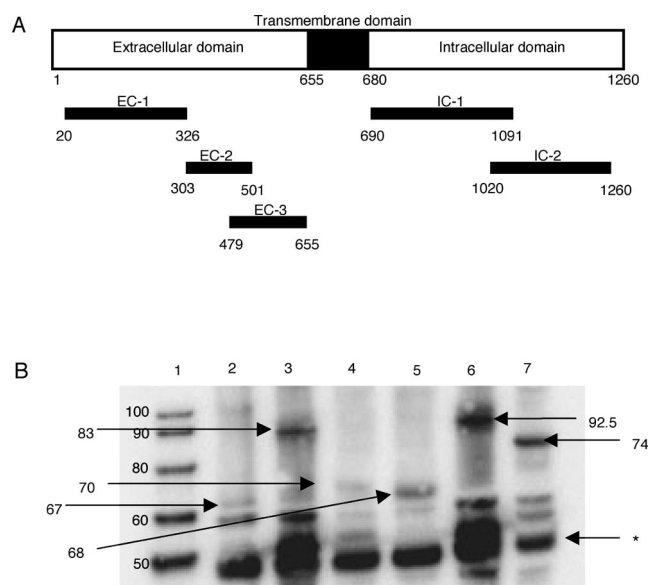
FIGURE 1. HER-2/neu has been broken into fragments, and recombinant $L.$ $monocytogenes$ is capable of secreting each of these vaccines as an LLO-fusion protein. A, Rat HER-2/neu was broken into fragments for the construction of a series of Lm-LLO-HER-2/neu vaccines as described under $Materials$ and $Methods$. B, Following the construction of each of these vaccines, the secretion of the fusion peptides was confirmed by Western blot analysis. Marker ($lane$ 1), Lm-LLO-E7 ($lane$ 2), Lm-LLO-EC1 ($lane$ 3), Lm-LLO-EC2 ($lane$ 4), Lm-LLO-EC3 ($lane$ 5), Lm-LLO-IC1 ($lane$ 6), and Lm-LLO-IC2 ($lane$ 7). * denotes the band for endogenous LLO secreted by all $L.$ $monocytogenes$. The $Listeria$ vaccines were grown overnight at 37°C in Luria-Bertani broth. Supernatants were TCA precipitated, and 15 μl of each sample were analyzed by Western blot analysis. The blot was probed with an anti-PEST rabbit serum, followed by HRP-conjugated anti-rabbit secondary Ab. The blot was developed using ECL detection reagents.

of the rat HER-2/neu-expressing tumor line, NT-2. FVB/N mice were given s.c. NT-2 tumor injections on the right flank that produced palpable tumors of 4–5 mm 7 days later. Mice were given weekly i.p. injections of 0.1 LD_{50} Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, Lm-LLO-IC2, or the controls Lm-LLO-E7 or PBS. Neither the PBS nor Lm-LLO-E7 injections had any impact on tumor growth, but within a week of the first vaccination with either Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, or Lm-LLO-IC2, NT-2 tumor growth became static and remained so until the experiment was terminated at 90 days (Fig. 2, *A* and *B*). This cessation in tumor growth remained well after the last HER-2/neu vaccination on day 21, and a subsequent regression of tumors in some of the mice was observed. Three of eight of the mice with Lm-LLO-EC2 and Lm-LLO-EC3 vaccinations were completely cured of their tumors, while one of eight mice vaccinated with Lm-LLO-EC1 or Lm-LLO-IC1 showed complete tumor regression. None of the tumors in the Lm-LLO-IC2 vaccinated group underwent a complete regression, but the

tumor growth of the mice in this group remained static and in some cases the tumor size decreased.

### CD8+ T cells are necessary for Lm-LLO-HER-2/neu vaccine effectiveness

For both Lm-LLO-E7 and Lm-LLO-NP, CD8+ T cells are absolutely necessary for vaccine effectiveness (19). FVB/N mice were depleted of CD8+ T cells after establishment of NT-2 tumors. Upon depletion of CD8+ T cells with the anti-CD8 Ab 2.43, each of the Lm-LLO-HER-2/neu vaccines lost all effectiveness (Fig. 3, *A* and *B*). Tumor growth in mice that were not depleted of CD8+ T cells could be controlled with vaccination of the mice with Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, and Lm-LLO-IC2 on days 7, 14, and 21. Growth of tumor in PBS mock-vaccinated mice from a similar experiment is shown in Fig. 6*C* for comparative purposes. The growth of tumor in PBS vaccinated mice is similar but slightly slower than the growth of tumors in the Lm-LLO-HER-2/neu-vaccinated mice that were depleted of CD8+ T cells (Fig. 3*C*). This suggests that endogenous CD8+ T cells induced by the tumor may be slowing tumor growth in unvaccinated mice as we have previously observed in other tumor models (21).

Lm-LLO-EC2 vaccination induces an increase in HER-2/neu-specific CD8+ T cells

The CD8+ T cell depletion tumor regression experiment shown in Fig. 3 clearly shows that CD8+ T cells have an essential role in the control of NT-2 tumor growth. Measurement of the HER-2/neu-specific CD8+ T cells activated through vaccination with the rLm constructs can be performed by tetramer analysis. This analysis is only possible for the Lm-LLO-EC2 construct because the only CD8+ T cell epitope known for the FVB/N mouse is from aa 420–429, which is contained within the EC2 fragment (26). Mice were vaccinated with 0.1 LD_{50} Lm-LLO-EC2 or Lm-LLO-E7 or injected with PBS and then boosted 21 days later. Splenocytes were then stained with the H-2q tetramer loaded with the PD-SLRDLVF peptide, and the activated CD8+ T cells were analyzed to determine the percentages that were tetramer positive (Fig. 4). A 5-fold increase in tetramer-positive cells in the spleens was observed in non-tumor-bearing FVB/N mice after vaccination with Lm-LLO-EC2 vs injection with either Lm-LLO-E7 or PBS.

CTL analysis shows the induction of CD8+ T cells against subdominant epitopes

Because each Lm-LLO-HER-2/neu vaccine is capable of impacting on tumor growth, yet only one FVB/N CD8+ T cell epitope has been described, CTL assays were performed using 3T3 cell lines expressing HER-2/neu fragments to determine which regions of HER-2/neu contain a potential epitope. Mice were immunized with one of the Lm-LLO-HER-2/neu vaccines, and the splenocytes were used as effector cells in a standard ^{51}Cr release assay. Initially each vaccine was tested with wild-type 3T3 cells as targets for a negative control and 3T3 cells transduced with full-length rat HER-2/neu (26) as a positive control. Each vaccine is capable of inducing an anti-HER-2/neu CTL response as evidenced by the specific lysis of 3T3-neu cells vs wild-type 3T3 cells (Fig. 5, *A* and *B*). Splenocytes from PBS-vaccinated mice produced little lysis regardless of the E:T ratio, while >20% lysis of 3T3-neu cells was produced by each of the Lm-LLO-HER-2/neu vaccine constructs at E:T ratios of 200:1 or 100:1. Splenocytes from PBS-vaccinated mice were used as a control for all subsequent CTL assays because no difference in lysis of 3T3-wt and 3T3-neu cells was seen using either PBS or Lm-LLO-E7-primed CTLs (Fig. 5, *C* and *D*). Both PBS and Lm-LLO-E7 vaccinations induced a very low level of
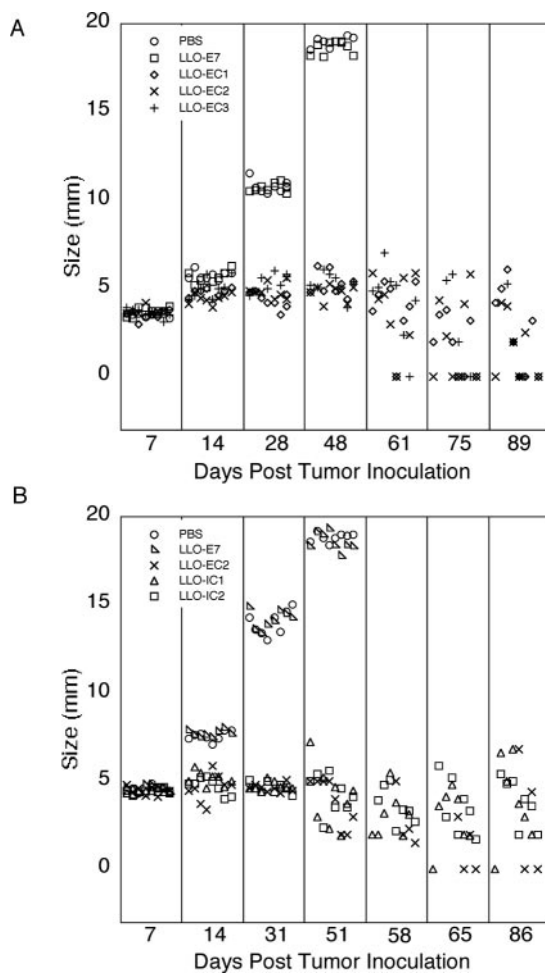
FIGURE 2. Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, and Lm-LLO-IC2 induce a halt in tumor growth of established NT-2 tumors. FVB/N mice (eight per group) were given 2×10^6 NT-2 cells by s.c. injection on the right flank. On days 7, 14, and 21 after tumor challenge, mice were treated with 0.1 LD_{50} Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, Lm-LLO-IC2, and Lm-LLO-E7 or 200 μl of PBS. Each data point represents the average tumor diameter of one mouse at a given time point as measured with calipers. Mice were sacrificed when the average tumor diameter reached 2.0 cm, and the tumor measurements for each time point are only shown for the surviving mice. Representative figures of two experiments are shown. Lm-LLO-EC1, Lm-LLO-EC2, and Lm-LLO-EC3 (*A*); Lm-LLO-IC1 and Lm-LLO-IC2 (*B*).

FIGURE 3. CD8^{+} T cells are necessary for Lm-LLO-HER-2/neu-induced tumor stasis. FVB/N mice (six mice per group) were given 2×10^6 NT-2 cells by s.c. injection on the right flank. Mice were treated with 0.1 LD$_{50}$ Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, or Lm-LLO-IC2 on days 7, 14, and 21 after tumor challenge. Mice were treated with 0.5 mg of 2.43 (anti-CD8 Ab) on days 6, 7, 8, 11, 14, 17, 20, and 23 following tumor challenge. Mice were sacrificed when tumor diameter reached ~2.0 cm, and the tumor measurements at each time point are shown only for the surviving mice. *A*, Lm-LLO-EC1, Lm-LLO-EC2, and Lm-LLO-EC3 both depleted and undepleted for CD8^{+} T cells. *B*, Lm-LLO-IC1 and Lm-LLO-IC2 both depleted and undepleted for CD8^{+} T cells. *C*, PBS-vaccinated mice that are not depleted of CD8^{+} T cells.
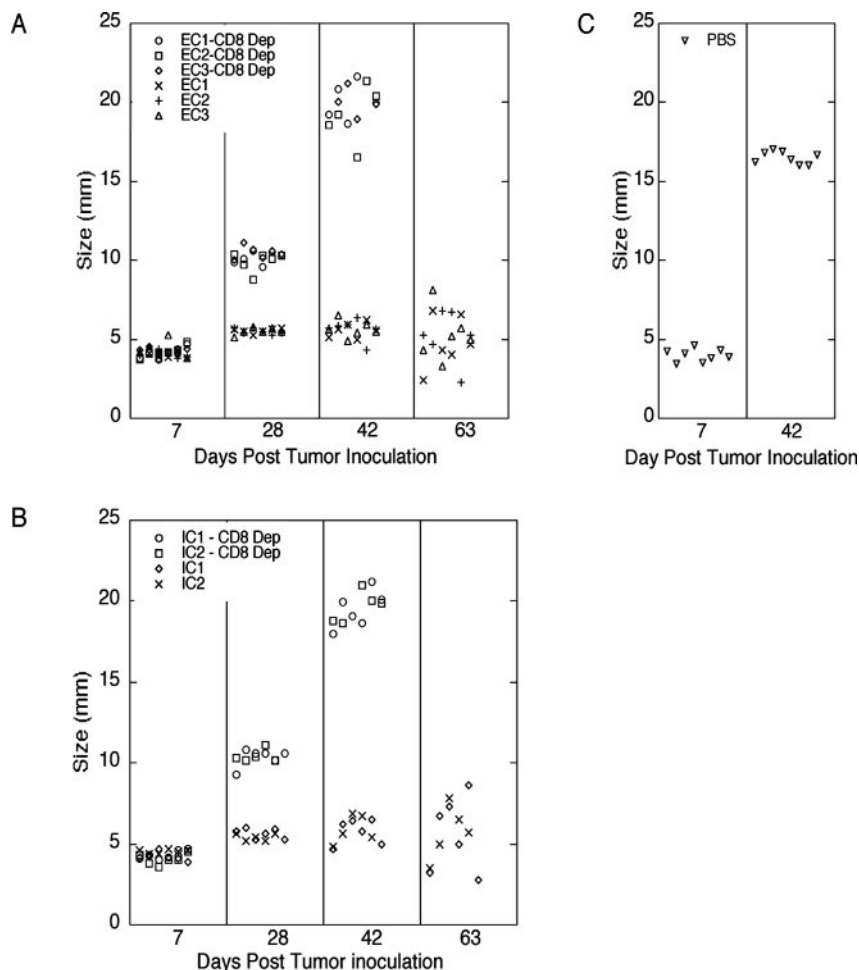
nonspecific lysis that was virtually equal. The levels of lysis seen from the Lm-LLO-HER-2/neu vaccinations with any of the HER-2/neu-expressing cell lines is similar to the lysis observed by pulsing 3T3-wt target cells with the known FVB/N CD8^{+} T cell epitope peptide at high E:T ratios (Fig. 5*E*). However, the lysis observed when not targeting a specific peptide titrates out by 50:1, whereas with a known peptide, a significant level of lysis can still be seen at an E:T ratio of 25:1 (Fig. 5*E*). This illustrates the fact that although these assays can be used as a guide to where in the HER-2/neu sequence potential CD8^{+} T cell epitopes are located, once a specific peptide sequence is identified, more sensitive CTL assays can be done.

Following the determination that each of the vaccine constructs was capable of eliciting an anti-HER-2/neu immune response in a CTL assay, a panel of 3T3 cells expressing fragments of HER-2/neu (26) were used as target cells for vaccinations with each of the corresponding rLm vaccines (Table I). Each vaccine is capable of eliciting a CTL response to at least one fragment of HER-2/neu. Several fragments of HER-2/neu likely contain a subdominant epitope as evidenced by lysis that was significantly different from controls ($p < 0.05$). Based on the CTL analysis of this panel of target cells, regions of HER-2/neu with potential subdominant epitopes for the FVB/N mouse have been identified.

Fusion to LLO and delivery by L. monocytogenes *enhances the immunogenicity of HER-2/neu*

There are several possibilities for why these recombinant *L. monocytogenes* vaccines are capable of revealing subdominant epitopes when other vaccination strategies have not. The Lm-LLO-HER-2/

neu vaccines may be revealing these subdominant epitopes through 1) delivery by *L. monocytogenes*, 2) fusion of the target tumor Ag to LLO, 3) breaking HER-2/neu into fragments, or 4) a combination of these factors. To determine which of these factors contributes to the enhanced immunogenicity of HER-2/neu in the Lm-LLO-HER-2/neu system, a series of DNA vaccines were constructed. Only the EC1 fragment was made to study the effect of breaking HER-2/neu into pieces because each Lm-LLO-HER-2/neu vaccine worked equally well in tumor regression studies. These vaccines consist of a pcDNA 3.1 backbone containing one of the following inserts: full-length HER-2/neu (pcDNA neu), full-length HER-2/neu fused to LLO (pcDNA LLO-neu), the EC1 fragment (pcDNA EC1), or the EC1 fragment fused to LLO (pcDNA LLO-EC1).

Following the construction of the pcDNA-HER-2/neu vaccines, a tumor regression experiment was done comparing these constructs with Lm-LLO-EC1. Mice were given 7×10^5 NT-2 cells s.c. and were vaccinated i.m. with the DNA vaccines plus GM-CSF on a separate pcDNA 3.1 plasmid and i.p. with Lm-LLO-EC1 or PBS on days 3, 10, and 18. GM-CSF was included as an adjuvant for the DNA vaccines because of its ability to enhance the efficacy of DNA vaccines (29). The best vaccine based on this experiment was Lm-LLO-EC1 (Fig. 6*A*). Two Lm-LLO-EC1-vaccinated mice never developed tumors and two more later regressed their tumors compared with the pcDNA LLO-EC1 group in which one mouse never developed a tumor. By day 62, the differences in tumor sizes of the Lm-LLO-EC1 and pcDNA LLO-EC1 groups are statistically different ($p < 0.05$) and remain so on day 76 by which time there are four mice in the Lm-LLO-EC1 group without
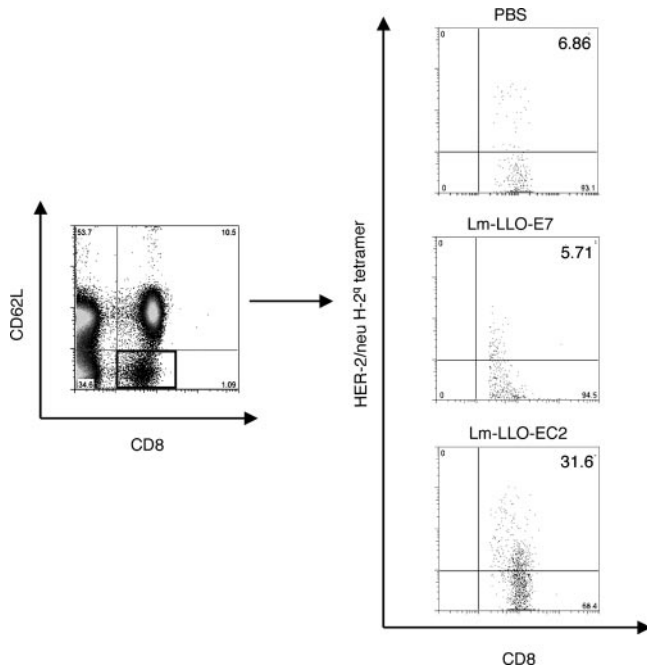
FIGURE 4. Lm-LLO-EC2 induces a 3-fold increase in tetramer$^+$,CD8$^+$ T cells. FVB/N mice were immunized and boosted 21 days later with 0.1 LD$_{50}$ Lm-LLO-EC2 and Lm-LLO-E7 or 200 μl of PBS. Five days after the boost, splenocytes were stained with an H-2^q HER-2/neu tetramer, anti-CD8, and anti-CD62L.

tumors, compared with only one mouse in the pcDNA LLO-EC1 group. Fusion to LLO was also compared through pcDNA EC1 vs pcDNA LLO-EC1 and pcDNA neu vs pcDNA LLO-neu (Fig. 6, *B* and *C*). The constructs where either the full-length HER-2/neu or the EC1 fragment are fused to LLO are clearly better in terms of tumor regression as compared with the unfused versions. Mice vaccinated with either of the unfused vaccines developed tumors that grew at the same rate as those in control mice vaccinated with just GM-CSF, which grew slightly slower than the tumors in mice vaccinated with only PBS. These differences in tumor sizes for pcDNA EC1 vs pcDNA LLO-EC1 and pcDNA neu vs pcDNA LLO-neu vaccinated mice are statistically significant ($p < 0.01$) on day 62 posttumor inoculation. The effect of breaking HER-2/neu up into smaller pieces was tested by comparing pcDNA LLO-neu vs pcDNA LLO-EC1 and pcDNA neu vs pcDNA EC1 (Fig. 6, *D* and *E*). Although there does seem to be minor differences in the tumor sizes between pcDNA LLO-EC1 and pcDNA LLO-neu between days 62 and 90 (Fig. 6*D*), this difference is not statistically significant. No difference could be seen between the pcDNA neu and pcDNA EC1-vaccinated mice, and tumors in both of these groups grew out as rapidly as the GM-CSF controls (Fig. 6*E*).

Broad CTL response to LLO-fused DNA vaccines vs unfused DNA vaccines

Each of the DNA vaccines was further analyzed using the standard ^{51}Cr release assay as described above. The LLO-fused versions of either full-length HER-2/neu or the EC1 fragment were capable of inducing T cells that lysed more of the 3T3-neu target cell lines than did the non-LLO-fused DNA vaccines (Table II). pcDNA neu induced a strong CTL response against only the 3T3-neu-4 target cell line, the line that contains the H-2^q epitope previously described by Ercolini et al. (26). The fusion of the full-length HER-2/neu to LLO resulted in a broadening of the regions that could be recognized by CTLs, and the same result was seen with the EC1
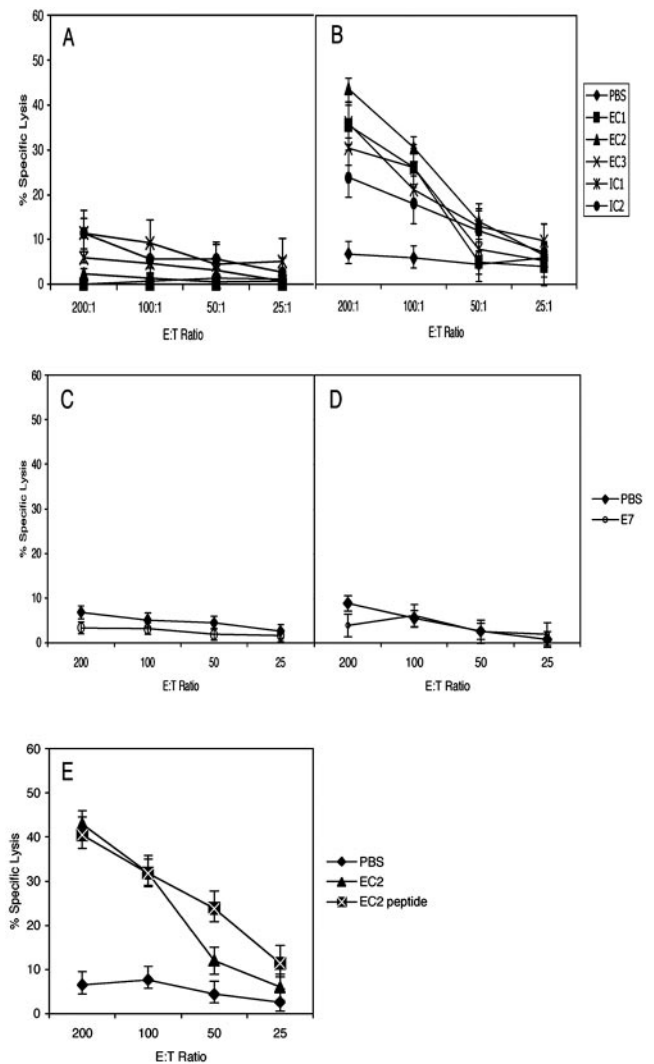
FIGURE 5. Each of the Lm-LLO-HER-2/neu vaccine constructs induces similar levels of anti-HER-2/neu CTL activity. FVB/N mice were immunized with 0.1 LD$_{50}$ Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, and Lm-LLO-IC2. Splenocytes were harvested 9 days later and cultured with irradiated NT-2 cells for 4 days. Following this restimulation, CTL activity was measured in a 4-h ^{51}Cr release from wild-type 3T3 (*A*) or 3T3-neu (full-length HER-2/neu) (*B*) targets. The ability of splenocytes from PBS-vaccinated mice was compared with splenocytes from Lm-LLO-E7 mice with either wild-type 3T3 (*C*) or 3T3-neu (*D*) target cells. *E*, Maximal levels of lysis in this type of assay was determined by the use of wild-type 3T3 target cells pulsed with the H-2^q-specific peptide. Results are shown as the mean of triplicate cultures. These results are representative of five experiments.

fragment fused to LLO vs the non-LLO-fused EC1. Thus, whereby only 3T3-neu-1 and 3T3-neu-4 were targeted for lysis by pcDNA neu splenocytes, 3T3-neu-1, -4, -5, and -9 were targeted by splenocytes from pcDNA-LLO-neu.

Discussion

Fusion of fragments of HER-2/neu to a truncated form of the *L. monocytogenes* hemolysin, LLO, and the delivery of the fusion constructs by *L. monocytogenes* increased the immunogenicity of this self-Ag. Five vaccines were constructed containing overlapping fragments of the rat HER-2/neu gene fused to a membrane inactive form of LLO. Based on previous epitope mapping, only

Table I. *Regions of HER-2/neu with potential H-2^q epitopes based on percent-specific lysis from CTL analysis and the corresponding vaccines*

Listeria Strain	Neu Region Spanned	Percent-Specific	Lysis of Target Cells[a] 200:1	100:1	Neu Regions Containing an Epitope
Lm-LLO-EC1	20–326	3T3-neu-1	14.3*	0.7	20–148
		3T3-neu-2	0	0	
		3T3-neu-3	6.5*	3.5	291–326
Lm-LLO-EC2	303–501	3T3-neu-3	10.9*	7.4	303–426
		3T3-neu-4	23.8*	8.4*	410–501
Lm-LLO-EC3	479–655	3T3-neu-4	1	0	
		3T3-neu-5	34.4*	25.3*	531–655
Lm-LLO-IC1	690–1081	3T3-neu-6	6.9*	9*	690–797
		3T3-neu-7	0	2.3	
		3T3-neu-8	18.2*	6.4	952–1081
Lm-LLO-IC2	1020–1260	3T3-neu-8	10.3*	8.2*	1020–1085
		3T3-neu-9	16.5	0	1063–1260

[a] Lysis of target cells shown as percent lysis minus background lysis by PBS control vaccinated mice for the E:T ratios of 200:1 and 100:1. Each well was set up in triplicate, and the results of a representative experiment are shown. * denotes statistically significant lysis above background level at $p < 0.05$. Percent-specific lysis before subtracting background PBS lysis was calculated as percent-specific lysis = 100 ((experimental lysis − spontaneous lysis)/(total lysis − spontaneous lysis)).

the Lm-LLO-EC2 vaccine was expected to generate an anti-HER-2/neu tumor response because this vaccine contains the only previously described rat HER-2/neu epitope for the FVB/N mouse, although there is evidence that this may not be the immunodominant epitope in the HER-2/neu-transgenic mouse (26, 30). Surprisingly, each of the five vaccine constructs generated an antitumor immune response that resulted in stasis of tumor growth and eventually a late onset of regression in a subset of the vaccinated mice. The antitumor immunity observed absolutely required CD8$^+$ T cells. The fact that each of these vaccine constructs could generate HER-2/neu-specific CD8$^+$ T cells and an antitumor response suggests that subdominant MHC class I-restricted HER-2/neu epitopes are being revealed through the use of these vaccines. This study shows that these epitopes emerge not only through the use of *L. monocytogenes* as a vaccine vector but also through the fusion of the HER-2/neu fragments to LLO.

Fusion to LLO was originally performed to ensure that the target Ag would be secreted by recombinant *L. monocytogenes*, resulting in presentation of the secreted target Ag by the MHC class I pathway and the generation of a CD8$^+$ T cell response (17). However, subsequent to these earlier experiments, we found that fusion of target Ags to either LLO or a PEST-like sequence contained in LLO resulted in an increased antitumor response (31). The exact reason for this enhancement in immunogenicity of fused Ags is not yet clear, but we hypothesize that these fused Ags are more readily degraded by the proteasome and subsequently targeted to the class I pathway. Decatur and Portnoy (32) have shown that deletion of a PEST-like sequence toward the N terminus of LLO results in an accumulation of LLO in the cytosol of the host cells, which suggests that this sequence may target LLO for degradation. However, we believe that LLO is doing more to increase the immunogenicity of target Ags than simply increasing the degradation and subsequent presentation of these Ags. We compared vaccines where the tumor Ag is fused to LLO vs fusion to PEST and showed that both elicited similar antitumor immune responses (31). However, even fusing the Ag to a LLO molecule from which the PEST sequence has been deleted resulted in better antitumor efficacy than using the Ag alone, suggesting that LLO may have an adjuvant effect quite apart from enhancing Ag processing. We have shown previously using vaccines that deliver the HPV-16 E7 Ag that Lm-LLO-E7 can mature bone marrow-derived dendritic cells and up-regulate

costimulatory molecules, whereas Lm-E7 does not, which also points to an adjuvant effect for LLO (33).

Potential regions of HER-2/neu with subdominant epitopes have been identified through CTL analysis following vaccination of mice with DNA vaccines consisting of either full-length or the EC1 fragment of HER-2/neu both fused to LLO and unfused. Lysis of regions of HER-2/neu not containing the identified dominant epitope (26) was seen with each of the four DNA vaccines. A broadening of the regions of HER-2/neu with potential epitopes based on this CTL analysis can be observed with fusion to LLO, further solidifying the theory that LLO enhances the immunogenicity of target Ags leading to the revelation of subdominant epitopes. Comparing the DNA vaccines, it is clear that fusion of the full-length neu to LLO increases the regions of neu that are recognized from two, with no LLO fusion, to four with LLO fusion (Table II). However, this expanded response was not equal to the responses seen with the *Listeria*-based HER-2/neu vaccines, as a total of nine regions with potential epitopes have been identified with these vaccines (Table I). Furthermore, the comparison of tumor regression induced by pcDNA LLO-EC1 with that of Lm-LLO-EC1 shows that Lm-LLO-EC1 is superior to pcDNA LLO-EC1 (Fig. 6*A*). Therefore, the increased epitope recognition of the *Listeria*-based vaccines vs the DNA vaccines is likely due to enhancement of the magnitude of the immune response through delivery by *Listeria*, which enhances the detection level of weak epitopes. These DNA vaccine results suggest that some epitopes, but not all, can be revealed through fusion to LLO, but some need the additional boost of delivery by *L. monocytogenes* as well, perhaps because of the added danger signals provided by a live vaccine vector (34) in addition to direct delivery to the MHC class I pathway of Ag processing by professional APCs.

DNA-based vaccines targeting HER-2/neu have been evaluated by other groups in transplantable tumor models (35–38). A major difference between the Lm-LLO- or DNA-LLO-HER-2/neu vaccine studies described here and those performed by other groups is that all of our vaccines were tested in a therapeutic, rather than a prophylactic, mode. In our studies, the first DNA vaccination was given 3 days after injection of the tumor but before the appearance of a palpable tumor. In the pcDNA LLO-EC1 group, one mouse never developed a tumor and one mouse later rejected its tumor, whereas there was a slow growth of tumor in the pcDNA LLO-neu
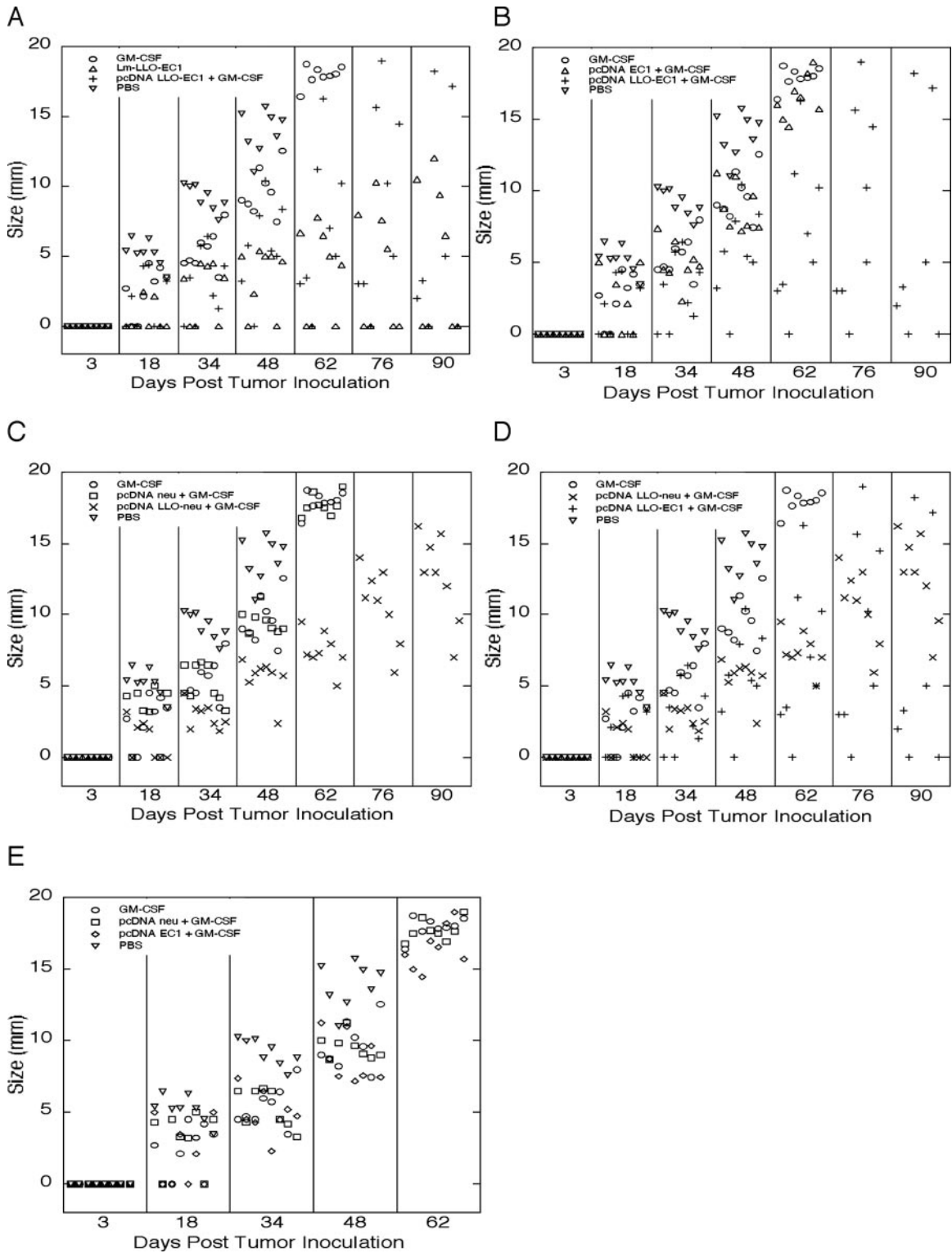
FIGURE 6. Delivery by *L. monocytogenes* and fusion to LLO increases the antitumor immune response of HER-2/neu vaccines. FVB/N mice (eight per group) received 7×10^5 NT-2 cells by s.c. injection in the right flank. Mice were treated on days 3, 10, and 18 with 0.1 LD_{50} Lm-LLO-EC1 or 50 μg of pcDNA neu + GM-CSF, pcDNA LLO-neu + GM-CSF, pcDNA EC1 + GM-CSF, pcDNA LLO-EC1 + GM-CSF, GM-CSF alone, or PBS. The average tumor diameter was measured with calipers and is shown for each mouse. Mice were sacrificed when tumor diameter reached ~2.0 cm, and tumor measurements are only shown for the surviving mice at each time point. Lm-LLO-EC1 vs pcDNA LLO-EC1 + GM-CSF (*A*), pcDNA EC1 + GM-CSF vs pcDNA LLO-EC1 + GM-CSF (*B*), pcDNA neu + GM-CSF vs pcDNA LLO-neu + GM-CSF (*C*), pcDNA LLO-neu + GM-CSF vs pcDNA LLO-EC1 + GM-CSF (*D*), and pcDNA neu + GM-CSF vs pcDNA EC1 + GM-CSF (*E*).

group (Fig. 6*D*). The Lm-LLO-HER-2/neu vaccines are given 7 days posttumor injection, at which point there is a 4- to 5-mm macroscopic tumor present. A subset of mice vaccinated with these

vaccines do completely regress their tumors, which is a very important result because it is extremely difficult to cause the regression or induce a halt in the growth of established tumors. The

Table II. *Regions of HER-2/neu with potential epitopes based on DNA vaccinations of wt FVB mice*

Listeria Strain	Neu Region Spanned	Percent-Specific	Lysis of Target Cells[a]		Neu Regions Containing an Epitope
			200:1	100:1	
pcDNA neu + GM-CSF	1–1260	3T3-neu-1	17.4*	12.4*	20–148
		3T3-neu-2	0	0.1	
		3T3-neu-3	0	1.6	
		3T3-neu-4	23.4*	19.5*	410–479
		3T3-neu-5	0	0.3	
		3T3-neu-6	2.6	0	
		3T3-neu-7	0	0.6	
		3T3-neu-8	0	1.2	
		3T3-neu-9	0	0.1	
pcDNA LLO-neu + GM-CSF	1–1260	3T3-neu-1	30.9*	20.5*	20–148
		3T3-neu-2	0.4	0	
		3T3-neu-3	1.9	1.7	
		3T3-neu-4	31.2*	25.5*	410–501
		3T3-neu-5	6.4*	6.4	479–531
		3T3-neu-6	0	0	
		3T3-neu-7	0	2.5	
		3T3-neu-8	0	4	
		3T3-neu-9	21*	15.8*	1085–1260
pcDNA EC1 + GM-CSF	1–326	3T3-neu-1	7.8	1.9	
		3T3-neu-2	0	2.1	
		3T3-neu-3	8.5*	0	291–326
pcDNA LLO-EC1 + GM-CSF	1–326	3T3-neu-1	14.6*	8.5*	20–148
		3T3-neu-2	0	0.5	
		3T3-neu-3	9.9*	5.1	291–326

[a] Lysis shown of each of the identified cell lines is identified, and lysis is noted as percent lysis minus background lysis from PBS control vaccinated mice. * denotes significant lysis above background at $p < 0.05$. Percent-specific lysis before the subtracting background levels was calculated as percent-specific lysis = 100 ((experimental lysis − spontaneous lysis)/(total lysis − spontaneous lysis)).

majority of HER-2/neu DNA vaccines produced by other groups were used in the less stringent prophylactic mode to determine whether vaccination will lead to protection against a subsequent tumor challenge (35–38). In addition, the majority of these studies have been performed in a BALB/c HER-2/neu tumor model, which shows a vastly different disease progression than the FVB/N mouse (36–38). Prophylactic DNA vaccination in the FVB/N mouse has not been shown to induce a complete rejection of a subsequent tumor challenge but will protect 25–100% of animals from developing tumors depending on the treatment used (35). In this study, the DNA vaccines studied were comprised of the extracellular domain of HER-2/neu, the transmembrane domain of HER-2/neu, or the intracellular domain of HER-2/neu. The extracellular domain DNA vaccine protected 25–35% of mice and that percentage was increased to 60–75% with the addition of IL-12. Approximately 50% of the mice were protected from tumor challenge after treatment with the intracellular DNA vaccine, and the administration of IL-12 increased this percentage of protected animals to between 80 and 100%. Sixty to 75% of mice were protected from tumor challenge with the administration of the transmembrane DNA vaccine, and this percentage remained the same with the addition of IL-12 (35).

In our study, each of the vaccines resulted in the stasis of tumor growth immediately following the first vaccination (Fig. 2). However, none of the tumors decreased in size until >1 mo following the last vaccination. Ag loss or mutation and the down-regulation of MHC class I molecules frequently result in the lack of tumor regression (39). In the case of HER-2/neu-overexpressing tumors, where the overexpression of the tumor Ag contributes to the transformed phenotype of the tumors, the loss of the tumor Ag could also result in a lack of tumor growth. Neu-negative variants have been observed after anti-HER-2/neu therapies (40). We are currently attempting to determine whether the tumors that do not decrease in size or undergo regression in response to the *Listeria*-based vaccines are down-regulating HER-2/neu.

The induction of tumor regression at >1 mo after the final boost is in contrast with our observations using other tumor Ags where tumor regression will often begin after the first immunization and is completed within 28 days (19, 21, 31). The delay in tumor regression observed with the Lm-LLO-HER-2/neu vaccines is not likely due to the reactivation of a memory response. Although, it is likely that memory cells are generated following the recombinant *L. monocytogenes* vaccines, no additional vaccination or stimulus is given to reactivate these memory cells at the time that the tumor regression begins. We think it is more plausible that the increase in antitumor reactivity at a delayed time point is due to epitope spreading by cross-presentation of tumor-derived HER-2/neu or other Ags.

Epitope spreading has been seen previously in many different tumor models and can result in the generation of a specific antitumor response to cryptic and subdominant epitopes that were not initially part of the treatment administered (41–43). The *Listeria*-based vaccines containing fragments of HER-2/neu may be generating sufficient initial antitumor CD8$^+$ T cells to limit the growth of the tumor early after vaccination. Although the tumor growth is stabilized with new tumor cells being generated at the same rate that tumor cells are dying, APCs such as dendritic cells may be acquiring pieces of the dying cells, traveling to the draining lymph nodes, and cross-presenting these Ags to activate a much broader CD8$^+$ T cell response than could be generated by vaccination targeting one region of the HER-2/neu molecule. This phenomenon has been observed previously, and cross-presentation by CD11c$^+$ dendritic cells is seen as a key factor in the control of tumor growth because the CD8$^+$ T cell response elicited by a specific immunotherapy can be significantly enhanced by this mechanism (44, 45). We intend to explore this hypothesis by testing the reactivity of the

lymphocytes in the tumor-draining lymph nodes to see whether T cells specific to a region of HER-2/neu that was not included in that particular vaccine construct emerges at the time that regression begins. However, this task will be easier once we have completed our epitope mapping of the entire HER-2/neu molecule, which is currently underway. We have already determined that there are a large number of CD11c$^+$ dendritic cells infiltrating the tumors following the vaccinations before the onset of regression (data not shown) that could cross-present Ags.

The most effective antitumor response will likely be generated by enhancing low-affinity anti-HER-2/neu-specific CD8$^+$ T cells that were not deleted during thymic selection (46). This will be particularly important in transgenic mouse models of cancer in which the mice are tolerant to the tumor Ag and the high-affinity T cells that can be generated to target the tumor in wild-type (nontolerant) mice will not be present (47, 48). Makki et al. (49) and Kedl et al. (50) have shown that targeting an immunodominant tumor epitope can fail to result in a strong enough antitumor response that will result in the eradication of a tumor. Anti-HER-2/neu therapies that can lead to the amplification of subdominant epitopes are likely be more effective than vaccination against dominant epitopes.

Potentially confounding the efficacy of these vaccines is the fact that the vaccines are based on the rat HER-2/neu gene but that these vaccines are being tested in mice. In this study, the wild-type FVB/N mice are not specifically tolerant to the rat HER-2/neu gene, although the mouse and rat HER-2/neu genes are >90% homologous (51), so some degree of tolerance is expected. Although these sequences are extremely homologous, there are differences in the degree of homology for the different vaccine constructs. The IC1 construct containing the highly conserved kinase domain is 98% homologous to the mouse protein sequence, and this sequence shows the highest level of homology among the five vaccine constructs. Both the EC1 and EC2 regions are 94% homologous with the corresponding mouse regions, and the EC3 and IC2 regions are 93% homologous. In all of the vaccines, excluding Lm-LLO-IC1, the amino acid differences are spread throughout the entire fragment, whereas in IC1 the differences flank the kinase domain with only one nonhomologous amino acid in the kinase domain making the kinase domain 99.6% homologous. Interestingly, the CTL analysis of the *Listeria*-based vaccines (Table I) shows that of the three 3T3 lines that can be targeted by this fragment only two, 3T3-neu-6 and 3T3-neu-8, were capable of being lysed by effector cells induced by Lm-LLO-IC1 vaccination. Although we do not yet know the exact sequences of the MHC class I epitopes that are recognized in these fragments, it is possible that there are no epitopes present in the kinase domain, as this region is almost entirely homologous between the mouse and the rat. In addition, the portion of HER-2/neu contained in 3T3-neu-7 falls entirely in the kinase domain, and there is no CTL response against this region.

We do not observe a strong correlation between CTL activity and tetramer analysis of HER-2/neu-specific T cells from mice immunized with Lm-LLO-EC2, for which we have a tetramer available. This is because for most of the vaccines, the only source of Ag available to stimulate bulk populations of T cells in splenocytes is irradiated tumor cells. The NT-2 cells do not express a high level of Ag and thus are not very potent stimulators of effector cells. Furthermore, the effector cells used are a polyclonal T cell population that is derived from whole splenocytes, so CD8$^+$ T cells are not the only cells being counted as effector cells. In contrast, tetramer-positive T cells can be analyzed in the activated CD8$^+$ T cell population. Despite these limits to the CTL assay, we do see statistically significant levels of killing of the target cells

from which we are able to narrow down potential areas that contain CTL epitopes.

We have shown in this study that vaccines generated from fragments of HER-2/neu that do not contain the dominant epitope are as effective as the vaccine that does result in CD8$^+$ T cells specific to the dominant epitope when fused to LLO and delivered by *L. monocytogenes*. Work is now underway to identify these subdominant epitopes and to test these vaccines in a HER-2/neu-transgenic mouse model for breast cancer (30).

Acknowledgments

Disclosures

References

1. Bargmann, C. I., M. C. Hung, and R. A. Weinberg. 1986. The neu oncogene encodes an epidermal growth factor receptor-related protein. *Nature* 319: 226–230.
2. Kim, H., and W. J. Muller. 1999. The role of the epidermal growth factor receptor family in mammary tumorigenesis and metastasis. *Exp. Cell Res.* 253: 78–87.
3. King, C. R., M. H. Kraus, and S. A. Aaronson. 1985. Amplification of a novel v-erB-related gene in human mammary carcinoma. *Science* 229: 974–976.
4. Yarden, Y., and M. X. Sliwkowski. 2001. Untangling the ErbB signaling network. *Nat. Rev. Mol. Cell Biol.* 2: 127–137.
5. Disis, M. L., and M. A. Cheever. 1997. HER-2/neu protein: a target for antigen-specific immunotherapy of human cancer. *Adv. Cancer Res.* 71: 343–371.
6. Knutson, K. L., K. Schiffman, K. Rinn, and M. L. Disis. 1999. Immunotherapeutic approaches for the treatment of breast cancer. *J. Mammary Gland Biol. Neoplasia* 4: 353–365.
7. Li, B. D., S. P. Harlow, R. M. Budnick, D. L. Sheedy, and C. C. Stewart. 1994. Detection of HER-2/neu oncogene amplification in flow cytometry-sorted breast ductal cells by competitive polymerase chain reaction. *Cancer* 73: 2771–2778.
8. Cohen, B. D., C. B. Siegall, S. Bacus, L. Foy, J. M. Green, I. Hellstrom, K. E. Hellstrom, and H. P. Fell. 1998. Role of epidermal growth factor receptor family members in growth and differentiation of breast carcinoma. *Biochem. Soc. Symp.* 63: 199–210.
9. Coronella, J. A., P. Telleman, G. A. Kingsbury, T. D. Truoung, S. Hays, and R. P. Junghans. 2001. Evidence for an antigen-driven humoral immune response in medullary ductal breast cancer. *Cancer Res.* 61: 7889–7899.
10. Peoples, G. E., R. C. Smith, D. C. Linehan, I. Yoshino, P. S. Goedegeburre, and T. J. Eberlein. 1995. Shared T cell epitopes in epithelial tumors. *Cell. Immunol.* 164: 279–286.
11. Tuttle, T. M., B. W. Anderson, W. E. Thompson, J. E. Lee, A. Sahin, T. L. Smith, K. H. Grabstein, J. T. Wharton, C. G. Ioannides, and J. L. Murray. 1998. Proliferative and cytokine responses to class II HER-2/neu-associated peptides in breast cancer patients. *Clin. Cancer Res.* 4: 2015–2024.
12. Southwick, F. S., and D. L. Purich. 1996. Intracellular pathogenesis of listeriosis. *N. Engl. J. Med.* 334: 770–776.
13. Dussurget, O., J. Pizarro-Cerda, and P. Cossart. 2004. Molecular determinants of *Listeria monocytogenes* virulence. *Ann. Rev. Microbiol.* 58: 587–610.
14. Mackaness, G. B. 1962. Cellular resistance to infection. *J. Exp. Med.* 116: 381–406.
15. Gedde, M. M., D. E. Higgins, L. G. Tilney, and D. A. Portnoy. 2000. Role of listeriolysin O in cell-to-cell spread of *Listeria monocytogenes*. *Infect. Immun.* 68: 999–1003.
16. Hsieh, C. S., S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra, and K. M. Murphy. 1993. Development of Th1 CD4$^+$ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science* 260: 547–549.
17. Ikonomidis, G., Y. Paterson, F. J. Kos, and D. A. Portnoy. 1994. Delivery of a viral antigen to the class I processing and presentation pathway by *Listeria monocytogenes*. *J. Exp. Med.* 180: 2209–2218.
18. Paterson, Y., and R. S. Johnson. 2004. Progress towards the use of *Listeria monocytogenes* as a live bacterial vaccine vector for the delivery of HIV antigens. *Expert Rev. Vac.* 3(4 Suppl.): S119–S134.
19. Gunn, G. R., A. Zubair, C. Peters, Z. K. Pan, T. C. Wu, and Y. Paterson. 2001. Two *Listeria monocytogenes* vaccine vectors that express different molecular forms of human papilloma virus-16 (HPV-16) E7 induce qualitatively different T cell immunity that correlates with their ability to induce regression of established tumors immortalized by HPV-16. *J. Immunol.* 167: 6471–6479.
20. Pan, Z. K., L. M. Weiskirch, and Y. Paterson. 1999. Regression of established B16F10 melanoma with a recombinant *Listeria monocytogenes* vaccine. *Cancer Res.* 59: 5264–5269.
21. Pan, Z. K., G. Ikonomidis, A. Lazenby, D. Pardoll, and Y. Paterson. 1995. A recombinant *Listeria monocytogenes* vaccine expressing a model tumour antigen

protects mice against lethal tumour cell challenge and causes regression of established tumours. *Nat. Med.* 1: 471–477.

22. Machiels, J. P. H., R. T. Reilly, L. A. Emens, A. M. Ercolini, R. Y. Lei, D. Weintraub, F. I. Okoye, and E. M. Jaffee. 2001. 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.

23. Wolope, M. E., E. R. Lutz, A. M. Ercolini, S. Murata, S. E. Ivie, E. S. Garrett, L. A. Emens, E. M. Jaffee, and R. T. Reilly. 2003. HER-2/neu-specific monoclonal antibodies collaborate with HER-2/neu-targeted granulocyte macrophage colony-stimulating factor secreting whole cell vaccination to augment CD8$^+$ T cell effector function and tumor-free survival in Her-2/neu-transgenic mice. *J. Immunol.* 171: 2161–2169.

24. Manjili, M. H., X. Y. Wang, X. Chen, T. Martin, E. A. Repasky, R. Henderson, and J. R. Subjeck. 2003. HSP110-HER-2/neu chaperone complex vaccine induces protective immunity against spontaneous mammary tumors in HER-2/neu transgenic mice. *J. Immunol.* 171: 4054–4061.

25. Reilly, R. T., J. P. H. Machiels, L. A. Emens, A. M. Ercolini, R. I. Okoye, R. Y. Lei, D. Weintraub, and E. M. Jaffee. 2001. 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.

26. Ercolini, A. M., J. P. Machiels, Y. C. Chen, J. E. Slansky, M. Giedlen, R. T. Reilly, and E. M. Jaffee. 2003. Identification and characterization of the immunodominant rat HER-2/neu MHC class I epitope presented by spontaneous mammary tumors from HER-2/neu-transgenic mice. *J. Immunol.* 170: 4273–4280.

27. Reilly, R. T., M. B. C. Gottlieb, A. M. Ercolini, J. P. H. Machiels, C. E. Kane, F. I. Okoye, W. J. Muller, K. H. Dixon, and E. M. Jaffee. 2000. HER-2/neu is a tumor rejection target in tolerized HER-2/neu transgenic mice. *Cancer Res.* 60: 3569–3576.

28. Weiskirch, L. M., Z. K. Pan, and Y. Paterson. 2001. The tumor recall response of antitumor immunity primed by a live, recombinant *Listeria monocytogenes* vaccine comprises multiple effector mechanisms. *Clin. Immunol.* 98: 346–357.

29. McKay, P. F., D. H. Barouch, S. Santra, S. M. Sumida, S. S. Jackson, D. A. Gorgone, M. A. Lifton, and N. L. Letvin. 2004. Recruitment of different subsets of antigen-presenting cells selectively modulates DNA vaccine-elicited CD4$^+$ and CD8$^+$ T lymphocyte responses. *Eur. J. Immunol.* 34: 1011–1020.

30. Guy, C. T., M. A. Webster, M. Schaller, T. J. Parsons, R. D. Cardiff, and W. J. Muller. 1992. Expression of the *neu* protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc. Natl. Acad. Sci. USA* 89: 10578–10582.

31. Sewell, D. A., V. Shahabi, G. R. Gunn 3rd, Z. K. Pan, M. E. Dominiecki, and Paterson Y. 2004. Recombinant *Listeria* vaccines containing PEST sequences are potent immune adjuvants for the tumor-associated antigen human papillomavirus-16 E7. *Cancer Res.* 64: 8821–8825.

32. Decatur, A. L., and D. A. Portnoy. 2000. A PEST-like sequence in listeriolysin O essential for *Listeria monocytogenes* pathogenicity. *Science* 290: 992–995.

33. Peng, X., S. F. Hussain, and Y. Paterson. 2004. The ability of two *Listeria monocytogenes* vaccines targeting human papillomavirus-16 E7 to induce an antitumor response correlates with myeloid dendritic cell function. *J. Immunol.* 172: 6030–6038.

34. Gallucci, S., and P. Matzinger. 2001. Danger signals: SOS to the immune system. *Cur. Opin. Immunol.* 13: 114–119.

35. Chen, Y., D. Hu, D. J. Eling, J. Robbins, and T. J. Kipps. 1998. DNA vaccines encoding full-length or truncated neu induce protective immunity against neu-expressing mammary tumors. *Cancer Res.* 58: 1965–1971.

36. Chang, S. Y., K. C. Lee, S. Y. Ko, H. J. Ko, and C. Y. Kang. 2004. Enhanced efficacy of DNA vaccination against Her-2/neu tumor antigen by genetic adjuvants. *Int. J. Cancer* 111: 86–95.

37. Rovero, S., A. Amici, E. Di Carlo, R. Bei, P. Nanni, E. Quagliano, P. Porcedda, K. Boggio, A. Smorlesi, P. L. Lollini, et al. 2000. 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.

38. Lin, C. C., C. W. Chou, A. L. Shiau, C. F. Tu, T. M. Ko, Y. L. Chen, B. C. Yang, M. H. Tao, and M. D. Lai. 2004. Therapeutic HER2/Neu DNA vaccine inhibits mouse tumor naturally overexpressing endogenous neu. *Mol. Ther.* 10: 290–301.

39. Ahmad, M., R. C. Rees, and S. A. Ali. 2004. Escape from immunotherapy: possible mechanisms that influence tumor regression/progression. *Cancer Immunol. Immunother.* 53: 844–854.

40. Knutson, K. L., B. Almand, Y. Dang, and M. L. Disis. 2004. neu antigen-negative variants can be generated after neu-specific antibody therapy in neu transgenic mice. *Cancer Res.* 64: 1146–1151.

41. Duraiswamy, J., M. Bhardwaj, J. Tellam, G. Connolly, L. Cooper, D. Moss, S. Thomson, P. Yotnda, and R. Khanna. 2004. Induction of therapeutic T cell responses to subdominant tumor-associated viral oncogene after immunization with replication-incompetent polyepitope adenovirus vaccine. *Cancer Res.* 64: 1483–1489.

42. Disis, M. L., V. Goodell, K. Schiffman, and K. L. Knutson. 2004. Humoral epitope-spreading following immunizations with a HER-2/neu peptide based vaccine in cancer patients. *J. Clin. Immunol.* 24: 571–578.

43. van der Most, R. G., K. Murali-Krishna, J. G. Lanier, E. J. Wherry, M. T. Puglielli, J. N. Blattman, A. Sette, and R. Ahmed. 2003. Changing immunodominance patterns in antiviral CD8 T cell responses after loss of epitope presentation or chronic antigenic stimulation. *Virology* 315: 93–102.

44. Stumbles, P. A., R. Himbeck, J. A. Frelinger, E. J. Collins, R. A. Lake, and B. W. S. Robinson. 2004. Cutting edge: Tumor-specific CTL are constitutively cross-armed in draining lymph nodes and transiently disseminate to mediate tumor regression following systemic CD40 activation. *J. Immunol.* 173: 5923–5928.

45. Nowak, A. K., R. A. Lake, A. L. Marzo, B. Scott, W. R. Heath, E. J. Collins, J. A. Frelinger, and B. W. S. Robinson. 2003. Induction of tumor cell apoptosis in vivo increases tumor antigen cross-presentation, cross-priming rather than cross-tolerizing host tumor-specific CD8 T cells. *J. Immunol.* 170: 4905–4913.

46. Lyman, M. A., C. T. Nugent, K. L. Marquardt, J. A. Biggs, E. G. Pamer, and L. A. Sherman. 2005. The fate of low affinity tumor-specific CD8$^+$ T cells in tumor-bearing mice. *J. Immunol.* 174: 2563–2572.

47. Lustgarten, J., A. L. Dominguez, and C. Cuadros. 2004. The CD8$^+$ T cell repertoire against HER-2/neu antigens in neu transgenic mice is of low avidity with antitumor activity. *Eur. J. Immunol.* 34: 752–761.

48. Slifka, M. K., J. N. Blattman, D. J. Sourdive, F. Liu, D. L. Huffman, T. Wolfe, A. Hughes, M. B. Oldstone, R. Ahmed, and M. G. Von Herrath. 2003. Preferential escape of subdominant CD8$^+$ T cells during negative selection results in an altered antiviral T cell hierarchy. *J. Immunol.* 170: 1231–1239.

49. Makki, A., G. Weidt, N. E. Blachere, L. Lefrancois, and P. K. Srivastava. 2002. Immunization against a dominant tumor antigen abrogates immunogenicity of the tumor. *Cancer Immun.* 2: 4–12.

50. Kedl, R. M., J. W. Kappler, and P. Marrack. 2003. Epitope dominance, competition and T cell affinity maturation. *Curr. Opin. Immunol.* 15: 120–127.

51. Gallo, P., S. Dharmapuri, M. Nuzzo, D. Maldini, M. Iezzi, F. Cavallo, P. Musiani, G. Forni, and P. Monaci. 2005. Xenogeneic immunization in mice using HER2 DNA delivered by an adenoviral vector. *Int. J. Cancer* 113: 67–77.