

Antibody and CD8⁺ T Cell Responses against HER2/neu Required for Tumor Eradication after DNA Immunization with a Flt-3 Ligand Fusion Vaccine

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Abstract **Purpose:** HER2/neu is frequently overexpressed in breast cancer. In a mouse model, vaccination with HER2/neu DNA elicits antibodies that confer partial protection against tumor challenge. **Experimental Design:** To enhance antitumor immunity, we fused cDNA encoding Flt-3 ligand (FL) to the rat HER2/neu extracellular domain (neu), generating a chimeric FLneu molecule. FLneu and neu DNA vaccines were compared for immunogenicity and their ability to protect mice from tumor challenge. **Results:** The neu vaccine generated a HER2/neu-specific antibody response. In contrast, vaccination with FLneu induced CD8⁺ T cells specific for HER2/neu but a negligible anti-HER2/neu antibody response. The switch from an antibody-mediated to T cell – mediated response was due to different intracellular localization of neu and FLneu. Although the neu protein was secreted, the FLneu protein was retained inside the cell, co-localizing with the endoplasmic reticulum, facilitating processing and presentation to T cells. The neu and FLneu vaccines individually conferred only weak tumor immunity. However, efficient tumor rejection was seen when neu and FLneu were combined, inducing both strong anti-HER2/neu-specific antibody and T cell responses. Adoptive transfer of both immune CD8⁺ T cells and immune sera from immunized mice was required to confer tumor immunity in naïve hosts. **Conclusions:** These results show that active induction of both humoral and cellular immunity to HER2/neu is required for efficient tumor protection, and that neither response alone is sufficient.

HER2/neu is a tyrosine-kinase receptor and a member of the epidermal growth factor receptor (EGFR) family, which functions to transmit signals regulating cell growth and differentiation (1, 2). This receptor plays a central role in the pathogenesis of several human cancers, including breast, ovarian, renal, colon, and lung carcinomas (3–5). When overexpressed or mutated, HER2/neu forms homo- and heterodimers with other members of the EGFR family, which results in the transduction of positive growth signals in a ligand-independent manner (6). HER2/neu is amplified and

overexpressed in about 20% to 30% of invasive breast cancers (7), and overexpression is associated with aggressive disease and poor prognosis (8).

The involvement of the adaptive immune system in the eradication of tumor cells is well documented (9, 10). Many cancer immunotherapy strategies are designed to activate specific CTLs, which play a major role in tumor rejection. In addition, treatment with antibodies against CD20 (11), EGFR (12), and HER2/neu (13) have been successful and generated new interest in the contribution of humoral effector molecules. Trastuzumab, a humanized monoclonal antibody specific for HER2/neu, is active in combination with chemotherapy in patients with breast cancer overexpressing HER2/neu (14, 15). Antibody and T cell responses specific for HER2/neu are detected in patients with HER2/neu+ breast cancer (16). Although these natural responses do not apparently prevent tumor development and progression, they suggest that immune responses to HER2/neu can occur.

Better strategies are needed to enhance the immunogenicity of inherently weak immunogenic self-antigens such as HER2/neu. We have used xenogeneic immunization to overcome immune ignorance or tolerance to self-antigens expressed by cancer (17–20). Our results show that immunization with DNA encoding orthologues can generate both humoral and cellular immunity against tumors (21–23). The xenogeneic DNA vaccination strategy has been efficacious in outbred companion animals (pet dogs) with spontaneous melanoma, and a human tyrosinase DNA vaccine has received conditional

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Food and Drug Administration approval in dogs as the first licensed tumor DNA vaccine (24, 25). In humans, xenogeneic DNA vaccines are being used in multiple clinical trials for patients with melanoma and prostate and breast cancer.

In this study, we report the preclinical evaluation of two DNA-based vaccines against HER2/neu+ breast cancer. The first encodes the rat HER2/neu extracellular domain (neu), whereas the second contains the mouse Flt-3 ligand extracellular domain (FL) fused upstream to the neu sequence (FLneu). The FL cytokine has been shown to augment the function and quantity of dendritic cells (DC) *in vivo* (26, 27) by binding to and activating a tyrosine kinase receptor, Flt3R, which has homology to c-Kit and c-Fms (the receptor for M-CSF; ref. 28). Flt3R is expressed by multipotent hematopoietic progenitor cells, common lymphoid and myeloid progenitors, including DC precursors, and mature steady-state DCs (29). Furthermore, in previous studies, the potency of DNA vaccination was augmented by the covalent linkage of the E7 viral antigen to FL (30).

Our results reveal that the neu vaccine generated predominantly antibody-mediated immunity, whereas the FLneu triggered a predominant T cell-mediated immune response. Neither vaccine alone protected mice from challenge with a syngeneic HER2/neu+ mammary tumor. In contrast, a combination vaccine that activates both the humoral and cell-mediated immune responses conferred excellent tumor immunity, demonstrating that synergy between antibodies and T cells is necessary to achieve potent tumor protection in this model.

Materials and Methods

Cell lines. The N202.1A and N202.1E (31) cell lines were derived from two independent mammary carcinomas in FVB-neuN mice (H-2^d) transgenic for the rat neu proto-oncogene (32). The 233-VSGA1 tumor cell line was also derived from mouse mammary carcinoma in FVB-neuNT mice transgenic for the activated rat neu (33, 34). These cell lines were cultured as described (31). All three cell lines were a kind gift from Pier Luigi Lollini (University of Bologna, Italy).

The T2D^d cell line expressing the mouse H2-D^d was graciously donated by Elizabeth Jaffee (Johns Hopkins University, Baltimore, MD) and was maintained as described (35). The COS-7 cells were purchased from American Type Culture Collection (CRL-1651) and maintained in culture according to standard guidelines.

Mice and route of injection. Female FVB/N mice (Taconic), 6 to 8 weeks of age, were maintained and treated according to guidelines of the Animal Health Services of Memorial Sloan-Kettering Cancer Center (MSKCC). All mouse experiments were done under a protocol approved by the Institutional Animal Care & Utilization Committee of MSKCC.

Mice were immunized once a week for 3 weeks by particle bombardment using a gene gun provided by PowderMed, Inc. as previously described (22, 36). DNA (typically 100 µg) was coated onto plastic tubing. The total amount of DNA was doubled when mice received the combination of two plasmids (neu+empty vector, FLneu+empty vector, and neu+FLneu). Abdominal hair of mice was removed, and DNA-gold complexes containing 4 µg DNA per plasmid were injected into the dermis of the mouse abdomen by particle bombardment using 400 psi of pressure.

DNA constructs. All cDNAs were cloned into the expression vector pCDNA3.1(+) (Invitrogen). The extracellular domain of rat HER2/neu (neu) was amplified by PCR from the pCMVneuNT (37) plasmid using the primers forward: 5'-CGAAGCTTACCATGGAGCTGGCGGCTGG-3' and reverse: 5'-CGGAATTCCTATGTCACCGGGCTGGC-3'. The *Hind*III-*Eco*RI fragment was cloned into pCDNA3.1(+). A fragment encoding the

signal peptide and the extracellular domain of FL was isolated by PCR using a mouse FL DNA template, pNGVL-mFL (University of Indiana), and the following primers: forward 5'-GCAAGCTTACCATGACAGTGTGGCGC-3' and reverse 5'-GCGAATTCCTACTGCCTGGGCCGAGGCTCT-3'. A second FL fragment that does not include the stop codon was also generated by PCR using conditions described by Hung et al. (30), digested with *Hind*III and *Kpn*I and inserted in pCDNA3.1(+). A DNA fragment encoding neu was isolated and then inserted in frame, downstream of the FL sequence, generating the chimeric plasmid FLneu.

The QuickChange site-directed mutagenesis kit (Stratagene) was used to introduce mutations in FLneu. Oligonucleotides were designed according to the manufacturer's guidelines (mutated nucleotides are underlined): 5'-CTGTTACTTCAGCCGAGTCCCATCTCCTCC-3' (FL_H8R), 5'-CTTCAGCCACAGTCCCTACTCTCCAACCTTC-3' (FL_I11Y), 5'-CAGCTGCTTGGTCTGGAGCCCTGTATCGGG-3' (FL_K116E), 5'-GACTGTTACTTCAGCTACAGTCCCATCTCC-3' (FL_H8Y) and 5'-GATACATTTGTCACCGAATGTACCTCCAGCCCC-3' (FL_S84E).

All constructs were confirmed by sequencing (MSKCC core facility). The Qiagen Plasmid Maxi kit was used for large-scale preparation of plasmids.

Tumor challenge. Mice were challenged intradermally in the right flank with 50 µL (5×10^4 cells) of a single cell suspension of 233-VSGA1 tumor cells 5 days after the last DNA injection. The incidence and growth of tumors were then measured thrice a week with calipers in two perpendicular diameters for a minimum of 60 days. Mice were sacrificed when the tumor diameter reached 10 mm.

In vivo depletion of CD8⁺ T cells. Anti-CD8 monoclonal antibody produced by hybridoma clone 53-6.7.2 was obtained from the MSKCC Monoclonal Antibody Facility. The antibody was injected i.p. once a week for 4 weeks, starting 2 days before tumor challenge (250 µg were given with the first two injections and 500 µg with the last two).

Transient transfection experiments. A FLAG-tag peptide sequence was introduced at the carboxyl-terminus of neu and FLneu. cDNAs were amplified using the following primers: neu-forward 5'-CGAATTCACCATGGAGCTGGCGGCTGG-3' and FLneu-forward 5'-CGAATTCACCATGACAGTGTGGCGCC-3', both introducing an *Eco*RI site upstream of the start codon; reverse 5'-CGAAGCTTTGTCACCGGGCTGGCTCTC-TGC-3', removing the stop codon and introducing the *Hind*III site. *Eco*RI/*Hind*III-digested PCR products were cloned in the pCMVTag4A vector (Stratagene). Both constructs were confirmed by sequencing.

COS-7 cells were plated in six-well plates and transfected with both FLAG-tagged constructs using Fugene 6 (Roche Diagnostic Corp.) and 2 µg of DNA. Twenty-four hours after transfection, cells were treated with muconomycin A (0.6 mmol/L; Sigma-Aldrich) for 1 h. Cells were washed and cultured either in the presence of the proteasome inhibitor MG132 (20 nmol/L; Sigma-Aldrich) or the Golgi inhibitor Brefeldin A (BFA; 2 µg per well; BD Bioscience) or without further treatment. At each time point, cells were lysed in 10 mmol/L Tris-HCl (pH, 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% NP40, and 0.5% deoxycholate plus protease inhibitors (Roche Diagnostic Corp.; lysis buffer) for 30 min on ice. Lysate protein concentration was determined by the BCA Protein Assay (Pierce Biotechnology). Cell lysates (20 µg total protein per sample) were then loaded in a 7.5% SDS-PAGE gel (Bio-Rad), electrophoresed, and transferred to a nitrocellulose membrane (Whatman Inc.). FLAG-tagged proteins were detected using peroxidase-conjugated M2 anti-FLAG monoclonal antibody (Sigma-Aldrich) and the enhanced chemiluminescence reagent (Amersham Biosciences) according to the manufacturer's instructions.

ELISPOT assay. Inguinal and axillary lymph nodes (LNs) from immunized and control FVB/N mice were harvested 5 days after the last DNA injection. LNs were mechanically disrupted in complete RPMI. CD8⁺ T cells were positively selected by incubation with magnetic anti-CD8 beads (Miltenyi Biotec). HER2/neu-specific IFN-γ production was determined by a standard ELISPOT assay (38) following a 20-h incubation of CD8⁺ T cells (10^5 per well) with T2D^d target cells

(10^4 per well) pulsed with RNEU₄₂₀₋₄₂₉ peptide (10 μ g/mL). Alternatively, splenocytes isolated from syngeneic, naive mice were used as targets. The RNEU₄₂₀₋₄₂₉ epitope has been shown to be immunodominant for the H2-D^q haplotype (35), and it maps to the extracellular region of HER2/neu. As a negative control, target cells were either left unpulsed or pulsed with the irrelevant NP₁₁₈₋₁₂₆ peptide (39).

Analysis of antibody response. Sera were obtained from naive and immunized mice 5 days after the last DNA immunization and analyzed by flow cytometry. The N202.1A cell line (HER2/neu-positive) was used as the source of antigen; the N202.1E line (HER2/neu-negative) was used as negative control. Cells were incubated with the anti-c-ErbB2/c-Neu (Ab-4) mouse monoclonal antibody (Calbiochem/EMD Biosciences; positive control) or with mouse sera diluted in fluorescence-activated cell sorting buffer (1% FCS in PBS) for 45 min on ice. FITC-conjugated goat anti-mouse immunoglobulin G (IgG) was used as the secondary antibody (1/500 dilution; BD Pharmingen). A FACScan flow cytometer and CellQuest Software (Becton Dickinson Immunocytometry Systems) were used to acquire data, and analysis was done using Flow Jo software (Tree Star, Inc.).

Immunofluorescence staining. To introduce a green fluorescent protein (GFP) sequence at the COOH terminus of neu and FLneu, cDNAs were amplified using the following primers: neu-forward 5'-CGAATTCACCATGGAGCTGGCGGCCTGG-3' and FLneu-forward 5'-CGAATTCACCATGACAGTGTGGCGGC-3', both introducing a *Eco*RI site upstream of the start codon; neu-reverse 5'-GCGGTACCGTGT-CACCGGGC-3' and FLneu-reverse 5'-GCGTGCAGCTGCACCGGGC-3' removing the stop codon and introducing the *Kpn*I and *Sal*I sites, respectively. PCR products were cloned in the pEGFP-N2 vector (Clontech). Both constructs were confirmed by sequencing.

COS-7 cells were plated in four-well chamber slides and transfected with neu-GFP or FLneu-GFP using Fugene 6 reagent. Forty-eight hours after transfection, cells were fixed in Cytofix/Cytopherm (BD Pharmingen), washed in permeabilization buffer (PBS, 5% FCS, 0.5% saponin, 10 mmol/L Gly), and incubated with rabbit anti-calnexin or anti-giantin antibodies (Abcam), diluted 1:500 and 1:1,000, respectively. Subsequently, cells were washed and incubated with Alexa-594-conjugated goat anti-rabbit (Invitrogen). Slides were then coverslipped using Prolong anti-fading mounting media (Invitrogen) and visualized on a confocal laser scanning microscope (Leica TCS SP2).

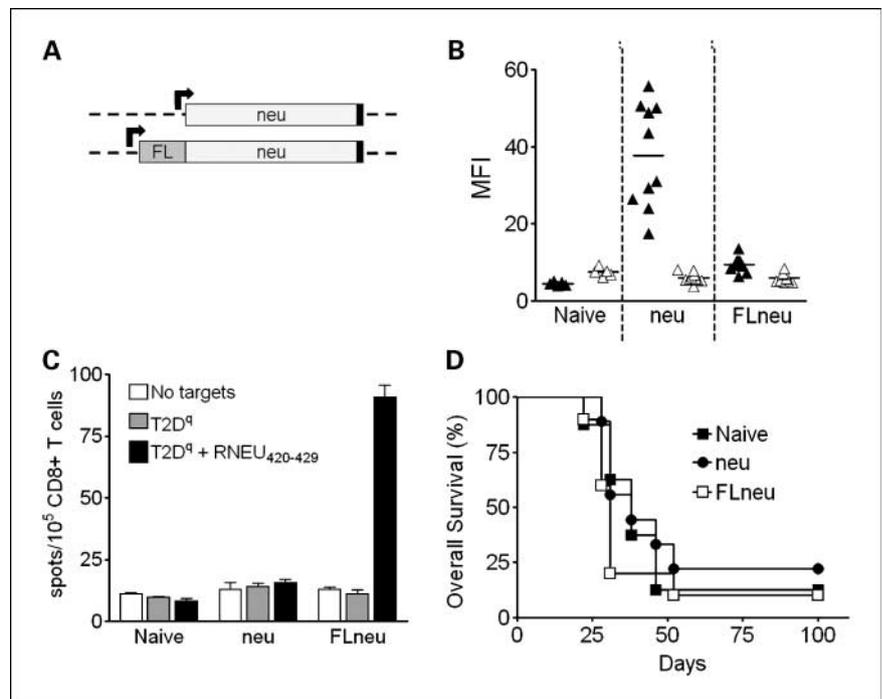
Adoptive transfer experiments. FVB/N donor mice were immunized by particle bombardment with a combination of neu and FLneu DNA; immunizations were repeated weekly for 5 weeks. A second group of FVB/N naive donor mice were used as control animals. Serum and T cells were obtained 5 days after the last immunization from groups of donors. Sera were collected and pooled for each group. Inguinal and axillary LNs were harvested, and CD8⁺ T cells were isolated by incubation with CD8-magnetic beads. Recipient mice (naive FVB/N) were sublethally irradiated 1 day before the adoptive transfer (300 cGy) to deplete the lymphoid population and facilitate engraftment of transferred CD8⁺ T cells. The experiment used a donor/recipient ratio of 2:1. Recipient mice were challenged with 5×10^4 233-VSGA1 tumor cells on the day of the adoptive transfer. Following tumor challenge, 700 μ L of total serum and 10^7 CD8⁺ T cells from the donor mice were injected into each recipient mouse (i.p and i.v., respectively). Details on experimental groups are found in Results. On the day of the transfer and 4 consecutive days thereafter, recombinant human interleukin 2 (IL-2) was given to recipient mice (50,000 IU per mouse). Mice were assessed thrice a week for the appearance of palpable tumor masses and sacrificed when the tumor reached a mean diameter of 10 mm or became ulcerated.

Statistical analysis. Statistical analysis was done using Prism Software (GraphPad Software, Inc.). The Kaplan-Meier method was used to plot survival experiments, and statistical significance was determined by log-rank analysis. For the analysis of antibody responses and T-cell responses, a one-way ANOVA test was used with a Bonferroni correction test for multiple comparisons.

Results

Design of DNA-based vaccines that target HER2/neu+ breast cancer. We designed and tested two HER2/neu DNA vaccines for their ability to elicit both humoral and T cell responses to HER2/neu. Neu consists of the extracellular domain of rat HER2/neu, whereas FLneu contains the mouse FL extracellular domain fused upstream of the neu sequence (Fig. 1A). Because the neu vaccine generates a predominantly antibody-mediated immune response, the FLneu vaccine was created with the

Fig. 1. DNA-based vaccines against HER2/neu elicit antibody and T cell immune responses. **A**, schematic representation of the neu and FLneu constructs is shown. **B** and **C**, female FVB/N mice were vaccinated thrice a week for 3 wk by particle bombardment with neu or FLneu or left untreated as a negative control (Naive). Five days after the last DNA injection, antibody assays (10 mice per group) or T cell assays (3 mice per group) were done. **B**, only mice immunized with neu produced antibodies (tested at 1/50 dilution) that bind N202.1A cells (HER2/neu+, filled symbols), but not N202.1E cells (HER2/neu-, empty symbols). MFI, mean fluorescence intensity. Symbols refer to individual mice; horizontal bars in **B** indicate mean values for each group. **C**, results of IFN- γ ELISPOT assay show that only mice immunized with FLneu produced CD8⁺ T cells specific for the immunodominant RNEU₄₂₀₋₄₂₉ peptide. Bars, SE. **D**, mice (10 per group) were challenged with 5×10^4 233-VSGA1 mammary tumor cells 5 d after the last DNA immunization. Cutaneous tumor masses were measured thrice weekly with calipers in two perpendicular diameters.



intent to enhance HER2/neu-specific CD8⁺ T cell responses (30).

FVB/N mice generate a HER2/neu-specific immune response that is not sufficient for tumor protection. Mice (15 per group) were immunized by particle bombardment once a week for 3 weeks with the neu or the FLneu vaccine. Five days after the last immunization, three mice per group were sacrificed for T cell analysis, and the remaining mice were challenged cutaneously with 5×10^4 233-VSGA1 mammary tumor cells.

Sera collected at the time of tumor challenge were used to stain HER2/neu-positive (N202.1A) and control HER2/neu-negative (N202.1E) cells. The mean fluorescence intensity obtained with sera from mice immunized with neu was significantly higher ($P < 0.001$) compared with untreated mice, confirming that the neu vaccine elicited an anti-HER2/neu-specific antibody response (Fig. 1B). There was no binding of sera to the control cells. Mice receiving FLneu displayed little or no anti-HER2/neu antibody in comparison to mice immunized with neu ($P < 0.001$). An IFN- γ ELISPOT assay revealed that strong, HER2/neu-specific CD8⁺ T cell responses were elicited in mice immunized with FLneu (Fig. 1C). No significant T cell responses were observed in mice immunized with neu or in control animals. Although vaccination resulted in either T cell or humoral immune responses, animals challenged with the 233-VSGA1 mammary tumor cell line had progressive tumor growth without significant evidence of tumor protection (Fig. 1D).

FL functional activity is not required for the generation of HER2/neu-specific T-cell responses. FL administration in mice has been shown to induce consistent expansion of DCs that are capable of inducing an antigen-specific T cell response *in vivo* (27). Therefore, fusion of FL to neu could induce proliferation of DC progenitors and maturation of DCs, thereby enhancing T cell responses to the HER2/neu antigen. To test this hypothesis, we analyzed the number and the activation state of DCs in LNs of mice vaccinated with neu or FLneu. No differences were found in the number of CD11c⁺/MHC-class II⁺ DCs between mice immunized with empty vector (negative control), neu or FLneu (data not shown). DCs of mice vaccinated with neu or FLneu also showed no enhanced expression of the activation marker CD86 (data not shown). These findings indicate that DNA immunization with the FL domain does not generate detectable changes in DCs, consistent with results reported by Hung et al. (30).

The ability of FL to stimulate proliferation and/or maturation of DC precursors depends on its capacity to bind the receptor, Flt3R, expressed by multipotent hematopoietic progenitor cells. Single amino acid substitutions in human FL have been characterized by Graddis et al. (40): some FL mutations lead to decreased capacity to bind the Flt3R, and others are able to enhance binding to Flt3R, resulting in reduced or augmented FL biological activity, respectively. Given the high homology between human and mouse FL, most of the described single-point mutations were mapped in conserved regions of the FL molecule. To further investigate the role of FL biological activity in inducing a HER2/neu-specific T cell response when fused to neu, we used site-directed mutagenesis to introduce five mutations into FLneu: FLneu_H8R, FLneu_I11Y, FLneu_K116E, FLneu_H8Y, and FLneu_S84E. The numbers reflect the amino acid position of the mutation and correspond to the nomenclature used by Graddis et al. The first three constructs (FLneu_H8R, FLneu_I11Y, and FLneu_K116E) contain muta-

tions that are known to decrease FL capacity to bind Flt3R, whereas the latter two (FLneu_H8Y and FLneu_S84E) contain mutations that are known to increase FL biological activity. Quantitation of FLneu protein levels after transient transfection in COS7 cells showed comparable expression of wild-type and mutant proteins (data not shown).

Mice were immunized with the wild-type or the mutant versions of FLneu, and HER2/neu-specific T cell responses were analyzed by IFN- γ ELISPOT assay. Each of the mutated FLneu constructs generated similar HER2/neu-specific CD8⁺ T cell responses when compared with wild-type FLneu (Fig. 2). Therefore, FL biological activity is not required for the induction of cellular immunity against HER2/neu, and we conclude that the FL domain is not acting directly to activate Flt3R⁺ DC precursors.

Neu protein is more efficiently secreted than FLneu. Another possible explanation for the predominantly cellular-mediated immunity generated by the FLneu vaccine is a different pattern of intracellular localization, stability, and/or degradation of the chimeric FLneu protein compared with neu. To address this, we ligated a FLAG-tag peptide coding sequence at the carboxyl-terminus of neu or FLneu. The purpose of the FLAG peptide was to track the two proteins intracellularly and determine their stability and cellular localization.

We transiently transfected neu-FLAG or FLneu-FLAG constructs into COS-7 cells, and 24 h post-transfection, cells were treated for 1 h with muconomycin A. Subsequently, after different time intervals (Fig. 3), cell lysates were analyzed by Western blot for protein fate. A distinct difference was observed between the fates of neu and FLneu; FLneu is a very stable protein that was not degraded in a chase following inhibition of new protein synthesis by muconomycin treatment, whereas the neu protein was progressively degraded and was almost undetectable 18 h after muconomycin treatment (Fig. 3A). We then repeated the experiment with incorporation of a proteasome inhibitor (MG132) to determine if neu was degraded through a proteasome-dependent mechanism. Results revealed that the decrease in neu was largely or completely proteasome

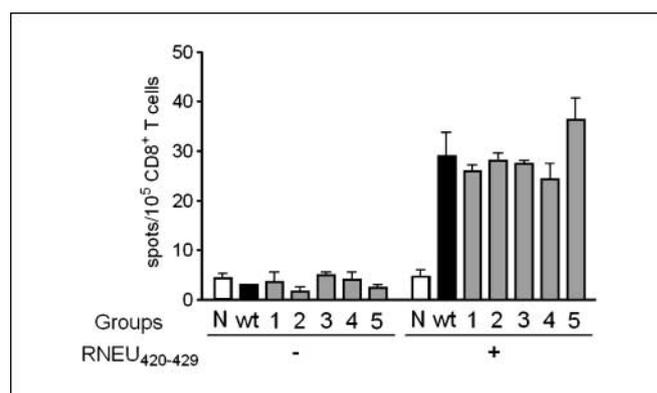


Fig. 2. FLneu mutant variants elicit comparable T cell responses against HER2/neu compared with wild-type FLneu. Mice (three per group) were immunized once a week for 3 wk by particle bombardment with the wild-type version of FLneu (wt) or with mutated FLneu constructs described in Materials and Methods: FLneu_H8R (1), FLneu_I11Y (2), FLneu_K116E (3), FLneu_H8Y (4), and FLneu_S84E (5). A group of mice was left untreated (N) as negative control. Five days after the last immunization, inguinal and axillary LNs were harvested, and CD8⁺ T cell response was measured by IFN- γ ELISPOT assay. Splenocytes from naive mice were used as target cells and were pulsed with the immunodominant RNEU₄₂₀₋₄₂₉ peptide or left unpulsed as negative control.

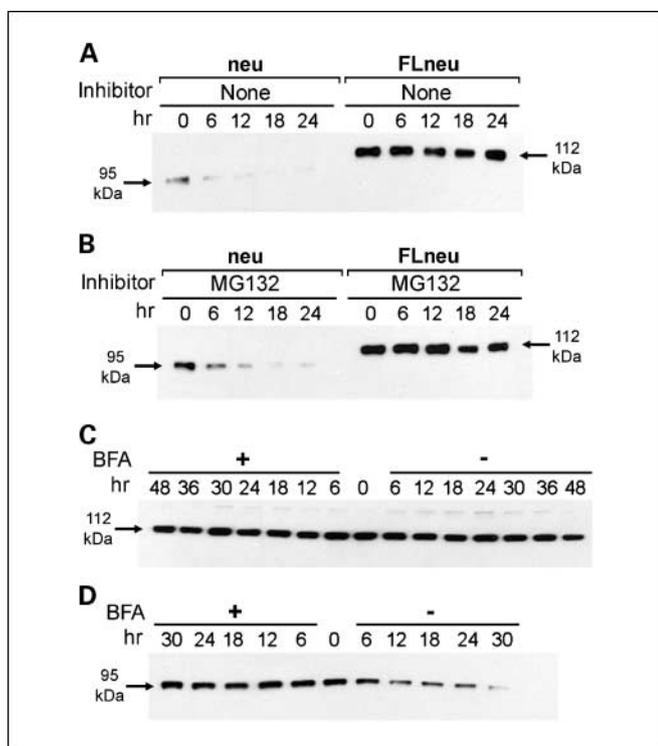


Fig. 3. Intracellular stability of neu and FLneu. COS-7 cells were transiently transfected with plasmid DNA encoding neu and FLneu linked to the FLAG peptide DNA sequence at the carboxyl terminus. At 24 h after transfection, cells were treated with muconomycin A (0 time point) to inhibit new protein synthesis and chased for an additional 24 h in the absence (A) or presence (B) of the proteasome inhibitor MG132. Samples were assayed at 6, 12, 18, and 24 h post-chase by Western blot analysis using an anti-FLAG-HRP antibody for detection. In similar experiments, after treatment with muconomycin A, COS-7 cells transfected with FLneu-FLAG (C) or neu-FLAG (D) were incubated with the inhibitor of protein secretion BFA. Samples were assayed at different time points post-chase as described for (A) and (B).

independent (Fig. 3B), and detection of neu-FLAG in the supernatant suggested that neu is efficiently secreted (data not shown).

As shown in Fig. 3C, BFA, an inhibitor of transport vesicles in the secretory pathway, had no effect on intracellular levels of FLneu, whereas it led to intracellular retention of neu (Fig. 3D), resulting in a ≥ 30 -h half-life for both proteins. Therefore, neu is secreted much more efficiently than FLneu. Secreted proteins are known to preferentially induce antibody responses, whereas cytoplasmic proteins elicit T cell responses (41). The subcellular localizations of neu and FLneu, thus, are consistent with the type of adaptive immune response following delivery as DNA vaccines.

Intracellular localization of neu and FLneu. A GFP sequence was fused to the carboxyl terminus of neu and FLneu to study the intracellular localization of each protein. The neu-GFP or FLneu-GFP were transfected in COS-7 cells and subsequently stained with antibodies specific for calnexin [endoplasmic reticulum (ER) marker] and giantin (Golgi complex marker). As shown in Fig. 4A, neu and FLneu patterns of expression were substantially different. Although FLneu was broadly expressed throughout the cytoplasm, co-localizing with calnexin, the neu protein was confined in small vesicles, presumably secretory vesicles. Neither neu nor FLneu protein co-localized with giantin (Fig. 4B).

The combination of neu and FLneu vaccines protects mice from HER2/neu+ mammary tumors. It has been reported that infusion of HER2/neu-specific IgG combined with adoptive transfer of *in vitro* generated, HER2/neu-specific T cells function together to eradicate HER2/neu+ mammary tumor in a severe combined immunodeficiency mouse model (42). We observed in initial experiments that the neu vaccine generates a predominantly antibody-mediated immune response to HER2/neu, whereas FLneu generates T cell-mediated responses to the same antigen. Therefore, we combined vaccines that activate both the cellular and humoral pathways of the adaptive immune system against HER2/neu.

To administer the combined vaccine, we injected neu and FLneu DNA constructs simultaneously. Additional groups of mice received neu or FLneu alone, and the quantity of injected DNA was normalized using the control empty vector in these groups. Five days after the last immunization, some mice were used for T cell analysis, and the remaining were challenged with 233-VSGA1 mammary tumor cells and followed for tumor growth. For detection of antibody response, all mice were bled 1 day before tumor challenge.

Mice immunized with neu and with the combination of neu and FLneu generated anti-HER2/neu-specific antibodies, whereas control mice and mice receiving FLneu did not (Fig. 5A). Anti-HER2/neu-specific CD8⁺ T cells were produced only in mice immunized with FLneu and in mice receiving the combination vaccine (Fig. 5B).

Long-term survival was observed only in mice immunized with the combined vaccine (neu+FLneu). The overall survival rate was low in mice immunized either with neu or FLneu (2 out of 16, 12%), whereas about 45% of mice (7 out of 16) that received the combination neu+FLneu were still alive 3 months after tumor challenge (Fig. 5C; neu versus neu+FLneu: $P = 0.032$; FLneu versus neu+FLneu: $P = 0.01$).

To assess the involvement of CD8⁺ T cells, we immunized a second cohort of mice as previously described and depleted CD8⁺ T cells at the effector phase before tumor challenge. Mice depleted of CD8⁺ T cells showed a significant decrease in tumor protection compared with control mice ($P = 0.02$), indicating that CD8⁺ T cells are required for enhanced tumor immunity (Fig. 5D). Tumor immunity was not completely abrogated in mice depleted of CD8⁺ T cells, suggesting that antibodies or other effector mechanisms are also playing a role.

Adoptive transfer of immune sera and immune CD8⁺ T cells. To confirm that antibodies and T cells cooperate in eradicating HER2/neu+ tumors, we adoptively transferred antibodies and/or CD8⁺ T cells from immunized mice. Half of FVB/N donor mice were immunized with the combination of neu and FLneu, whereas the other half was left untreated. Serum and CD8⁺ T cells were then transferred into naive recipient FVB/N mice by i.p. and i.v. injection, respectively. Recipient mice were divided in four groups receiving (a) serum and CD8⁺ T cells from naive donors (Naive, $n = 13$), (b) serum from immunized donors and CD8⁺ T cells from naive donors (Serum, $n = 12$), (c) serum from naive donors and CD8⁺ T cells from immunized donors (T cells, $n = 12$), and (d) both serum and CD8⁺ T cells from immunized donors (Serum+T cells, $n = 13$). Recipient mice were then challenged with HER2/neu+ tumor cells on the day of adoptive transfer. Mice were given IL-2 daily for 4 days post-transfer to stimulate expansion of transferred CD8⁺ T cells. Figure 6 shows the overall survival of

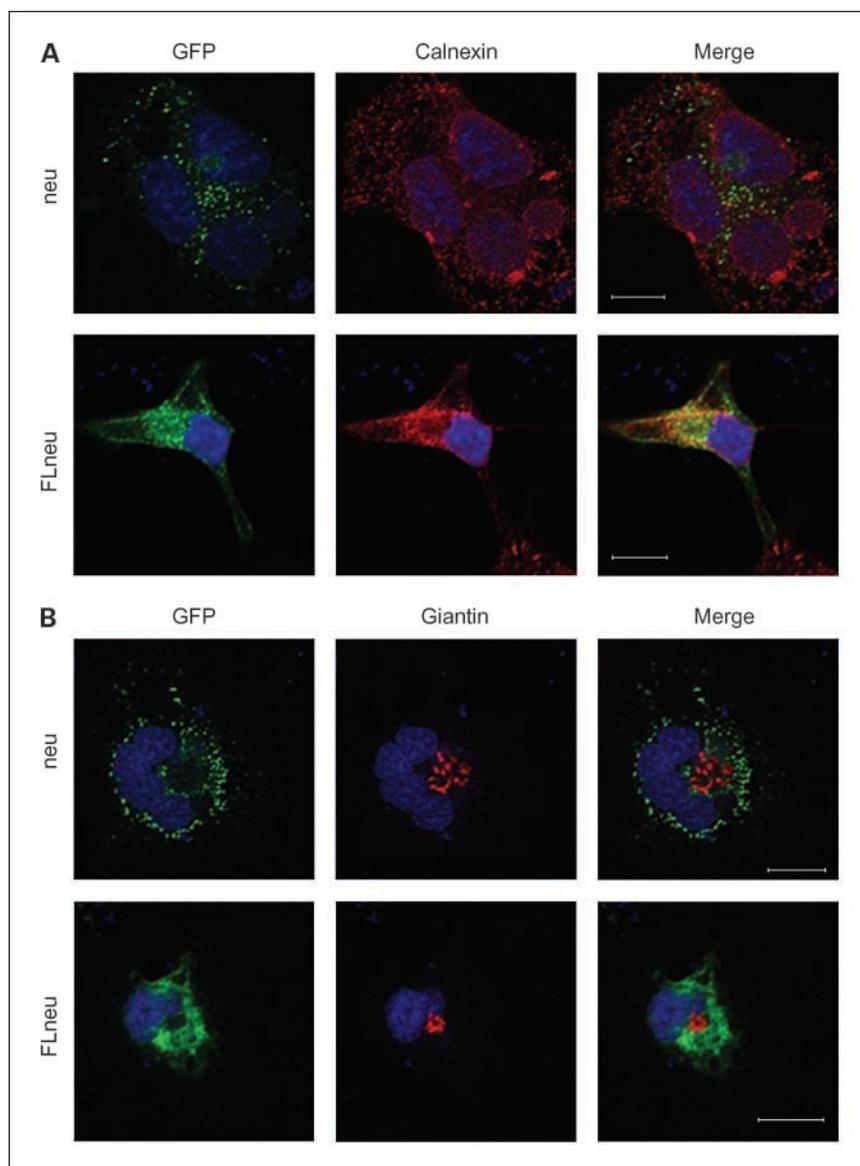


Fig. 4. Intracellular localization of neu and FLneu. COS-7 cells were transfected with neu-GFP or FLneu-GFP using Eugene 6 and stained 48 h later with anti-calnexin (A) or anti-giantin (B) antibody as described in Material and Methods. Bar, ~15 μ m.

recipient mice after challenge with HER2/neu+ breast cancer cells. Only mice that received the combination of immune serum and immune T cells showed significantly increased survival compared with control mice ($P = 0.004$). These results indicate that both antibodies and T cells are required for effective elimination of HER2/neu+ breast tumor cells, and that protective immunity generated by active immunization can be transferred to naive recipients.

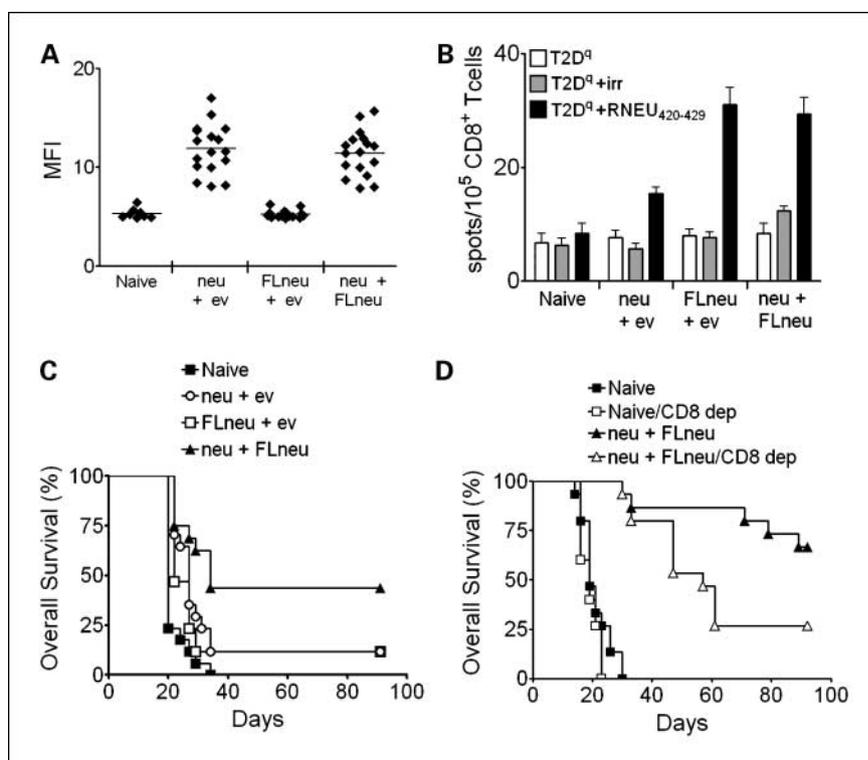
Discussion

We have developed and assessed two DNA vaccines in a transplantable mouse tumor model of breast cancer, neu and FLneu. Our results revealed that the neu vaccine generated a HER2/neu-specific antibody response. In contrast, vaccination with FLneu produced a CD8⁺ T cell response against HER2/neu but a negligible anti-HER2/neu antibody response (Fig. 1B and C). Despite the induction of HER2/neu-specific antibodies or T cells, mice immunized with either vaccine were not

protected from the outgrowth of HER2/neu+ mammary tumors (Fig. 1D). These results indicate that neither humoral nor cellular responses alone are sufficient to provide tumor protection and led us to ask whether a combination of B and T cell responses might be necessary to achieve complete tumor eradication.

We hypothesized that the presence of the FL domain would produce a direct effect on DC progenitors and DCs. FL plays an important role in the differentiation and maturation of hematopoietic precursors (43) and is known to promote accumulation of mature DCs *in vivo* (27). Our data, however, revealed that mutations in FLneu that have previously been reported to alter (either reduce or enhance) FL biological activity had no impact on HER2/neu-specific T cell responses when compared with the native FLneu vaccine (Fig. 2). These results reveal that fusing FL to neu does not activate DCs directly, implicating an indirect mechanism by which the FL domain facilitates induction of a T cell response to HER2/neu.

Fig. 5. Antibodies and T cells cooperate in HER2/neu+ breast cancer rejection. FVB/N female mice (20 per group) were immunized by particle bombardment with the indicated plasmids. An empty vector (*ev*) was used as a control plasmid in combination for immunization with neu or FLneu alone. Three mice per group were sacrificed for T cell analysis; the rest were assessed for antibody response and underwent tumor challenge with 5×10^4 233-VSGA1 tumor cells, and tumor growth was monitored thrice a week. **A**, flow cytometry analysis was done as in the caption to Fig. 1 to determine the presence of anti-HER2/neu antibodies in the sera (1:50 dilution). **B**, IFN- γ expression by CD8⁺ T cells measured by ELISPOT assay is shown. T2D^q target cells were pulsed with the immunodominant RNEU₄₂₀₋₄₂₉ peptide or with the NP₁₁₈₋₁₂₆ irrelevant peptide (*irr*). **C**, overall survival after challenge with the 233-VSGA1 mammary tumor cells is presented using Kaplan-Meier curves. **D**, to assess the involvement of CD8⁺ T cells in tumor rejection, the indicated group of mice were treated with an anti-CD8 antibody (hybridoma 53-6.7.2) to deplete CD8⁺ T cells at the effector phase just before tumor challenge as described in Materials and Methods.



Cellular localization and stability of antigens influences the type of adaptive immune response. For instance, gene-gun immunization with DNA encoding cytoplasmic ovalbumin resulted in strong CTL-mediated immune responses, which were not seen with a secreted version of the same antigen (41). We propose that the distinct cellular fate of the neu and FLneu proteins explains the different types of immune response generated by each vaccine, and specifically that the FL domain facilitates antigen presentation by MHC molecules.

Our results show that the intracellular half-life of the neu product is relatively short, and that the protein is secreted (Fig. 3). Following inhibition of protein synthesis, only ~50% of neu protein was detected inside the cell at 6 h and almost none by ≥ 30 h. This decline in cellular neu levels was at least partially dependent on the secretory pathway, evidenced by intracellular retention following BFA blockade and by recovery of neu in the supernatant. In contrast, FLneu was remarkably stable within the cell after biosynthesis, with approximately the same amount of FLneu detected more than 30 h following inhibition of protein synthesis.

We further propose that the secreted neu antigen is readily available for interaction with immunoglobulin receptors on B cells, leading to antibody responses. On the other hand, FLneu is retained inside the cell, mostly in the ER. This scenario is supported by studies with GFP-labeled neu and FLneu proteins. The neu protein was specifically located in intracellular vesicles, and immunostaining for the lysosome marker Lamp-1 did not show co-localization with the neu protein (data not shown), further supporting the notion that neu efficiently move through the secretory pathway. The FLneu protein instead was retained inside cells, localizing to the ER marker, supporting the notion that the FL fusion domain does lead to retention in the ER, perhaps due to incomplete or altered protein folding during

translations (Fig. 4). These results are also supported by previous work with a chimeric FL-E7 construct, which induced a potent T cell-mediated response; the E7 protein fused to FL localized in the ER compartment, whereas the non-chimeric E7 protein showed a cytoplasmic/nuclear distribution (30). Presumably in both cases, the FL domain leads to misfolding of the chimeric proteins, such that they are not able to exit the ER, perhaps due to retention by ER chaperones. This protein product would generate peptides for loading on MHC-class I molecules, following degradation either in the ER (where processing is limited largely to aminopeptidase trimming) or

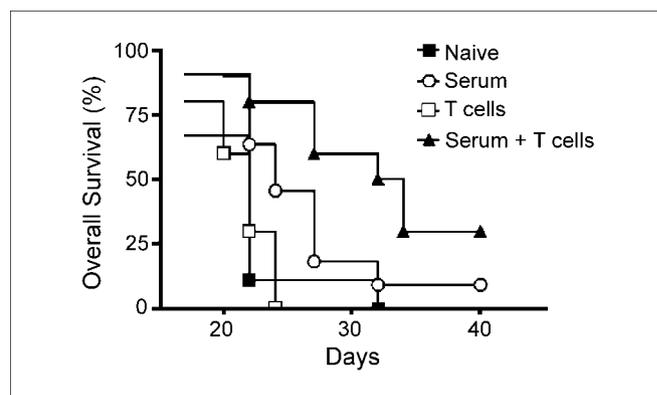


Fig. 6. Adoptive transfer of immune sera and immune T cells delays the growth of HER2/neu+ mammary tumors. Recipient mice (12 or 13 per group) were sublethally irradiated (300 cGy) 1 d before adoptive transfer. The day of the transfer, recipient mice were challenged with 5×10^4 233-VSGA1 mammary tumor cells and subsequently received sera and CD8⁺ T cells from untreated donors (*Naive*), sera from immunized donors and CD8⁺ T cells from untreated donors (*Serum*), sera from untreated donors and CD8⁺ T cells from immunized donors (*T cells*), or sera and CD8⁺ T cells from immunized donors (*Serum + T cells*). Tumor growth and survival was assessed as described in Materials and Methods.

more likely after translocation of the misfolded protein into the cytosol for processing by the proteasome machinery, followed by transport of peptides back to the ER (44).

Passively transferred antibodies and T cells can cooperate to effectively hamper tumor progression (42). We found that the combined neu and FLneu vaccine activated both the cellular and humoral arms of the adaptive immune system (Fig. 4). Most importantly, the overall survival rate of mice that received the combined vaccine was significantly higher when compared with control mice or mice receiving vaccines with the individual components, and this effect was partially CD8 dependent (Fig. 5). The adoptive transfer experiments (Fig. 6) further shows that antibody and T cells are required to achieve antitumor effects.

Passive transfer of antibody combined with active immunization to induce T cell responses has been described for generating tumor immunity in different HER2/neu+ breast cancer models (45, 46), as well as in the mouse EL-4 lymphoma model (47). Reilly et al. (42) combined passive administration of HER2/neu-specific IgG with adoptive transfer of a HER2/neu-specific CD8⁺ T cell clone. The approach of Reilly et al. can be translated into clinical application, but is expensive and technically challenging, requiring the expansion of T cell lines from individual patients. We have focused on developing active immunotherapies to elicit both B and T cell responses against HER2/neu. DNA-based vaccines are relatively inexpensive to prepare, straightforward to produce and store, and easy to administer. Furthermore, injection of even small quantities of DNA by gene gun can generate immunity against the corresponding antigen. However, the potency of DNA vaccines needs to be improved to be potentially useful in a clinical treatment setting, and chimeric constructs such as FLneu need to be further investigated.

Several mechanisms could be involved in the synergy between antibodies and immune T cells. Anti-HER2/neu antibodies are

able to induce HER2/neu down-regulation, which results in increased endocytic degradation of the receptor, potentially providing peptide epitopes for MHC-class II presentation. Furthermore, antibodies to HER2/neu can enhance ubiquitination of HER2/neu (48) and, thus, increase the availability of HER2/neu-derived peptides for loading on MHC-class I molecules. Recently, Kono et al. (49) showed that Trastuzumab can increase MHC-class I presentation of endogenous HER2/neu, resulting in augmented susceptibility of HER2/neu+ cancer cells to lysis by anti-HER2/neu CTL.

Other potential mechanisms link adaptive humoral responses to innate cellular immunity through antibody-dependent cell cytotoxicity (ADCC). ADCC involves recognition of antibodies bound on the surface of target cells by Fc receptors present on effector cells, such as macrophages, mast cells, and natural killer (NK) cells. Fc receptor signaling can lead to production of effector molecules such as perforin, reactive oxygen species, tumor necrosis factor, and other mediators to kill target cells. NK cells have not been implicated previously in immunity against HER2/neu+ tumors (42), but a role for macrophages and monocytes is possible. In addition, antibodies bound to cell surface antigens can induce Fc receptor-positive antigen-presenting cells, such as DCs or macrophages, to opsonize tumor cells for processing antigens for presentation to T cells.

In conclusion, our results show that active immunization with plasmid DNA must induce both cellular and humoral immunity to HER2/neu to achieve complete protection from HER2/neu+ mammary tumors.

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Antibody and CD8⁺ T Cell Responses against HER2/neu Required for Tumor Eradication after DNA Immunization with a Flt-3 Ligand Fusion Vaccine

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