Plasmid-based vaccines encoding rat *neu* and immune stimulatory molecules can elicit rat *neu*-specific immunity

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Abstract

DNA vaccines are ideally suited for immunizing against tumor antigens because constructs can be formulated that not only encode the tumor antigen but also encode molecules chosen to improve the ability to elicit an antitumor response. Ligands expressed on antigen-presenting cells associated with stimulating a robust T-cell response are excellent candidates for inclusion in a DNA vaccine. Mice transgenic for the HER-2/*neu* homologue, rat *neu*, were immunized with full-length rat *neu* cDNA given alone or in combination with plasmids encoding costimulatory molecules CD80 or CD86 and the ligand for CD137 (CD137L). Intradermal injection of the plasmid constructs resulted in both plasmid transcript and antigen protein expression being detected in lymph nodes draining the injection site. Immunization with plasmids encoding the *neu* antigen along with plasmids encoding CD137L and either CD80 or CD86 resulted in the generation of *neu*-specific antibodies that induced phopshorylation of the *neu* tyrosine kinase and inhibited the growth of cultured tumor cells overexpressing *neu*. Survival of animals was significantly prolonged after immunization with vaccines encoding *neu* together with the costimulatory molecules. Although tumors eventually occurred in the vaccinated animals, they were markedly infiltrated with CD4+ T cells. DNA vaccines encoding *neu*, when given in combination with both CD137L and either CD80 or CD86, can induce cellular and humoral immunity and result in an antitumor effect. (Mol Cancer Ther. 2003;2:995–1002)

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Introduction

The development of tumor antigen vaccines in the form of bacterial plasmid DNA encoding an immunogenic protein has several potential advantages in clinical application as compared with more standard forms of immunization (1). First, DNA vaccines are not MHC restricted, in contrast to peptide vaccines. Secondly, DNA is easy to produce and transport and it is stable in long-term storage, in contrast to protein or viral vector-based vaccines. Finally, plasmid-based strategies may allow the delivery of multiple antigens simultaneously. Vaccinating against several tumor-related proteins would be beneficial as human cancers express multiple immunogenic proteins (2). Plasmid-based DNA vaccines, however, have been only minimally effective when translated to human clinical use. There are several reasons for the lack of success such as the low local transduction of DNA into antigen-presenting cells (APC) as most vaccines are delivered to muscle (3). Furthermore, monocytes and macrophages are more commonly transduced than dendritic cells (DC). In addition, APC transfected *in vivo* by DNA, generally in muscle, do not home effectively to draining lymph nodes; thus, systemic immunity is not efficiently stimulated (4). Intradermal (i.d.) immunization may be a more effective route of vaccination for plasmid DNA. Many APC are present in the epidermis and dermis including Langerhans cells and skin DC. *In vivo* transfection of skin cells with DNA encoding antigen as well as immune stimulatory molecules may allow the generation of more potent APC (*i.e.*, transform Langerhans cells or even dermal fibroblasts, monocytes, and macrophages into highly effective APC).

We have evaluated the efficacy of DNA immunization in the *neu* transgenic mouse. In this animal, rat *neu* is a homologue of human HER-2/*neu*, a well-defined tumor antigen in many solid tumors. HER-2/*neu* is a member of the epidermal growth factor receptor family and is presumed to function as a growth factor receptor (5). HER-2/*neu*, a transmembrane protein, consists of a cysteine-rich extracellular domain, which functions in ligand binding, and an intracellular cytoplasmic domain, which has kinase activity involved in growth regulation (6). In normal cells, it is present as a single copy (7). In contrast, the HER-2/*neu* gene is amplified in 20–40% of breast carcinomas and ~20–30% of ovarian carcinomas, and amplification occurs in other tumors, including carcinomas of uterus, stomach, and lung (8). The HER-2/*neu* protein is overexpressed in these tumors, and overexpression has been associated with a poor prognosis in carcinomas of the breast and ovary (9). In the mouse model described here, the *neu* transgene is expressed in the mammary gland and females spontaneously develop breast carcinomas (10).

From an immunologic standpoint, *neu* transgenic mice are tolerant to *neu*. Recent studies in the *neu* transgenic mouse suggest that both humoral and cellular *neu*-specific immunities are needed for tumor eradication (11).

We combined cDNA encoding full-length rat *neu* with cDNA encoding ligands of the costimulatory molecules CD28 and CD137. CD137 is expressed on both CD8+ and CD4+ T cells and recent studies demonstrate the costimulatory role of CD137 for CD4+ T cells (12–14). The CD137 costimulatory pathway is synergistic and yet independent of that mediated via CD28. In addition, CD137 engagement, by either antibody (15) or its ligand (16), can cause the rejection of established tumors, including those of low antigenicity (17). Intradermal vaccination with constructs encoding rat *neu* resulted in antigenic protein expression in the draining lymph node. The addition of costimulatory molecules to the vaccine induced the generation of both *neu*-specific antibodies and T cells, which resulted in prolonged survival in mice challenged with syngeneic *neu*-overexpressing tumors.

Materials and Methods

Construction of Plasmid-Based Vaccines

A plasmid encoding the *neu* antigen was constructed using pLNCX containing a cytomegalovirus (CMV) promoter (Fig. 1, A and B). Rat *neu* sequence was obtained by excising rat *neu* from pSV2 (5) with *Hin*dIII and *Afl*III. pLNCX-rat *neu* (RN) was constructed by cloning rat *neu* into *Hpa*I-cut pLNCX (Fig. 1B). Vaccination with the rat *neu* plasmid alone was used to establish the immunization route. Constructs encoding costimulatory molecules were generated using the multigenic cloning vector, pMG (InvivoGen, San Diego, CA), which contains two cloning sites, MCS1 and MCS2 (Fig. 1C), allowing a single plasmid to encode two costimulatory molecules (Fig. 1, D and E).

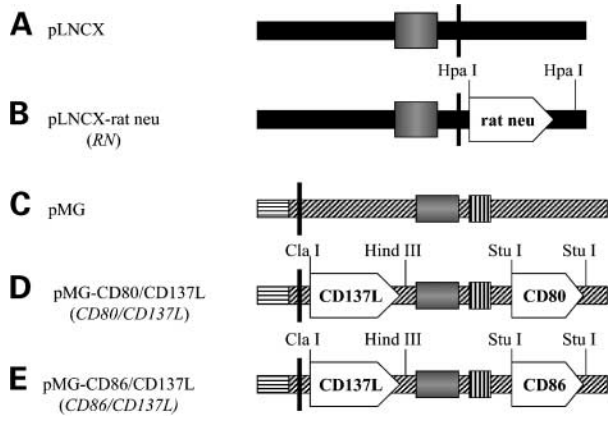

Figure 1. Plasmid construction. **A,** pLNCX expression cassette with MCS (*black bar*) and CMV promoter (*gray box*). **B,** pLNCX coding for full-length rat *neu* (*white arrow*). **C,** pMG expression cassette with MCS (*black bar*), hCMV-intron A promoter (*horizontal stripes*), encephalomyocarditis virus-internal ribosome entry site (*gray box*), and hEF-1-HTLV promoter (*vertical stripes*). **D** and **E**, pMG encoding CD80/CD137L and CD86/CD137L (*white arrows*).

The first transcriptional unit is under the control of an EF-1a/HTLV hybrid promoter, and the second unit contains a CMV promoter with intron A (18) and a modified internal ribosome entry site from encephalomyocarditis virus (19). Murine CD80 (20) was obtained by reverse transcription-PCR amplification of 5-day murine *Con*A blast RNA with the primers 5′ mCD80: TAAGCTTATGGCTTGCAATTGTCAGTTG and 3′ mCD80: GTATCGATCTAAAGGAAGACGG TCTGTTC. Murine CD86 (alternative variant B7.2I; 21) was obtained by reverse transcription-PCR amplification of 5-day murine *Con*A blast RNA with the primers 5′ mCD86: CGAAGCTTGTTCCAGAACTTACGGAAG and 3′ mCD86: CGATCGATCTTTCC TCAGGCTCTCAC. Murine CD137L was obtained by excision from pLXSHD (a gift from Dr. L. Chen, Mayo Clinic, Rochester, MN; 17) by *Sfi*I and *Eco*RI. Constructions of pMG CD80/CD137L (CD80/CD137L; Fig. 1D) and pMG CD86/CD137L (CD86/CD137L; Fig. 1E) required two cloning steps. First, linearization of pMG in MCS2 with *Pst*I-*Bam*HI and ligation of CD137L and then linearization of pMG CD137L in MCS1 with *Stu*I and ligation of Klenow-blunted CD80 or CD86 cDNA.

Immunization of *neu* Transgenic Mice with Plasmid-Based Vaccines

Specific pathogen-free breeder FVB/N-TgN (mouse mammary tumor virus-*neu*) 220 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred at the University of Washington under specific pathogen-free conditions. Female mice 8–16 weeks old were used in the experiments described. Plasmids for DNA vaccination were prepared with Qiagen Plasmid Maxi Kit (Qiagen, Inc., Valencia, CA). The DNA was dissolved in water, stored at 4°C, and used within a month. In initial studies to define the dose and route of vaccine administration, mice were immunized s.c. in the flank and i.p., im., or i.d. in the flank or ear with varying doses of RN. Lymph node chains draining those injection sites were defined by sham injections with blue dye and the identification of the sentinel node (data not shown). In subsequent experiments where lymph node analyses were performed, those previously identified nodal chains were the focus of the evaluation. Groin nodes on the same side as the vaccination were harvested for flank immunizations, and cervical lymph nodes on the same side as the vaccination were harvested when injections were given in the ear. For vaccination experiments, groups of six mice were vaccinated once a week for 3 weeks with 30 μg of plasmid-based vaccine (30 μg of RN alone or 30 μg each of RN and CD80/CD137L or CD86/CD137L) diluted in 1× PBS (Dulbecco's PBS, Life Technologies, Inc., Grand Island, NY) and adjusted to a final volume of 50 μl. Mice were injected i.d. in either the flank or the ear. One week after the last vaccination, mice were transplanted sc. with 5×10^5 tumor cells from freshly removed spontaneous mammary carcinomas in *neu* transgenic mice. Tumor growth was assessed every 2 days via caliper measurement and the tumor surface was recorded (mm^2). Median survival times were compared with a two-tailed, paired *t* test using GraphPad

Instat (Version 3.05). Survival times were considered significantly different at $P < 0.05$. In addition, a mouse mammary carcinoma cell line (MMC) established from a spontaneous tumor and expressing high levels of rat *neu* was propagated *in vitro* and used for experiments described below.

Evaluation of Vaccine-Specific Transcription and Translation in Lymph Nodes Draining the Injection Site

The detection of pLNCX transcripts in lymph nodes by reverse transcription-PCR was calibrated *in vitro* using PE501, an ecotropic packaging cell line (22), retrotransfected with pLNCX (23). Transcripts were amplified from PE501 pLNCX cDNA with the primers P1: CCATCCACGCTGTTTTGACC and P2: CGTTACTTCCGCTAGCTTGCC using standard thermal cycling conditions. *In vivo*, DNA vaccine transcripts were detected by reverse transcription-PCR amplification of lymph nodes from immunized mice. After 48 h, the draining lymph nodes were harvested and immediately stored in RNA Later Solution (Ambion, Inc., Austin, TX) or directly processed. RNA was extracted with Trizol reagent (Life Technologies) following the manufacturer's protocol and 5 μg of the total RNA were reverse transcribed with Superscript II RNase H-Reverse Transcriptase (200 units/μl). The cDNA was amplified by PCR with the primers P1 and P2. Cytoplasmic expression of rat *neu* in lymph nodes before and 48 h after DNA vaccination was assessed by histologic techniques. Lymph nodes were harvested and embedded in paraffin. For staining, the sections were deparaffinized with HemoDe reagent (Fisher Scientific, Fairlawn, NJ), cleared with a graded alcohol series, and blocked with goat serum for 20 min. Sections were then incubated overnight at 4°C with 5 μg/ml of a rabbit anti-rat *neu* polyclonal antibody, c-*neu* Ab-1 (Oncogene Research Products, Cambridge, MA). After washing, the sections were incubated for 30 min in biotinylated anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) and stained by 3,3′diaminobenzidine peroxidase substrate according to the manufacturer's instructions.

Quantitation and Functional Evaluation of Rat *neu*-Specific Antibodies

Rat *neu*-specific antibodies in serum were detected by modifications to an ELISA as previously described (24). Antibodies were functionally evaluated by phosphorylation assays and by their ability to inhibit tumor cell growth in soft agar. In the phosphorylation assays, MMC cells were seeded in 60 mm Petri dishes and grown to 70% confluence in culture medium. Sixteen hours before the assay, the cells were starved in RPMI supplemented with 0.2% FCS. After being washed once, the cells were incubated on ice for 5 min with 1:10 diluted sera from immunized mice and then immediately lysed with 10-mM Tris (pH 7.5), 150-mM NaCl, 1-mM EDTA, 1% Triton, Protease Inhibitor Cocktail Tablets (Complete, Mini, Roche Molecular Biochemicals, Indianapolis, IN) supplemented with 1 mM of sodium orthovanadate (Sigma). Nuclei were removed by centrifugation, 12,000 × *g* at 4°C, and the supernatants were immunoprecipitated with 10 μg/ml of c-*neu* Ab-4 (Oncogene Research Products) overnight at 4°C. Immunoprecipitated proteins were eluted following 3 h of incubation with Protein A-Sepharose and boiled in 2× SDS sample buffer (Novex, San Diego, CA) with 5% of 2-β-mercaptoethanol. Proteins were separated by electrophoresis on a 4–12% gradient gel (Novex), transferred to polyvinylidene fluoride membranes (Novex), and probed with horseradish peroxidase-conjugated antiphosphotyrosine antibody (4G10, Upstate Biotechnology, Inc., Lake Placid, NY) used in combination with the kit Western Breeze (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Signal was detected by enhanced chemiluminescence (Amersham, Piscataway, NJ) and quantified by using OptiQuant Version 03.00, May 12, 1998 program (Packard Instrument Co., Meriden, CT). After analysis for phosphorylated proteins, the blot was stripped and reprobed with c-*neu* Ab-4 (10 μg/ml) and horseradish peroxidase-conjugated anti-murine IgG (Jackson ImmunoResearch Laboratories, West Grove, PA).

Inhibition of tumor growth by rat *neu*-specific antibodies was assessed using a modified protocol from Rapaport *et al.* (25). MMC were resuspended with a prewarmed solution of 0.6% ultralow gelling temperature agarose, Type IX-A (Sigma) in RPMI supplemented with 10% FCS and with 2-fold serial dilutions of sera from immunized mice. The cells were seeded into 60 mm Petri dishes coated with 1.2% low gelling agar suspension and incubated at 37°C for 15 days. Each sample was seeded in triplicate. Colonies comprising >30 cells were counted independently by two persons blinded to the experimental group.

Evaluation of CD4+ T-Cell Infiltration in Tumors

Sections of tumors from vaccinated animals were examined for infiltration of CD4+ T lymphocytes. Immunohistochemical staining was performed as described above, except that the sections were blocked with rabbit serum incubated with biotinylated anti-CD4 antibody (5 μg/ml; PharMingen, San Diego, CA). Furthermore, the second incubation step was conducted with 3,3′diaminobenzidine Vecta-Red peroxidase substrate (Vector Laboratories). CD4+ areas exhibit green fluorescence under dark-field microscopy.

Results

Intradermal Plasmid DNA Immunization Will Result in Both Plasmid Transcript and Antigenic Protein Expression in Draining Lymph Nodes

To initiate an immune response, antigen must be processed by APC, which traffic to a regional lymph node where antigen-specific T cells are stimulated. Fig. 2A demonstrates that DNA vaccination by the s.c. or i.p. route does not result in detectable plasmid in the lymph node draining the injection site. However, after the plasmid is given i.m. or i.d., transcript can be detected in the lymph nodes of immunized mice. Even at the lowest dose of plasmid (1 μg), pLNCX could still be identified in the draining lymph node of mice vaccinated via the i.d. route. To determine whether trafficking cells expressed rat *neu*

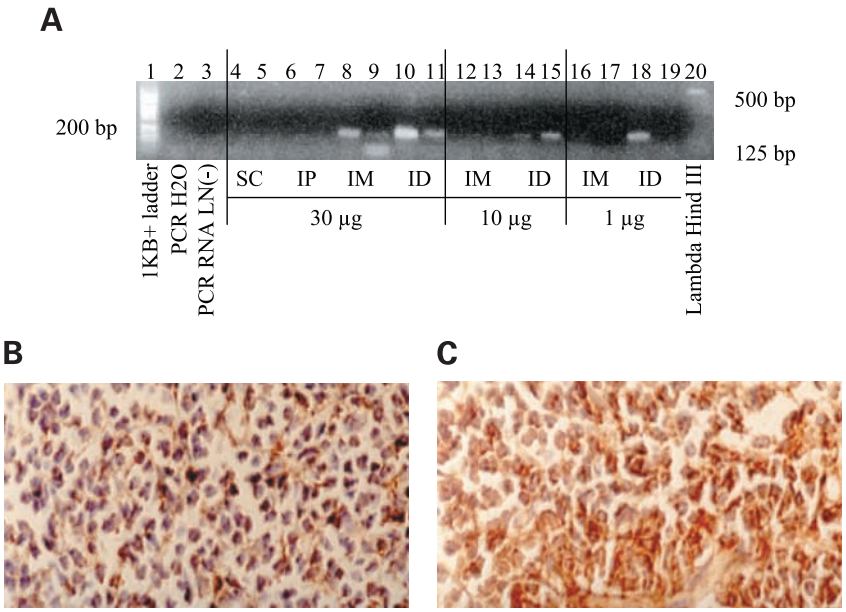

Figure 2. Intradermal plasmid DNA immunization will result in both plasmid transcript and antigenic protein expression in draining lymph nodes. **A,** PCR of cDNA from draining lymph nodes 48 h after injection. *Lanes 4–11,* individual animals immunized with 30-µg RN: s.c. vaccination (*4 and 5*), i.p. (*6 and 7*), i.m. (*8 and 9*), and i.d. (*10 and 11*). For subsequent doses, only the i.m. and i.d. routes were evaluated. *Lanes 12–15,* lymph nodes from mice that received 10-µg RN: i.m. (*12 and 13*) and i.d. (*14 and 15*). *Lanes 16–20,* 1-µg vaccinations: i.m. (*16 and 17*) and i.d. (*19 and 20*). *Lanes 1 and 20,* size markers. Negative controls were identical PCR reactions with H_2O (*lane 2*) or cDNA from lymph nodes of naïve mice (*lane 3*). Representative of three independent experiments (2 mice/condition). **B** and **C**, sections from lymph nodes stained for rat *neu* protein expression at 48 h after injection. Lymph nodes were derived from animals vaccinated with pLNCX (**B**) or RN (**C**). Representative of three independent experiments.

protein, we performed immunohistochemical staining of lymph nodes derived from mice immunized i.d. with either 30 µg of pLNCX or RN. Fig. 2B demonstrates that mice receiving plasmid alone did not show evidence of protein expression in the draining lymph node. However, mice receiving plasmid that encoded rat *neu* had significant expression of the rat *neu* protein in the cytoplasm of cells in the lymph node (Fig. 2C).

Addition of Costimulatory Molecules to Plasmid DNA Immunization Elicits a Functional *neu*-Specific Antibody Response after Vaccination

Antibodies to rat *neu* were assessed by ELISA. Fig. 3 demonstrates that rat *neu*-specific antibodies could not be detected in animals immunized with the pLNCX or pMG vectors alone. A significant antibody response was defined as the mean ± 2 SD of the *neu*-specific antibody response in six control (PBS-immunized) animals (0.03 ± 0.02). Low-titer (≤1:200) rat *neu*-specific antibodies could be detected in mice immunized with RN or RN + pMG, although the addition of pMG to RN did not enhance the response (Fig. 3). In contrast, significantly higher-titer (>1:800) *neu*-specific antibodies could be detected in mice vaccinated with RN + costimulatory molecules as compared with mice immunized with RN or RN + pMG, RN + CD80/CD137L ($P < 0.001$ at all dilutions), or RN + CD86/CD137L ($P < 0.001$ at all dilutions). Activated *neu* induces a phosphorylation cascade in cytoplasmic kinases (26, 27). To determine whether the rat *neu*-specific antibodies elicited with active immunization could affect signaling via the tyrosine kinase, MMC cells, which overexpress rat *neu*, were incubated with sera from naïve mice and from mice immunized with RN + either CD80/CD137L or CD86/CD137L. As shown in Fig. 4A, serum from mice immunized with rat *neu* with costimulatory molecules induced tyrosine phosphorylation of the rat *neu* protein, while sera from naïve mice or from mice immunized with RN (and RN + pMG, data not shown) did not have an effect on phosphorylation. The increase in phosphorylation was not related to different concentrations of the *neu* receptor in the preparations (Fig. 4A).

We then assessed whether sera from immunized mice, which affected *neu* receptor tyrosine phosphorylation, could also impact anchorage-independent tumor cell growth. MMC tumor cells were exposed to sera and colony formation was assessed with soft agar assays. Fig. 4B shows that sera from animals immunized with RN ($P = 0.01$ at 1:40 dilution), RN + CD80/CD137L ($P = 0.003$), or RN + CD86/CD137L ($P = 0.001$) could significantly inhibit colony formation when compared with sera from naïve mice. While immunization with RN was effective at

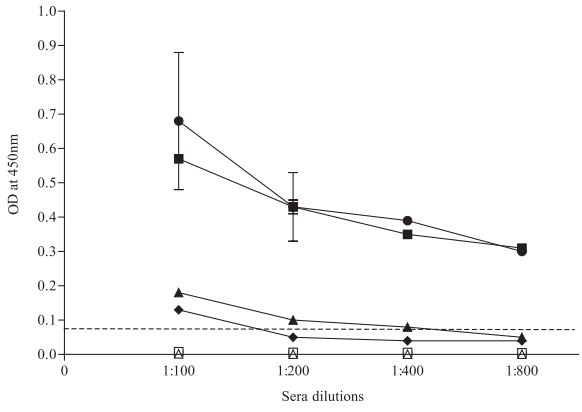

Figure 3. Rat *neu*-specific antibody immunity can be generated after immunization with plasmid-based vaccines encoding costimulatory molecules. Mice were immunized thrice with vaccine constructs and sera were obtained 45 days after the last vaccine. Antibody titers for mice immunized with PBS (△), pLNCX (□), or pMG (○). Antigen-containing vaccines included RN (◆), RN + pMG (▲), RN + CD80/CD137L (■), or RN + CD86/CD137L (●). *Dotted line,* mean ± 2 SD of the nonimmunized controls. *Lines,* mean; *bars,* SD (6 mice/group).

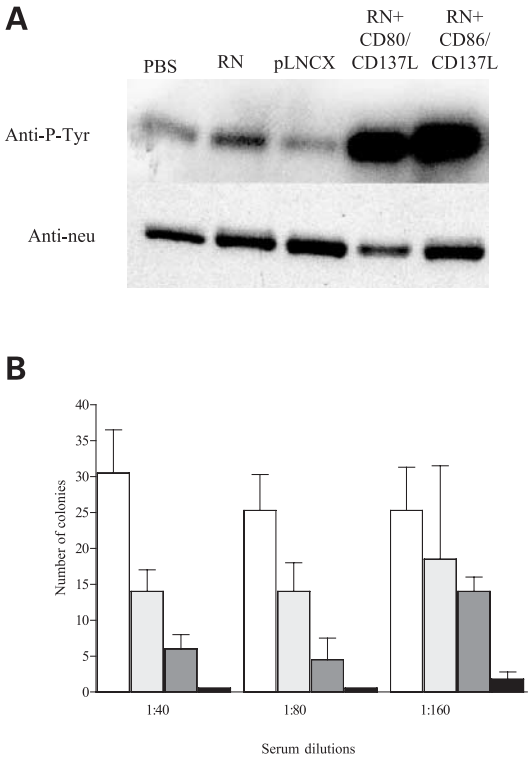

Figure 4. Rat *neu*-specific antibodies can induce rat *neu* tyrosine phosphorylation and inhibit the growth of *neu*-overexpressing tumor cells in vitro. **A,** (*top*) phosphorylation of *neu* expressed in MMC after treatment with sera derived from animals immunized with PBS, RN, pLNCX, RN + CD80/CD137L, and RN + CD86/CD137L; (*bottom*) *neu* protein from treated cells is present at similar levels. Representative of three independent experiments. **B,** MMC cells incubated with varying dilutions of experimental sera: naïve mice (*white*), RN (*light gray*), RN + CD80/CD137L (*dark gray*), and RN + CD86/CD137L (*black*). *Columns,* mean of triplicate cultures; *bars,* SD. Representative of three individual experiments.

inhibiting colony formation, the addition of either CD80/CD137L ($P = 0.02$ *versus* RN) or CD86/CD137L ($P = 0.002$ *versus* RN) significantly improved the inhibitory effects of RN immunization. Furthermore, CD86/CD137L was superior to CD80/CD137L ($P = 0.01$).

Plasmid DNA Immunization Targeting Rat *neu* Delays Tumor Development *in Vivo* and the Effect Is Augmented by the Addition of Costimulatory Molecules to the Vaccine

Mice were immunized with PBS alone, RN, RN + CD80/CD137L, or RN + CD86/CD137L, and a month after the final vaccination, 5×10^5 syngeneic tumor cells were implanted. Forty-five days after implantation, tumors could be detected in the PBS control group and measurements were taken every 2 days thereafter (Fig. 5). Although all the immunized groups developed tumors, animals vaccinated with RN + CD86/CD137L had a significantly prolonged survival over both PBS-immunized mice ($P < 0.0001$) and mice immunized with RN ($P = 0.0007$). The difference in survival between animals immunized with RN + CD80/CD137L and RN + CD86/CD137L was not significant ($P = 0.12$). Tumors from immunized mice contained marked CD4+ T-cell infiltrates. Fig. 6 depicts immunochemically stained sections of tumors from mice given PBS (Fig. 6A) or immunized against RN (Fig. 6B), RN + CD80/CD137L (Fig. 6C), or RN + CD86/CD137L (Fig. 6D). In animals immunized with RN + CD86/CD137L, tumor destruction and necrosis is evident (Fig. 6D).

Discussion

Potent methods of immunization are particularly important when contemplating vaccinating against cancer. Most of the recently identified tumor antigens are self-proteins and tolerance to self is one of the major mechanisms of tumor immune escape (28–30). Cancer vaccine strategies must be developed that are capable of augmenting or generating immune responses against self-tumor antigens (*i.e.*, circumvent tolerance). Considerable effort has been focused on using potent APC such as DC to process and present tumor antigens to the immune system (31–33). *Ex vivo* generation of DC, however, is laborious and may not be practical for wide-scale vaccine applications (34, 35). Our strategy for vaccinating against a self-tumor antigen, rat *neu*, in the *neu* transgenic mouse focuses on *in vivo* modification of skin cells with plasmid DNA constructs encoding costimulatory molecules chosen to enhance immune recognition. Data presented here demonstrate that (1) i.d. injection of plasmid DNA encoding *neu* will result in antigen expression in the local draining lymph node, indicating cells that traffic from skin to lymph node have been transfected *in vivo*; (2) functional *neu*-specific antibody immunity can be generated when *neu* encoding constructs are used in active immunization in conjunction with constructs encoding costimulatory molecules CD137L and CD80 or CD86; and (3) use of CD137L and CD80 or CD86 as costimulatory molecules in plasmid DNA vaccines will result in marked CD4+ T-cell infiltrates in tumors and significantly prolong survival in vaccinated mice.

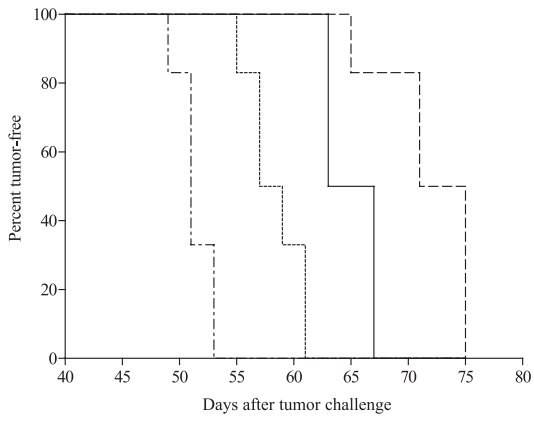

Figure 5. Plasmid DNA immunization targeting rat *neu* can delay tumor growth and the effect is augmented with the addition of costimulatory molecules in the vaccine. Groups of six mice were immunized with DNA vaccines. pLNCX (-●-), RN (—), RN + CD80/CD137L (– –), and RN + CD86/CD137L (– – –). Percent survival of mice is plotted as a function of the time after tumor implantation.

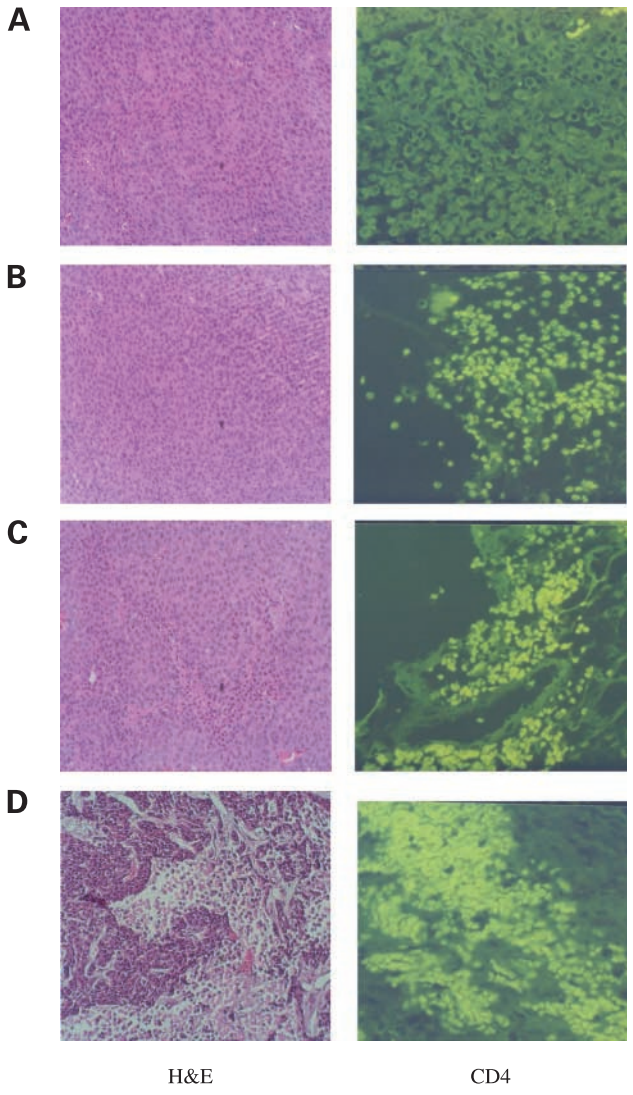

Figure 6. Tumors derived from mice immunized with rat *neu*-specific DNA vaccines contain CD4+ T cells. Tumors derived from immunized mice were stained with H&E (*first column*) or anti-CD40 monoclonal antibody (*second column*). Tumors from mice immunized with pLNCX (**A**), RN (**B**), RN + CD80/CD137L (**C**), and RN + CD86/CD137L (**D**). Data from an individual tumor are representative of 6 animals/experimental group.

For DNA vaccination to be successful, antigen encoded must be effectively taken up and processed by APC and presented to T cells in the draining lymph node. Therefore, activated APC must internalize the antigen at the site of injection and have the ability to migrate to the local lymph node. In addition, immunostimulatory molecules should be up-regulated on antigen-bearing APC to enhance presentation of antigen to T cells, thus stimulate a systemic immune response. In our model, i.d. immunization was the most effective route of vaccination for demonstrating antigen expression in the lymph node draining the injection site. Even at the lowest doses of plasmid DNA delivered, vector could still be detected in the lymph node. Vector transcript was only found at the injection site and in the draining lymph node and not in brain, heart, lungs, spleen, liver, digestive tract, or kidney (data not shown). Therefore, detection of plasmid and *neu* antigen expression in the lymph node is most likely due to locally transfected cells in the skin migrating to the node rather than a nonspecific generalized tissue distribution of the plasmid. APC in the skin capable of trafficking to lymph nodes include Langerhans cells or other nonprofessional APC such as macrophages. The use of costimulatory molecules in the plasmid constructs may ensure that even nonprofessional APC express molecules capable of triggering T-cell activation.

We chose to evaluate CD137L, CD80, and CD86 as costimulatory molecules to include in plasmid constructs to be delivered along with the *neu* antigen. CD137 is a member of the tumor necrosis family receptors and is expressed on activated T cells as well as on NK cells and monocytes for which it serves as a survival factor (36). CD137L is expressed on activated APC and has been shown to stimulate both CD4+ and CD8+ T cells (37). Although the CD137 axis is associated with the generation of a CD8+ T-cell response, it has been shown that CD137L can enhance the function of either CD4+ or CD8+ T cells with similar efficacy (12, 38). Furthermore, CD137L interactions with activated T cells will result in continued numerical and functional expansion of these immune effector populations *in vitro* (37). The CD137 signaling axis may be particularly important in the generation of an antitumor response. Recent studies have demonstrated in a poorly immunogenic murine tumor model that triggering signaling via CD137 in the presence of specific tumor antigen will result in tumor regression (39, 40).

Important for stimulating a naïve T-cell response, T-cell costimulatory receptors CD28 and CTLA-4 bind CD80 and CD86 ligands on APC (41). In addition, the CD28 pathway is critical in the maturation of the B-cell response (42). Several strategies have been developed to use these ligands in combination to enhance antigen-specific immune responses *in vivo* or *in vitro*. Investigators have transfected lymphoma cells with CD80, CD86, or CD137L and evaluated whether the intact tumor cell could act as a cancer vaccine (43). Although tumor cells transfected with CD80 or CD86 conferred partial protection, those including the CD137L resulted in long-lasting tumor protection. More recent studies have demonstrated that transfecting cells with the ligands for CD28 and CD137 results in an artificial APC that is capable of directly stimulating or recruiting cells that could stimulate the expansion of human T lymphocytes *in vitro* (44). Our strategy was to create such artificial APC *in vivo* in the skin by direct i.d. transection.

The *neu* transgenic mouse is a model for simulating human malignancy as mice are tolerant to *neu*. Plasmid-based vaccines encoding *neu* and CD137L and either CD80 or 86 were effective in generating both an antibody and a T-cell response specific for *neu*; therefore, tolerance was circumvented. The tumor antigen in our system, *neu*, is a growth factor receptor and antibodies directed against *neu* may mediate a therapeutic effect. Indeed, *neu*-specific antibodies elicited after active immunization induced tyrosine phosphorylation. Many *neu* ligand mimicking

antibodies will stimulate phosphorylation of the *neu* tyrosine kinase and their associated antitumor effect correlates with accelerated endocytic degradation (45, 46). The antibodies elicited after active immunization could suppress the growth of *neu*-overepxressing tumor cells *in vitro*. Although survival was prolonged in mice vaccinated with DNA encoding *neu* and CD137L and CD80 or CD86, tumors eventually developed. In studies of active immunization in human melanoma, it has been noted that there can be a dissociation between local and systemic immune responses against a specific tumor antigen (47). For this reason, we assessed the ability of T cells to home to antigen-bearing tumor as a functional measure of immunity. The tumors that did develop in the immunized animals were infiltrated predominantly with CD4+ T cells and tumors derived from the mice vaccinated with constructs encoding CD137L and CD86 demonstrated areas of necrosis. Although one could expect that CD8+ T cells could also be generated by endogenous processing via class 1 pathway by internally expressed protein, the infiltrating T cells were primarily CD4+. An immune response was generated but it was not fully protective. Investigations by others have demonstrated that the immunostimulatory properties of CD137L can be enhanced by the addition of cytokines such as interleukin-12 (48). The experiments performed here used the plasmid DNA constructs without any additional adjuvant. Potentially, the vaccine could be made more effective by coadministration with soluble cytokines such as interleukin-12 or granulocyte macrophage colony-stimulating factor (49).

Multiple tumor antigens have been defined, especially for common malignancies such as breast cancer (2). Recent studies have shown that patients with breast cancer can be immunized against proteins expressed by their tumors including HER-2/*neu* (50, 51). Vaccine technologies must be developed that will allow an immune response to be generated to multiple antigens expressed in a patient's tumor. Plasmid-based vaccine strategies offer such a technology that is easily and clinically applicable in a broad population. Costimulatory molecules functioning to transform local APC in the skin to stimulate immunity to weak antigens, such as a self-tumor antigen, have the potential to make plasmid-based tumor antigen vaccines therapeutically effective.

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