Vaccination by Genetically Modified Dendritic Cells Expressing a Truncated neu Oncogene Prevents Development of Breast Cancer in Transgenic Mice

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ABSTRACT

Dendritic cells (DCs) are powerful antigen-presenting cells that process antigens and present peptide epitopes in the context of the major histocompatibility complex molecules to generate immune responses. DCs are being studied as potential anticancer vaccines because of their ability to present antigens to naive T cells and to stimulate the expansion of antigen-specific T-cell populations. We investigated an antitumor vaccination using DCs modified by transfer of a nonsignaling neu oncogene, a homologue of human HER-2/neu, in a transgenic model of breast cancer. BALB-neuT mice develop breast cancers as a consequence of mammary gland-specific expression of an activated neu oncogene. We vaccinated BALB-neuT mice with bone marrow-derived DCs transduced with Ad.Neu, a recombinant adenovirus expressing a truncated neu oncoprotein. The vaccine stimulated the production of specific anti-neu antibodies, enhanced interferon-γ expression by T cells, and prevented or delayed the onset of mammary carcinomas in the mice. Over 65% of vaccinated mice remained tumor free at 28 weeks of age, whereas all of the mice in the control groups developed tumors. When challenged with a neu-expressing breast cancer cell line, vaccinated tumor-free animals had delayed tumor growth compared with controls. The antitumor effect of the vaccine was specific for expression of neu. Studies showed that CD4+ T cells were required in order to generate antitumor immunity. Importantly, the effectiveness of the vaccine was not diminished by preexisting immunity to adenovirus, whereas the protection afforded by vaccination that used direct injection of Ad.Neu was markedly reduced in mice with anti-adenovirus antibody titers. DCs modified by recombinant adenoviruses expressing tumor-associated antigens may provide an effective antitumor vaccination strategy.

INTRODUCTION

Cancer cells express tumor-associated antigens (1, 2) that can be potentially targeted by the immune system. Dendritic cells (DCs) are powerful antigen-presenting cells that play a central role in generating and directing immune responses through the processing of antigens and presentation of epitopes in the context of surface MHC molecules to interact with T cells. DCs also express a full complement of costimulatory molecules and cytokines that are required to sustain and direct the immune response (3, 4). Major efforts at developing antitumor vaccines have focused on harnessing DCs to effectively present tumor antigens to the immune system. A number of clinical trials have examined the efficacy of epitope-modified DCs, most often by “pulsing” DCs ex vivo with synthetic peptides based on antigens (5–10), mutant oncogenes (11), or immunoglobulin idiotypes (12) expressed by tumors. Peptide loading has a number of potential drawbacks including a need for detailed knowledge of the antigen sequence, identification of the recipient MHC molecule, and a requirement for a favorable binding affinity of the peptide for the MHC molecule. Peptide binding half-lives may be brief (13, 14), and the ability of a peptide to induce an antigen-specific CTL response correlates with the stability of this complex (15). An alternative strategy is to load DCs by incubating them with protein lysates manufactured from tumors. Tumor lysates, however, contain many antigens, most of which are not tumor specific.

The introduction of a gene encoding a tumor-associated antigen into DCs may overcome many of these problems. Gene expression and the physiologic processing of intact antigens will likely generate greater numbers of epitopes and more favorable antigen presentation. In addition, constitutive gene expression provides for continuous replenishment of low affinity epitopes bound on MHC molecules. Among gene delivery systems, recombinant adenoviruses (rAds) can efficiently infect DCs and offer the advantage of accepting up to 8-kb cDNA inserts. In addition, viral-associated proteins may provide the “danger signals” required for the activation of DCs and up-regulation of their costimulatory molecules, which results in stronger immune responses (16, 17).

The HER-2/neu (ErbB2) oncogene, a member of the epidermal growth factor receptor tyrosine kinase family (18, 19), is frequently overexpressed in cancers of the breast (20, 21), ovary (22), uterus (23), lung (24), and gastrointestinal tract (25). Its overexpression in breast cancer is associated with a poorer prognosis (20). Trastuzumab, a humanized antibody targeting HER-2/neu is approved for the treatment of advanced breast cancer, indicating the ability of this antigen to serve as a target for immunotherapy (26). Vaccination of patients with advanced breast cancer using HER-2/neu peptides or peptide-pulsed autologous DCs has been examined in clinical trials (8). Few responses, however, have been reported, although increases in anti-HER-2/neu CTLs were seen in some patients (9).

To examine the efficacy of antitumor vaccination in a clinically relevant model, we studied the ability of DCs modified by a rAd expressing a truncated neu antigen to suppress the development of mammary carcinomas in a transgenic model of breast cancer in which the neu oncogene is expressed in tissue-specific fashion. We demonstrated that vaccination prevented or delayed the onset of cancers in this aggressive tumor model. Importantly, the efficacy of antitumor vaccination with rAd-infected DCs was unaffected by preexisting immunity to adenovirus.

MATERIALS AND METHODS

Cell Lines. TUBO cells derived from a breast cancer of a BALB-neuT mouse (27), and N202.1A cells isolated from a breast cancer of a FVB-neuN mouse, were the kind gift of Dr. Patrizia Nanni (University of Bologna, Bologna, Italy; refs. 28, 29) and were grown in Dulbecco’s modified Eagle’s medium (DMEM; BioSource, Rockville, MD) supplemented with 10% fetal bovine serum (Gemini, Woodland, CA). Both TUBO and N202.1A cells constitutively express neu. The neu-negative cell line, TS/A (also a gift of Dr. Patrizia Nanni), established from a spontaneous breast cancer from a BALB/c mouse (30) was grown in RPMI 1640 (BioSource) and 10% fetal bovine serum. Human 293 embryonic kidney and A549 lung cancer cells were obtained from American Type Culture Collection (ATCC; Manassas, VA) and were grown in DMEM with 10% fetal bovine serum.
Adenoviral Vectors. The cDNA encoding the extracellular and transmembrane domains of the rat neu oncogene was provided by Dr. Augusto Amici (University of Camerino, Camerino, Italy; ref. 27). Ad.Neu, an E1-, E3-deleted rAd expressing neu, and Ad.null, an “empty” control vector were generated by homologous recombination with the AdMax system (Microbix, Toronto, Canada; ref. 31). Ad.GFP, an adenovirus expressing green fluorescent protein (GFP) was obtained from Quantum Biotechnologies (Québec, Canada). All viruses were double plaque-isolated, expanded on 293 cells, purified on a cesium chloride gradient, titered by serial dilution as plaque forming units (pfu)/mL and stored at −70°C.

Animals. All animals were approved by the Animal Care and Use Committee of the National Cancer Institute. BALB-neuT mice expressing the rat neu oncogene under the control of a chimeric mouse mammary tumor virus (MMTV) promoter provide an aggressive model of mammary carcinogenesis as the transgene is overexpressed in the rudimentary mammary glands of 3-week-old mice (32–34). By 6 weeks, atypical lobular hyperplasia is seen that progresses to multiple in situ carcinomas that enlarge and converge to form rapidly growing, invasive, and metastasizing tumors in all 10 glands by 25 weeks of age (35). Female BALB/c mice were obtained from the Division of Cancer Treatment, National Cancer Institute (Frederick, MD).

Generation of Dendritic Cells. DCs were generated with the method of Lutz et al. (36). Briefly, bone marrow was harvested from femurs of 8- to 10-week-old BALB/c mice. Erythrocytes were lysed with ammonium-chloride-potassium buffer (BioWhittaker, Walkersville, MD) and the nucleated cells were plated in plastic bacteriologic dishes in RPMI 1640 with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.-Invitrogen, Grand Island, NY) and 20 ng/mL murine granulocyte/macrophage-colony stimulating factor (GM-CSF; PeproTech, Rocky Hill, NJ). The cultures were refreshed with 10 mL of medium containing 20 ng/mL GM-CSF on days 3, 6, and 8. On day 8, the nonadherent DCs were collected and infected with rAds. The DCs were used for experiments on day 10 after washing three times in phosphate buffered saline.

Flow Cytometry. DCs were incubated with FITC- or phycoerythrin-labeled antimonue CD11c, CD11b, CD40, CD80, CD86, H-2K d, I-Ad, CD8 (BD Pharmingen, San Diego, CA) and analyzed on a FACSsort (Becton Dickinson, San Jose, CA). Forty-eight hours after infection with Ad.Neu or Ad.null, the DCs were incubated with antirat neu monoclonal antibody (OncoGene Research, La Jolla, CA) followed by incubation with secondary FITC-labeled rabbit antimouse immunoglobulin and analyzed by FACSort. To detect cytoplasmic neu, the cells were permeabilized with Cytofix/Cytoperm (BD Pharmingen) and similarly stained.

Mixed Lymphocyte Proliferation and CTL Assay. Lymphocytes obtained from the spleens of 8- to 10-week-old naïve female BALB-neuT mice were incubated with irradiated unmodified DCs or DCs infected with either of the rAds. Four days later, the incorporation of [3H]thymidine was measured as described previously (37). CTL assays with chromium-51 release were performed as described previously (38).

Vaccination of Mice with Ad.Neu-Modified Dendritic Cells. Groups of 5- to 6-week-old female BALB-neuT mice received three weekly subcutaneous injections of 1 × 10³ DCs infected with Ad.Neu (DC-Ad.Neu) or Ad.null (DC-Ad.null) at a MOI 30, or of unmodified DCs. The mice were examined twice weekly for the development of tumors. Tumor volumes were calculated with the estimated volume of a rotational ellipse (39). Mice free of tumor at 28 weeks were challenged with a subcutaneous injection of 1 × 10⁶ neuTUBO cells. Another group of BALB-neuT mice received Ad.null 1 × 10⁶ pfu subcutaneously before anti-neu vaccination and was followed for the development of tumors after undergoing vaccination as described above. This group was compared with mice similarly pre Vaccinated with Ad.null and then vaccinated against neu by direct subcutaneous injections of Ad.Neu 1 × 10⁶ pfu, weekly for 3 weeks.

In another set of experiments, groups of 5- to 6-week-old BALB/c mice were given injections subcutaneously of Ad.null 1 × 10⁷ pfu weekly for 4-weeks. Two weeks after the last injection of Ad.null, groups of mice were vaccinated either with 1 × 10⁶ DC-Ad.Neu subcutaneously or directly with 1 × 10⁷ pfu Ad.Neu subcutaneously, weekly for two weeks. One week after the final vaccination, the mice were challenged with 5 × 10⁶ TUBO cells subcutaneously and were monitored for tumor growth.

In another experiment, BALB/c mice vaccinated with DC-Ad.Neu were challenged with 5 × 10⁶ neuTUBO cells subcutaneously. All of the mice remained tumor free, and at 37 days, the mice were divided into two groups and rechallenged with an injection of 5 × 10⁶ neuTUBO cells or neuTUBO cells, and were followed for development of tumors. In a third experiment, groups of mice were vaccinated with Ad.null 10⁸ pfu, weekly for 4-weeks. Two weeks after the last injection of Ad.null, groups of mice were vaccinated with 1 × 10⁶ DC-Ad.Neu subcutaneously or directly with 1 × 10⁷ pfu Ad.Neu subcutaneously, weekly for two weeks. One week after the last vaccination, the mice were challenged with 5 × 10⁶ TUBO cells and then were monitored for tumor growth.

Detection of serum Anti-neu Antibodies. Blood was obtained by retro-orbital venipuncture from BALB-neuT mice before vaccination and one week after the third vaccination. The blood was allowed to clot, and the sera from each treatment group were pooled. N202.1A (sera [new]) were used to detect and quantify anti-neu antibodies as described previously with slight modification (28). Briefly, 2 × 10⁵ N202.1A cells were incubated with the sera diluted 1:10 in 2% bovine serum albumin in phosphate buffered saline at 4°C for 1 hour. The cells were washed and incubated with FITC-labeled rabbit antimouse immunoglobulin antibody (DAKO, Carpinteria, CA) and were analyzed to account for GFP expression by flow cytometry. Antibody titers were assessed as the serum dilution that resulted in 50% inhibition of GFP expression compared with controls. Enzyme-Linked Immunosorbent Assay. Sera were obtained pre- and postvaccination from the mice. Anti-adenovirus antibody titers were measured as described previously (40). Briefly, 5 × 10⁵ A549 cells were seeded in a 24-well plate. Sixteen hours later, 10 μL of 2-fold serially diluted heat-inactivated test sera were incubated with 10 μL of Ad.GFP (2.5 × 10⁷ pfu) at room temperature for 1 hour. The mixture was added to the A549 monolayer (MOI 30). Thirty-six hours later, the A549 cells were trypsinized, washed, and analyzed for GFP expression by flow cytometry. Antibody titers were assessed as the serum dilution that resulted in 50% inhibition of GFP expression compared with controls.

Antibody Depletion of Lymphocyte Populations. Groups of BALB/c mice were given intraperitoneal injections of 200 μg of anti-CD4, or anti-CD8 purified from the supernatants of hybridomas GK1.5 (ATCC) and 2.43 (ATTC), respectively, for 3 consecutive days, then every 3 days, or with anti-asialo-GM1 (WAKO, Richmond, VA). Five days after initiation of antibody treatment, the mice were vaccinated as described above. One week after the last vaccination, the mice were challenged with 5 × 10⁶ TUBO cells and then were monitored for tumor growth.

Statistical Analysis. StatView version 5.01 software (SAS Institute, Cary, NC) was used for log-rank survival analysis and comparisons of tumor multiplicity with the Mann–Whitney U test. Enzyme-linked immunospot (ELISPOT) assay was analyzed with Student’s t test.
RESULTS

Effect of Adenovirus on Dendritic Cell Phenotype. DCs generated from 10-day bone marrow cultures expressed CD11c and CD11b, and lacked CD8α, indicating myeloid differentiation (Fig. 1A). They also expressed CD80, CD86, CD40, and MHC class I and class II. Compared with unmodified DCs, those infected with Ad.Neu (DC\textsubscript{Ad.Neu}) or Ad.null (DC\textsubscript{Ad.null}) expressed higher levels of CD80, CD86, CD40, and both MHC classes, indicating maturation (Fig. 1B).

Dendritic Cells Infected with Ad.Neu-Expressed Truncated neu mRNA and Oncoprotein. Thirty-six hours after infection with Ad.Neu or Ad.null, Northern analysis of RNA isolated from DC\textsubscript{Ad.Neu} showed the expected 2.1-kb truncated \textit{neu} mRNA transcript (Fig. 2A), which was not detected in DC\textsubscript{Ad.null}. The transcript was absent in noninfected BALB-neu\textsuperscript{T} bone marrow-derived DCs, even when analyzed by reverse transcription-PCR, indicating tissue-specific \textit{neu} expression in the transgenic animals (data not shown). DCs, stained with antirat \textit{neu} antibody 48 hours after infection with Ad.Neu or Ad.null and examined by flow cytometry, showed \textit{neu} expression on the cell surface and in the cytoplasm (Fig. 2B).

Ad.Neu-Transduced Dendritic Cell-Stimulated Proliferation of Naı ¨ve BALB-neu\textsuperscript{T} Lymphocytes. The function of the DCs was examined in mixed lymphocyte culture. Irradiated DC\textsubscript{Ad.Neu}, DC\textsubscript{Ad.null}, or unmodified DCs were cultured in various ratios with lymphocytes from the spleens of nonimmunized BALB-neu\textsuperscript{T} mice and were pulsed with \textsuperscript{3}H-thymidine. Three to 4-fold greater stimulation of splenocyte labeling was seen with DC\textsubscript{Ad.Neu} compared with DC\textsubscript{Ad.null} or unmodified DCs (Fig. 3).

Vaccination of BALB-neu\textsuperscript{T} Mice with DC\textsubscript{Ad.Neu} Prevented Autochthonous Breast Cancers. Vaccination of BALB-neu\textsuperscript{T} mice with DC\textsubscript{Ad.Neu} significantly improved tumor-free survival compared with mice treated with DC\textsubscript{Ad.null} or unmodified DCs (Fig. 4A). The median tumor-free survival of the mice treated with unmodified DCs compared with those receiving DC\textsubscript{Ad.null} was not different (19.5 versus 20 weeks, \textit{P} = 0.438). In contrast, the median tumor-free survival was not reached by 28 weeks in mice receiving DC\textsubscript{Ad.Neu} (\textit{P} < 0.0001). Furthermore, in the groups of mice treated with the unmodified DCs or DC\textsubscript{Ad.null}, the onset of the first tumor occurred at 14.5 weeks, and all of the mice had developed at least one breast cancer by 23.5 weeks. In mice vaccinated with DC\textsubscript{Ad.Neu}, the first breast lesion occurred at 20 weeks, and 14 (66.7%) of 21 mice were still free of tumor at 28 weeks. In view of the mean tumor multiplicity, DC\textsubscript{Ad.Neu} also conferred significant protection (Fig. 4B). At 28 weeks, tumor was palpable in all 10 mammary glands of all of the mice treated with unmodified DCs or with DC\textsubscript{Ad.null}. In the group of mice vaccinated with DC\textsubscript{Ad.Neu}, the mean tumor multiplicity was only 2.9.

![Fig. 1. DC phenotype. In A, surface marker expression of DCs derived from BALB/c mouse bone marrow examined by flow cytometry. Open curves, isotype controls. In B, DC surface marker expression 48 hours after infection with rAds (MOI 30).](image1)

![Fig. 2. Expression of truncated \textit{neu} mRNA and antigen in DCs infected with Ad.Neu. In A, on day 8, DCs were infected with Ad.Neu or Ad.null (MOI 30), and RNA was isolated on day 10. Total RNA was probed with a \textsuperscript{32}P-fragment of rat \textit{neu} cDNA. In B, DCs were infected with Ad.null (MOI 300) or Ad.Neu (MOI 30 or 300); 48 hours later, the DCs were incubated with an antirat \textit{neu} primary antibody and were stained with FITC-labeled antimouse immunoglobulin. Open curves, isotype control.](image2)
(P < 0.0001). Vaccination of older groups of mice was less effective, and the vaccine had little effect in mice that had developed palpable tumors (data not shown).

**Vaccination of BALB-neuT Transgenic and BALB/c Mice with DC<sub>Ad.Neu</sub> Inhibited Growth of a Transplantable neu-Expressing Breast Carcinoma.** To test whether DC<sub>Ad.Neu</sub>-vaccinated BALB-neuT mice could successfully reject a challenge with a neu-expressing tumor cell line, mice vaccinated at 5 to 7 weeks of age with DC<sub>Ad.Neu</sub> that were tumor free at 28 weeks (n = 6) were challenged with the neu<sup>+</sup> TUBO cells. A small number (n = 3) of surviving similar-age mice that had been vaccinated with unmodified DCs or DC<sub>Ad.null</sub> were also challenged. At a mean of 10 days after injection with 1 x 10⁵ TUBO cells, tumors were evident in the mice receiving unmodified DCs or DC<sub>Ad.null</sub>. The time to the appearance of palpable TUBO tumors was similar to that of unvaccinated BALB/c or BALB-neuT mice (data not shown). In contrast, in the BALB-neuT mice vaccinated with DC<sub>Ad.Neu</sub> tumors occurred at a mean of 56 days. Notably, in the DC<sub>Ad.Neu</sub>-vaccinated mice, TUBO tumor growth occurred contemporaneous or subsequent to the development of an autochthonous breast cancer, suggesting a general loss of immunity to neu (Table 1). Immunohistochemical staining of the TUBO tumors and late-occurring autochthonous breast cancers were positive for neu expression, which indicated that tumor escape was not due to selection and outgrowth of neu-negative tumor cells.

To examine the specificity of protection derived from vaccination with DC<sub>Ad.Neu</sub>, BALB/c mice (n = 8) were vaccinated with 1 x 10⁶ DC<sub>Ad.Neu</sub> weekly for 2 weeks, and were then subcutaneously challenged with 5 x 10⁵ TUBO cells a week later. All of the mice remained free of tumor at 37 days. These mice were rechallenged with 5 x 10⁵ of either TUBO cells (n = 4) or neu-negative TS/A cells (n = 4). All of the vaccinated mice that received injections of TS/A cells developed tumors by day 10, whereas those receiving TUBO cells remained free of disease up to 121 days later (Table 2).

**DC<sub>Ad.Neu</sub>-Vaccination Induced Serum Anti-neu Antibodies and IFNγ Secretion by T Cells.** We measured the induction of anti-neu antibodies in groups of BALB-neuT mice pre-vaccination and one week after the final vaccination. Major increases of anti-neu antibody titers were detected in the serum of mice vaccinated with DC<sub>Ad.Neu</sub>-Minor changes were seen in the mice receiving DC<sub>Ad.null</sub>, and no titers were detected in mice vaccinated with the unmodified DCs (Fig. 5A).

Tumor-specific CTLs could not be demonstrated by chromium-release assay in the vaccinated mice; however, IFNγ production by splenocytes was seen on ELISPOT (Table 3). The numbers of IFNγ-expressing T cells were increased in DC<sub>Ad.Neu</sub> vaccinated mice compared with the mice vaccinated with DC<sub>Ad.null</sub> (P < 0.0001), or unmodified DCs (P < 0.0001). These results were confirmed by cytokine secretion assay. A more than 3.5-fold increase in the number of "Table 1 DC<sub>Ad.Neu</sub> vaccination protects BALB-neuT mice from challenge with neu-expressing TUBO breast cancer cells"

**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TUBO tumor (days*)</th>
<th>Breast cancer (days*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC&lt;sub&gt;Ad.Neu&lt;/sub&gt;</td>
<td>108</td>
<td>80</td>
</tr>
<tr>
<td>DC&lt;sub&gt;Ad.null&lt;/sub&gt;</td>
<td>56</td>
<td>50</td>
</tr>
<tr>
<td>DC&lt;sub&gt;Ad.null&lt;/sub&gt;</td>
<td>56</td>
<td>66</td>
</tr>
<tr>
<td>DC&lt;sub&gt;Ad.null&lt;/sub&gt;</td>
<td>28</td>
<td>7</td>
</tr>
<tr>
<td>DC&lt;sub&gt;Ad.null&lt;/sub&gt;</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>DC</td>
<td>11</td>
<td>NA</td>
</tr>
<tr>
<td>DC&lt;sub&gt;Ad.null&lt;/sub&gt;</td>
<td>9</td>
<td>NA</td>
</tr>
<tr>
<td>DC</td>
<td>10</td>
<td>NA</td>
</tr>
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</table>

Abbreviations: NA, not applicable.

† DC<sub>Ad.Neu</sub> Vaccinated BALB-neuT mice that were tumor free at the age of 28 weeks were challenged with syngeneic neu<sup>+</sup> TUBO breast cancer cells and were monitored for the development of tumors.

**Table 2** Vaccination induced specific immunity to neu-expressing tumors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TUBO</th>
<th>TS/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor-free</td>
<td>4/4</td>
<td>0/4</td>
</tr>
</tbody>
</table>

Note. DC<sub>Ad.Neu</sub> vaccinated BALB/c mice, tumor-free 37 days after subcutaneous injection of 5 x 10⁵ neu<sup>+</sup> TUBO cells, were rechallenged with 5 x 10⁵ TUBO cells, or 5 x 10⁵ neu<sup>+</sup> TS/A cells. All mice receiving TS/A cells developed tumors by 10 days after injection. Mice re-challenged with TUBO cells remained tumor-free 121 days later.

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Fig. 3. DCs infected with Ad.Neu stimulated the proliferation of naïve BALB-neuT lymphocytes. DCs infected with Ad.Neu (DC<sub>Ad.Neu</sub> or Ad.null (DC<sub>Ad.null</sub>) or with unmodified DCs (DC) were mixed with naïve BALB-neuT mouse splenic lymphocytes in various ratios, and the incorporation of [³H]thymidine was measured. Error bars, ± SEM.

Fig. 4. Effect of vaccination on tumors in BALB-neuT transgenic mice. In A, Kaplan–Meier plot of tumor-free survival. BALB-neuT mice (5–6 weeks old) were given injections in the flank with 1 x 10⁵ DC<sub>Ad.Neu</sub> (○, n = 21), DC<sub>Ad.null</sub> (□, n = 11), or unmodified DCs (○, n = 10), weekly for 3 weeks. Mice were followed for the development of breast tumors. In B, mean tumor multiplicity (mean number of tumors per mouse) in the treatment groups over time. Error bars, ± SEM; *, P < 0.0001.

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Efficacy of DCAd.Neu Vaccination Is Unaffected by Preexisting Antigen-Specific Immunity with DCAd.Neu. To explore the mechanism of DCAd.Neu vaccination to deplete these lineages. One week after the last vaccination, the mice were challenged with 5 × 10^5 TUBO cells. Twenty-one days later, all of the mice treated directly with Ad.Neu had developed tumors, whereas six of seven mice receiving DCAd.Neu remained tumor-free (Fig. 7B), which suggested that immunity to adenovirus abrogated the protection afforded by direct injection of Ad.Neu.

**DISCUSSION**

We showed that vaccination of BALB neuT-transgenic mice with bone marrow-derived DCs modified by a rAd-expressing truncated neu oncoprotein prevented the development, or significantly delayed the onset, of breast cancer in the mice. Antitumor vaccination with DCs modified by adenoviral-mediated transfer of genes encoding tumor-associated antigens offers potential advantages over antigen-loading strategies that use peptides or tumor lysates. These include long-term antigen expression, activation of the DCs by the vector, and induction of DC maturation with enhanced expression of MHC and costimulatory molecules. DCs modified by adenovirus are able to stimulate naïve splenocytes to a greater degree than are unmodified DCs, possibly because of activation by viral proteins (41). This may enhance the vaccine’s efficacy, because mature DCs are more effective at generating immune responses (42).

**Table 3 Interferon γ ELISPOT**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Spots/3 × 10^4 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC</td>
<td>106 ± 35.0</td>
</tr>
<tr>
<td>DCAd.null</td>
<td>214 ± 81.2</td>
</tr>
<tr>
<td>DCAd.Neu</td>
<td>461 ± 36.8</td>
</tr>
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</table>

Note: T cells isolated from spleens of BALB-neuT mice 1 week after completion of vaccination were assessed for IFNγ secretion by ELISPOT. The ELISPOT was performed in triplicate and repeated in two independent mice from each treatment group. Data are presented as a mean ± SD.

5 The numbers of IFNγ-expressing T cells were increased in DCAd.Neu-vaccinated mice compared with the mice vaccinated with DCAd.null (P < 0.0001) or unmodified DCs (P < 0.0001).

**Fig. 5. Antibody and cellular responses to vaccination. In A, vaccination with DCAd.Neu induced the production of anti-neu antibodies. Serum collected from BALB-neuT mice before vaccination and 1 week after the last (third) vaccination were analyzed for anti-neu antibodies, as described in Materials and Methods. The results represent the mean titers of five mice in each group. Error bars, ± SEM. In B, DCAd.Neu vaccination increased the secretion of IFNγ from CD4+ and CD8+ T cells. Splenic T cells isolated from BALB-neuT mice 1 week after completion of the DCAd.Neu vaccination were assayed for IFNγ secretion after restimulation by DCAd.Neu, DCAd.null, or unmodified DCs by fluorescent antibody capture flow cytometry.**

**Fig. 6. Infiltration of Mammary Glands with CD4+ and CD8+ T Cells.** Immunohistochemical staining of mammary tissue from BALB-neuT mice vaccinated with DCAd.Neu showed modestly increased numbers of CD4+ and CD8+ cells infiltrating the periphery of hyperplastic and dysplastic mammary glands (data not shown). The breast tissue of mice receiving the control vaccines showed little cellular infiltrate.

**CD4+ T Cells Are Required for Generation of Antitumor Immunity with DCAd.Neu.** To confirm these results, BALB/c mice were given subcutaneous injections weekly for 4 weeks with Ad.null 1 × 10^6 pfu. All of the mice receiving Ad.null showed significant increases in serum anti-adenovirus antibody titers, from a mean of less than 1:2 to a mean greater than 1:256, 1 week after the last injection. Untreated mice had a mean baseline titer of less than 1:2 that remained unchanged over 6 weeks (data not shown). Two weeks later, the mice were vaccinated with either 1 × 10^6 pfu Ad.Neu, or 1 × 10^6 DCAd.Neu. One week later, the mice were challenged with 5 × 10^5 TUBO cells. Twenty-one days later, all of the mice treated directly with Ad.Neu had developed tumors, whereas six of seven mice receiving DCAd.Neu remained tumor-free (Fig. 7A), which suggested that immunity to adenovirus abrogated the protection afforded by direct injection of Ad.Neu.
and given injections of Ad.Neu/H11003 and during DC Ad.Neu vaccination. One week after completion of vaccination, the mice vaccinated animals. The activation of CD4+ T cells increased the expression of costimulatory molecules, the direct inter-

Although the infection of DCs with rAds induced maturation and required for the complete activation of B cells by many antigens (43).

expressing CD4+/H11001 mice, and studies that depleted T-cell subsets demonstrated a critical role for CD4+ cells in the generation of antitumor immunity in the vaccinated animals. The activation of CD4+ helper T cells by DCs is required for the complete activation of B cells by many antigens (43). Although the infection of DCs with rAds induced maturation and increased the expression of costimulatory molecules, the direct interaction of CD8+ T cells with DCs may not be sufficient for the induction of tumoricidal activity. Preconditioning by CD4+ helper T cells may be required for CD8+ cells to be fully activated (44, 45).

In humans, a significant fraction of the population has been exposed to adenovirus; therefore, preexisting immunity to the vector might hinder the effectiveness of adenovirus-based vaccines (46–48). We found no attenuation of the DCAd.Neu vaccine in animals preimmunized with Ad.null. In contrast, the effectiveness of direct vaccination with Ad.Neu was diminished by prior adenovirus exposure, which suggested that vaccination with rAds to modify DC ex vivo can overcome the inhibition of gene expression seen in individuals previously exposed to adenovirus (49, 50).

Y. Chen et al. (51) and Z. Chen et al. (52) reported that vaccination with rAds modified with a rAd to express HER-2/neu, offered partial protection in a transplantable HER-2/neu-expressing tumor model. In the transplantable TUBO cell model, DCAd.Neu vaccination was fully effective (data not shown); we, therefore, focused efforts on the transgenic BALB-neuT model. We found that a series of vaccinations before the development of in situ carcinomas in the animals was effective in preventing or delaying the onset of breast tumors. The age at which the mice were vaccinated was critical. Vaccination of 5- to 6-week-old BALB-neuT mice resulted in 66.7% of the mice being tumor-free at 28 weeks, whereas all of the control animals developed tumor. Vaccination of older groups of transgenic mice was less efficacious, and little effect was noted in mice that had already developed tumors, which suggests that other factors such as immunologic escape or tolerance may play a role. DCAd.Neu-vaccinated BALB/c mice rejected repeated challenges with neu-expressing TUBO cells but were not protected when challenged with neu-negative TS/A cells, indicating that DCAd.Neu vaccination generated specific immunity. Vaccinated mice, challenged with neu-expressing TUBO cells, were protected as long as they did not develop a primary breast cancer, indicating immunologic memory against neu antigen. Once a primary breast cancer was detected, growth of the implanted TUBO cells followed. Immunohistochemical studies found that autono-

In our model, the induction of anti-neu antibodies and the inability to demonstrate specific CTLs suggests that the antibody may be the primary protective mechanism of the vaccine; however, a T-cell response is not excluded. Increased numbers of neu-specific IFNγ-expressing CD4+ and CD8+ T cells were seen in the vaccinated mice, and studies that depleted T-cell subsets demonstrated a critical role for CD4+ cells in the generation of antitumor immunity in the vaccinated animals. The activation of CD4+ helper T cells by DCs is required for the complete activation of B cells by many antigens (43).

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Fig. 6. Protection by DCAd.Neu vaccination requires CD4+ T cells. BALB/c mice were depleted of specific cell populations using anti-CD4, anti-CD8, or anti-asialo-GM1 before and during DCAd.Neu vaccination. One week after completion of vaccination, the mice were given subcutaneous injections with 1 × 10^6 TUBO cells. Tumor volumes at day 63 are shown.

Fig. 7. Effect of preexisting immunity to adenovirus on the efficacy of DCAd.Neu vaccination. In A, the protective effect of vaccination with DCAd.Neu in BALB-neuT mice was not affected by preexisting immunity to adenovirus, and the benefit of direct vaccination using Ad.Neu was lost. BALB-neuT mice were injected with Ad.null 1 × 10^8 pfu s.c. Two weeks later, mice were vaccinated with either 1 × 10^6 DCAd.Neu s.c. (n = 7), or directly with 1 × 10^8 pfu Ad.Neu s.c. (n = 7). BALB-neuT mice (n = 7), not previously treated with Ad.null and given injections of Ad.Neu 1 × 10^8 pfu subcutaneously, served as controls. In B, DCAd.Neu vaccination was effective in BALB/c mice with preexisting immunity to adenovirus in protecting against TUBO breast cancer, whereas the protection from direct vaccination with Ad.Neu was lost. BALB/c mice were injected weekly for 4 weeks with Ad.null 1 × 10^8 pfu subcutaneously, and serum anti-adenovirus antibody titers were measured. The mice were then vaccinated with 1 × 10^6 DCAd.Neu or with direct injection of 1 × 10^8 pfu Ad.Neu subcutaneously weekly for 2 weeks. Mice vaccinated with Ad.Neu or DCAd.Neu alone served as controls. The mice were challenged with 5 × 10^5 TUBO cells subcutaneously 1 week later. Twenty-one days after injection of the TUBO cells, the tumor volumes were compared.
manner. The fact that these lesions were controlled by vaccination with gene-engineered DCs in a transgenic animal model that displays an early and diffuse overexpression of the neu antigen and relentlessly develops cancers, suggests that a new and rational approach exists to secure immunologic control of HER-2/neu-expressing lesions. Vaccination with DCs that are modified by rAdS encoding nonfunctional tumor antigens, such as nonsignaling HER-2/neu, may offer a preventative strategy for patients at risk of metastases after primary treatment of a tumor, or for those at high risk of developing cancer.

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Yoshio Sakai, Brian J. Morrison, J. Douglas Burke, et al.


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