

## Targeting HER-2/*neu* in Early Breast Cancer Development Using Dendritic Cells with Staged Interleukin-12 Burst Secretion

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### Abstract

**Overexpression of HER-2/*neu* (c-erbB2) is associated with increased risk of recurrent disease in ductal carcinoma *in situ* (DCIS) and a poorer prognosis in node-positive breast cancer. We therefore examined the early immunotherapeutic targeting of HER-2/*neu* in DCIS. Before surgical resection, HER-2/*neu*<sup>pos</sup> DCIS patients ( $n = 13$ ) received 4 weekly vaccinations of dendritic cells pulsed with HER-2/*neu* HLA class I and II peptides. The vaccine dendritic cells were activated *in vitro* with IFN- $\gamma$  and bacterial lipopolysaccharide to become highly polarized DC1-type dendritic cells that secrete high levels of interleukin-12p70 (IL-12p70). Intranodal delivery of dendritic cells supplied both antigenic stimulation and a synchronized preconditioned burst of IL-12p70 production directly to the anatomic site of T-cell sensitization. Before vaccination, many subjects possessed HER-2/*neu*-HLA-A2 tetramer-staining CD8<sup>pos</sup> T cells that expressed low levels of CD28 and high levels of the inhibitory B7 ligand CTLA-4, but this ratio inverted after vaccination. The vaccinated subjects also showed high rates of peptide-specific sensitization for both IFN- $\gamma$ -secreting CD4<sup>pos</sup> (85%) and CD8<sup>pos</sup> (80%) T cells, with recognition of antigenically relevant breast cancer lines, accumulation of T and B lymphocytes in the breast, and induction of complement-dependent, tumor-lytic antibodies. Seven of 11 evaluable patients also showed markedly decreased HER-2/*neu* expression in surgical tumor specimens, often with measurable decreases in residual DCIS, suggesting an active process of "immunoeediting" for HER-2/*neu*-expressing tumor cells following vaccination. DC1 vaccination strategies may therefore have potential for both the prevention and the treatment of early breast cancer. [Cancer Res 2007;67(4):1842-52]**

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This article is dedicated to the memory of Charley Carter (1952-2006) who was instrumental in developing the elutriation procedure for preparing the dendritic cell vaccines.

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### Introduction

Therapeutic anticancer vaccines hold great promise for the control of malignancies, but there is no clear consensus about how they may best be optimized. For example, controversies exist relating to whether vaccines should be deployed primarily against early- versus late-stage (i.e., metastatic) disease (1). Additionally, there are unresolved issues surrounding the selection of appropriate tumor target antigens. With respect to dendritic cell-based vaccines, there is little agreement about which dendritic cell properties best promote therapeutic efficacy, the culture and activation regimens best suited to maximize these properties, or the optimal route of vaccine delivery (2-4).

Despite this general lack of consensus, it could be reasonably argued that early disease settings represent promising opportunities for successful immunotherapy, due in part to the absence of both bulky disease and the negative consequences of prior radiation treatments or chemotherapy (5). In addition, vaccine efficacy may be enhanced by antigen selection that includes proteins linked to tumor aggressiveness and survival, as well as the capacity to stimulate both CD8<sup>pos</sup> and CD4<sup>pos</sup> T cells. Furthermore, dendritic cells that secrete high levels of interleukin (IL)-12p70 may offer unique advantages, due to their ability to maximize IFN- $\gamma$  production and functional avidity in T cells (6), provided issues of "dendritic cell exhaustion" are addressed (7). Finally, because the immune system evolved primarily to respond against microbial invasion, dendritic cell-based anticancer vaccine strategies may be potentiated by the inclusion of Toll-like receptor (TLR) agonists as dendritic cell activators (8). This may be especially beneficial when overexpressed, but nonmutated proteins, such as HER-2/*neu*, are used as vaccine antigens because mimicking infectious nonself may facilitate the breaking of tolerance.

We report here preliminary results for the first 13 subjects in a clinical trial to vaccinate against ductal carcinoma *in situ* (DCIS), a preinvasive malignancy of the breast. This strategy focused on HER-2/*neu*-expressing tumors, due to this frequent association of protein with poorer prognosis (9), and the availability of both MHC class II- and class I-restricted immunogenic peptides. We also used a DC1 polarization culture technique, including TLR agonist exposure [bacterial lipopolysaccharide (LPS)], to exploit the potential benefits of signaling infectious nonself and to assure robust IL-12 secretion at the time of vaccination. This unique activation strategy actually preconditioned dendritic cells for an apparent second burst of IL-12 if these cells subsequently encountered CD40 ligand. Injecting the vaccines directly into lymph

nodes also delivered a defined quantity of freshly antigen-loaded dendritic cells directly to the site of T-cell sensitization and within the time frame of IL-12 secretion bursts. The prescheduled resection of tumor following vaccinations also afforded the opportunity for quantitative histologic analyses of the effects of immunotherapy.

To date, this vaccination strategy has yielded very high rates of T-cell sensitization against both peptide and tumor targets and generated complement-fixing, tumor-lytic antibodies. We have also observed reductions in extent of DCIS and levels of expression of HER-2 after vaccination in several of the subjects. These early results suggest that this strategy may have direct implications for both prevention and therapy of breast cancer.

## Materials and Methods

**Clinical trial design.** Patients with histologically confirmed DCIS with HER-2/neu overexpression (>2+ intensity) in at least 10% of cells [assayed by HercepTest and verified by single pathologist (P.J.Z.)] were recruited to this Institutional Review Board–approved clinical trial. Subjects were screened by magnetic resonance imaging (MRI) before enrollment to eliminate individuals with obvious areas of invasive disease in either breast. Only patients requiring further surgical therapy for DCIS were eligible for neoadjuvant administration of study vaccine. All patients underwent cardiac evaluation with multigated acquisition (MUGA) scan or echocardiography to document adequate baseline cardiac function. These scans were done before the first dose of vaccine and within 2 weeks of the final dose. All patients underwent HLA class I tissue typing pre-enrollment and had routine history, physical exams, EKG, blood work, and urinalysis before vaccination. After obtaining informed consent, all patients had a prevaccine leukapheresis done to obtain sufficient numbers of monocytes for vaccine preparation; in a few cases, a second pheresis was required for additional monocytes. A postvaccination pheresis was also done, usually within 2 weeks of the final vaccination, to obtain postimmunization lymphocytes for evaluation. All patients underwent postvaccine mammography, MRI, and surgical resection of DCIS with either lumpectomy or mastectomy. An interim analysis for feasibility was planned after the first nine patients were enrolled.

**Materials and reagents.** All HER-2/neu–derived peptides were purchased from the American Peptide Corp. (Sunnyvale, CA). Serum-free medium (SFM) monocyte-macrophage medium and Iscove's medium was purchased from Invitrogen (Carlsbad, CA). Lymphocyte separation medium was obtained from ICN Inc. (Aurora, OH). Human AB serum and FCS were purchased from Sigma Chemical (St. Louis, MO). Reagents for ELISA assays were obtained from PharMingen (San Jose, CA). Clinical grade IFN- $\gamma$  was purchased from Intermune (Brisbane, CA) and clinical grade LPS was obtained from NIH (Bethesda, MD) through generous gift from Dr. Anthony Suffredini. Granulocyte macrophage colony-stimulating factor (GM-CSF) for vaccine production was purchased from Berlex (Richmond, CA). The CD40 ligand trimer was a generous gift from Amgen. Antibodies for flow cytometry were purchased from Berlex (Richmond, CA). HER-2/neu (369–377) MHC tetramer was purchased from Beckman Coulter (Fullerton, CA).

**Vaccination procedure.** Vaccines were administered in the NIH General Research Center at the University of Pennsylvania. The vaccines consisted of 10 to 20 million HER-2/neu–pulsed DC1 cells suspended in 1-mL sterile saline. The vaccines were administered by Ultrasound guidance into a single lymph node in each groin as described previously (4), half of each vaccine was placed into each node with a 22-g needle. The first nine subjects were observed for 2 h after vaccination with routine vital signs obtained at 15-min intervals. Subsequent subjects were observed for 1 h. Vaccines were administered once weekly for 4 weeks. All subjects completed all four scheduled vaccines.

**Preparation of HER-2/neu–pulsed DC1.** All patients underwent initial leukapheresis on Baxter CS3000 using monocyte enrichment settings in the Apheresis Unit at the Hospital of the University of Pennsylvania.

The apheresis product was then elutriated using Beckman Elutriator in the Cell Processing Facility as described previously (10). DC1 were prepared under Investigational New Drug (IND) BB-11043 under good clinical practice conditions. Monocytes were transferred to the Clinical Cell and Vaccine Production Facility where they were washed, counted, and either cryopreserved for later use or vaccine was directly prepared. DC1 were prepared using a rapid activation protocol described previously (6). Monocytes were cultured at  $3 \times 10^6$ /mL in monocyte-macrophage SFM, in sterile 24-well plates with GM-CSF overnight at 37°C. The next morning, monocyte pools were pulsed with one of six HER-2/neu MHC class II binding peptides, three extracellular domain (ECD) peptides (42–56, 98–114, and 328–345), and three intracellular domain (ICD) peptides (776–790, 927–941, and 1166–1180). After 8 to 12 h of further incubation, IFN- $\gamma$  (1,000 units/mL) was added and cells were subsequently cultured overnight. Six hours before harvest, LPS was added at 10 ng/mL. If the patient was HLA-A2<sup>pos</sup>, the monocyte pools were divided in half and each was pulsed with either MHC class I binding peptide 369 to 377 (11) or 689 to 697. The cells were harvested 2 h later, washed, counted, assessed for viability, and placed in a final administration volume of 1 mL in sterile saline. Samples of the vaccine were sent for bacterial and endotoxin testing. Lot release criteria required a viability of >70% as assessed by trypan blue dye exclusion, gram stain negative, and an endotoxin level of <5 endotoxin units/kg. Vaccines were evaluated for functional activation by flow cytometry and aliquots of monocyte medium were cryopreserved to assess IL-12p70 production by ELISA as described previously (6). All vaccines prepared met all release criteria. Vaccines 2 to 4 were prepared as above using cryopreserved monocytes.

**Flow cytometry.** Analysis of surface markers CD14-FITC, CD80-PE, CD86-PE, and CD83-PE was done on a FACSCalibur and analyzed using CellQuest Pro software (BD Biosciences, San Jose, CA).

**Tetramer staining.** Prevacination or postvaccination CD8<sup>pos</sup> T cells were either thawed and analyzed or sensitized by dendritic cells pulsed with the peptide HER-2/neu p369 to 377 for 10 days as described below. They were then harvested and stained with allophycocyanin (APC)–labeled HER-2/neu p369 to 377 tetramer and anti-CD8-FITC, anti-CD28-PE, or anti-CTLA-4-PE for 30 min. Cells were washed and subjected to flow cytometry analysis. APC-labeled MART-1 p27-35 tetramer was used for background control.

**Enzyme-linked immunospot assay.** Anti-IFN- $\gamma$  antibody was purchased from Mabtech, Inc. (Mariemont, OH) and the enzyme-linked immunospot (ELISPOT) assay was done according to the manufacturer's instructions. Multiscreen filter plates (MAIPSWU10) were purchased from Millipore (Billerica, MA). Substrate solution (TMB-H) was purchased from Moss (Pasadena, MD). Coating antibody (1-D1K) was diluted to 12  $\mu$ g/mL in sterile PBS. The ELISPOT plates were prewet with 70% ethanol for 1 min at room temperature and washed five times with sterile water. After addition of the coating antibody (100  $\mu$ L/well), the plates were incubated overnight at 4°C to 8°C. The plates were washed five times with sterile PBS before the addition of 200  $\mu$ L/well of culture medium. After blocking for >30 min, the medium was removed. CD4 cells were added with either immature or mature dendritic cells, pulsed with the ICD and ECD peptides (total of 150  $\mu$ L/well) at a ratio of  $1 \times 10^5$  to  $2 \times 10^4$ . Tetanus was used as control stimulus. The cells were incubated at 37°C for 20 h. The plates were then washed five times with 200  $\mu$ L PBS. One hundred microliters of detection antibody (7-B6-1-biotin) diluted to 1  $\mu$ g/mL in PBS with 0.5% FCS were added to each well. The plates were incubated at room temperature for 2 h. After washing five times, 1:1,000 diluted streptavidin-horseradish peroxidase in PBS with 0.5% FCS was added before incubation for an additional hour. The plates were washed and 100  $\mu$ L of substrate solution were added to each well. After color development, wells were rigidly washed with tap water. The plates were dried at room temperature and read in an ELISPOT reader (Immunospot CTL, Cleveland, OH). At routine intervals, the relative coefficient of variance was determined. The maximum value was 23% (1,029  $\pm$  63.34 = 0.448%; 993  $\pm$  71 = 3.18%; 635  $\pm$  1.52 = 0.09%; 596  $\pm$  35.2 = 2.4%; 99  $\pm$  23.8 = 12%; and 120  $\pm$  55.25 = 23%).

**CD4<sup>pos</sup> T-cell *in vitro* sensitization.** CD4<sup>pos</sup> T cells from thawed prevaccination or postvaccination lymphocytes were prepared with

negative selection columns as described previously (6). CD4<sup>pos</sup> T cells were sensitized by autologous dendritic cells pulsed with either HER-2/*neu* ECD or ICD peptides at a ratio of 10:1. IL-2 (60 IU/mL) was added to the cultures the next day. T cells were harvested on day 10 and tested for their antigen specificity. CD4<sup>pos</sup> T cells (10<sup>5</sup>) were cocultured with dendritic cells (10<sup>4</sup>) pulsed with HER-2/*neu* peptides or dendritic cells pulsed with B-Raf kinase in 96-well plates. After 24 h of coculture, supernatants were harvested and IFN- $\gamma$  release was measured by ELISA.

**CD8<sup>pos</sup> T-cell sensitization.** Autologous dendritic cells were pulsed with HER-2/*neu* p369 to 377 or p689 to 697 at 10  $\mu$ g/mL 2 h before harvest. Dendritic cells were then cocultured with column-purified prevaccination or postvaccination CD8<sup>pos</sup> T cells at a ratio of 10:1 in 48-well plates. IL-2 (30 IU/mL) was added to the cultures on day 2. After 10 days of sensitization, the CD8<sup>pos</sup> T cells were harvested and restimulated with T2 cells pulsed with either relevant or irrelevant peptides or tested against breast cancer cell lines MDA-MB-231 and MCF-7 (HER-2/*neu*<sup>neg</sup> and HLA-A2<sup>pos</sup>), MDA-MB-435S and SK-BR-3 (HER-2/*neu*<sup>pos</sup> and HLA-A2<sup>neg</sup>), and ovarian cancer cell lines SKOV3 (HER-2/*neu*<sup>pos</sup>- and HLA-0201\*<sup>-</sup>-transfected or nontransfected cells; a kind gift of Dr. Mary Disis, University of Washington, Seattle, WA). Supernatants were harvested after 24 h and analyzed by ELISA.

**Immunohistochemical staining of DCIS lesions.** Formalin-fixed, paraffin-embedded tissue blocks were sectioned at 5  $\mu$ m on plus slides (Fisher Scientific, Hanover Park, IL), Hanover Park, IL. Sections were heated for 1 h at 60°C to remove excess paraffin, cooled for 10 min, and subsequently deparaffinized and rehydrated in a series of xylenes and alcohols. Immunohistochemistry was done using the DAKO Autostainer (DAKO, Carpinteria, CA). All tissues were stained for HercepTest (DAKO), CD1a (Novocastra Laboratories, Vision BioSystems, Inc., Norwell, MA), CD3 (Novocastra Laboratories), CD4 (Biocare Medical, Walnut Creek, CA), CD8 (DAKO), CD20 (DAKO), CD45RO (DAKO), CD56 (Monosan, Uden, the Netherlands), CD68 (DAKO), CD69 (LabVision Corp., Neomarkers, Fremont, CA), Granzyme (LabVision, Neomarkers), FoxP3 (BioLegend, San Diego, CA), HLA class II (DAKO), and IgG (DAKO).

**Complement-dependent cytotoxicity assay.** SK-BR-3, a highly positive HER-2/*neu*-expressing breast cancer cell line, was used as a positive target and the melanoma cell line MEL624-A2<sup>neg</sup> that does not express HER-2/*neu* was used as a negative target. Briefly,  $1 \times 10^4$  cells were plated and incubated overnight at 37°C. Cultures were done in quadruplets. Human serum was inactivated at 56°C for 30 min and diluted 1:2. Fifty microliters of serum were added to the cell cultures for 1 h. Twenty microliters of guinea pig complement (diluted 1:4; Sigma Chemical) was added to half of the wells. The other half served as antibody control. After 4 h, 15  $\mu$ L WST1 was added to the wells. The plates were analyzed by an ELISA reader at 3 and 4.5 h at a wavelength of 450. The percentage cytotoxicity was calculated using the following formula:  $[(a - b) / (a - c)] \times 100$  (where  $a$  = cells in antibody only;  $b$  = cells in antibody plus complement; and  $c$  = medium only).

**Statistical methods.** To compare the mean number of HER-2/*neu* 369 to 377 tetramer binding (in 10<sup>5</sup> CD8<sup>pos</sup> T cells), between healthy donors and DCIS patients, and to compare HER-2/*neu* expression on initial biopsy with postsurgical biopsies for the vaccine patients compared with a group of unvaccinated control subjects, a Wilcoxon rank test was used. To compare the initial burst with the additional burst of IL-12 by DC1, a Wilcoxon signed-rank test for paired data was used. All significance values were two sided. Statistical analyses were done with SPSS 12.0 software (SPSS Inc., Chicago, IL).

## Results

**Vaccine scheme and clinical trial design.** *Ex vivo* activated DC1 (high IL-12-secreting dendritic cells) were prepared from autologous monocytes under Food and Drug Administration IND BB-11043 (Fig. 1A). Dendritic cells were pulsed individually with six MHC class II peptides derived from HER-2/*neu* as described previously by Disis et al. (12). The dendritic cells of HLA-A2<sup>pos</sup>

subjects were additionally pulsed with two HLA-A2-binding peptides (369–377 and 689–697) shown previously to stimulate CD8<sup>pos</sup> T cells (13–15). The intention of the combined MHC class II and class I peptide design was to promote CD4<sup>pos</sup> antigen-specific help for CD8<sup>pos</sup> T cells, partly by fostering an additional *in vivo* burst of IL-12 production through CD40 ligation of the administered DC1 (Fig. 1B). All patients were treated with 4 weekly HER-2/*neu*-pulsed autologous DC1 (10–20 million per vaccine) immunizations administered by intranodal route. Following completion of the vaccines, patients underwent immunologic testing followed by definitive surgical therapy. All patients underwent prevaccine and postvaccine cardiac evaluation with MUGA or echocardiogram to assess cardiac changes and vaccine safety. All patients completed the four scheduled vaccinations and vaccines were well tolerated with only CTC grade 1 or 2 toxicity consisting of anticipated low-grade fever, fatigue, chills, nausea, or injection site tenderness. There was no evidence of cardiac toxicity manifested by significant postvaccine ejection fraction on MUGA scan, all of which remained in the reference range or any clinical symptoms during 1 year of follow-up.

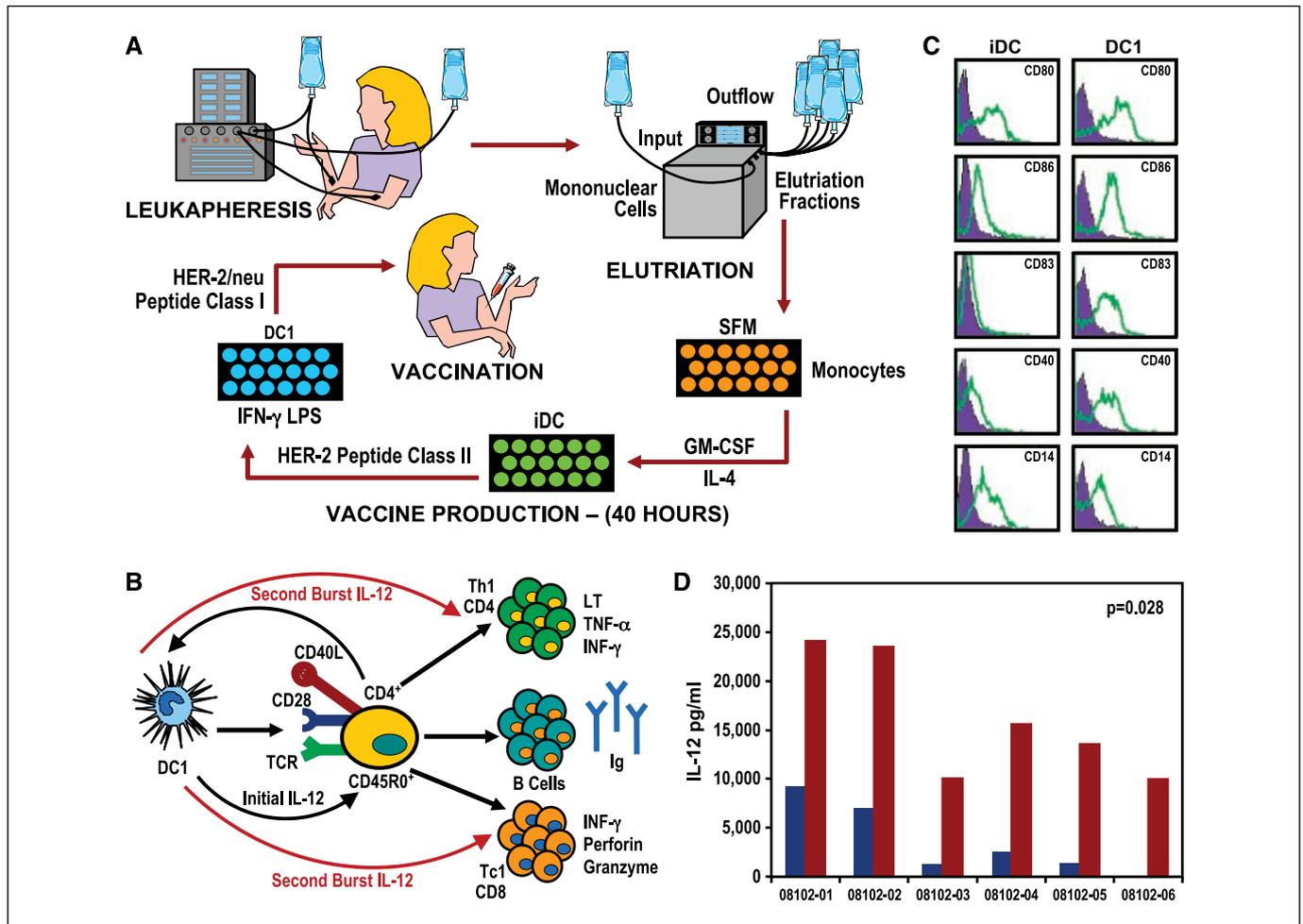
**Monocytes treated with IFN- $\gamma$  and TLR-4 agonist LPS show properties of mature polarized DC1.** As expected, monocyte-derived dendritic cells from DCIS patients displayed enhanced levels of CD80, CD86, and CD83 when activated with IFN- $\gamma$  and LPS (Fig. 1C). The dendritic cells also displayed CD40 up-regulation (Fig. 1C). An initial burst of IL-12p70 production (500–10,000 pg/mL) was observed for all patients' dendritic cells, consistent with DC1 polarization (16). To test whether these dendritic cells retained the potential to produce additional IL-12 should they encounter CD4<sup>pos</sup> CD40L<sup>pos</sup> T cells, IFN- $\gamma$ /LPS-treated dendritic cells were washed and incubated in fresh medium for 12 to 36 h before restimulation solely with recombinant CD40L. The prepared patient dendritic cells could consistently produce an additional IL-12p70 burst (range, 10,000–30,000 pg/mL) that even significantly exceeded the magnitude of the original burst ( $P = 0.028$ ; Fig. 1D). Hence, *in vitro* TLR agonist-induced DC1 polarization preconditioned dendritic cells for a second IL-12 burst through subsequent CD40-CD40L interactions (Fig. 1B).

**Evidence of negative regulation of anti-HER-2 T cells in patients with DCIS.** Peripheral blood CD8<sup>pos</sup> lymphocytes from patients with DCIS were assessed in the HLA-A2<sup>pos</sup> cohort before vaccination for preexisting anti-HER-2/*neu* responses. Compared with healthy donors, there were elevated numbers (mean  $77 \times 10^5$  for DCIS patients versus mean  $36 \times 10^5$  for healthy donors;  $P = 0.033$ ) of HER-2/*neu* 369 to 377 tetramer<sup>pos</sup> CD8<sup>pos</sup> T cells (Fig. 2A). We additionally examined T-cell expression of B7 ligands in this cohort. Before vaccination, tetramer-staining T cells expressed high levels of CTLA-4 (inhibitory signaling) relative to CD28 (activation signaling). After vaccination, however, this ratio became inverted (Fig. 2B). Additionally, in contrast to healthy donors, prevaccine CD8<sup>pos</sup> T cells sensitized using DC1 pulsed with 369 to 377 class I peptide did not secrete IFN- $\gamma$  in response to HER-2/*neu* 369 to 377 pulsed targets except in one subject. However, postvaccine T cells routinely acquired this property (Fig. 2C; Supplementary Figs. S1 and S2). Collectively, these results suggest a negative regulatory mechanism(s) for T cells naturally sensitized to HER-2/*neu* that was operative even at very early (preinvasive) stages of cancer, but which could be reversed by DC1 vaccination.

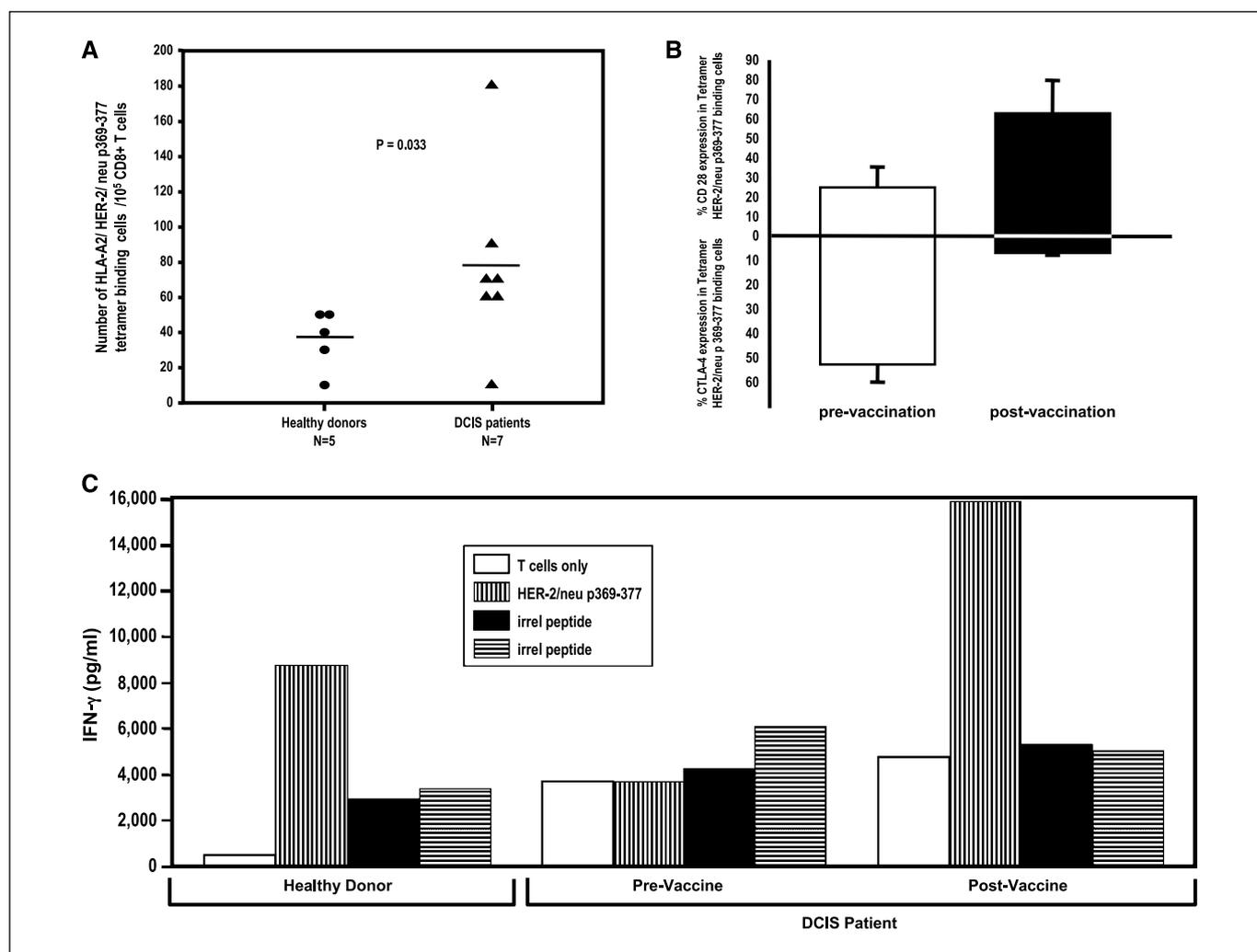
**DC1 vaccines induce HER-2/*neu*-reactive CD4<sup>pos</sup> T cells.** We evaluated CD4<sup>pos</sup> anti-HER-2/*neu* responses by two methods. The

first method used was ELISPOT to quantify T cells in peripheral blood without *in vitro* expansion. The second was an *in vitro* sensitization assay (IVS), in which lymphocytes were first stimulated and expanded for one round (10 days) *in vitro* and then restimulated and assessed by ELISA for secreted IFN- $\gamma$ . Both assays used HER-2/neu peptide-loaded dendritic cells as stimulators. A summary of all patient immune responses is summarized in Supplementary Table S1. Of 11 testable subjects, 10 (91%) had >5-fold postvaccination increases in the number of IFN- $\gamma$  spots against at least one MHC class II-restricted HER-2/neu peptide epitope, and the observed increases were usually of an even greater magnitude (Fig. 3A). A representative comparison of absolute numbers of IFN- $\gamma$  secretion spots is shown for subject 08102-05

(Fig. 3B). To determine whether such increases in spot number were a specific consequence of vaccination, we also tested the prevaccine and postvaccine response when tetanus-pulsed dendritic cells were used as stimulators. The ratios of postvaccine to prevaccine tetanus-specific IFN- $\gamma$  spots were consistently in the 1 to 2 range, indicating absent or scant modulation by the vaccine procedure (Fig. 3A). When assayed by IVS instead of by ELISPOT, 10 of 13 (77%) subjects responded to HER-2/neu peptides after vaccination with greater than a 2-fold increase in IFN- $\gamma$  secretion compared with control peptides (Fig. 3C and D). Most of the subjects developed responses to more than one of the six MHC class II peptides used (Fig. 3). As anticipated, ELISPOT and IVS results did not always precisely concur, due to repertoire disparities



**Figure 1.** Preparation of HER-2/neu-pulsed-DC1 vaccines. **A**, peripheral blood monocytes were obtained by combined leukapheresis and elutriation. The monocytes were cultured in SFM with GM-CSF and IL-4 overnight. The next day, immature dendritic cells (iDC) were pulsed in separate wells with one of six HER-2/neu MHC class II-derived peptides. IFN- $\gamma$  was added later in the day, and the following morning, LPS was added to complete maturation of DC1. For HLA-A2<sup>POS</sup> patients, half of the DC1 were pulsed with MHC class I binding peptide 369 to 377 and the other half with 689 to 697 for 2 h. The DC1 were harvested, release criteria were met, and 10 to 20 million HER-2/neu-pulsed DC1 were administered by intranodal injection into normal groin nodes. One injection was given in each side of the groin (half the vaccine in 0.5 cc each groin node). **B**, schematic of proposed affect of HER-2/neu-pulsed DC1 on the cellular immune response. Primed DC1 produce IL-12 and present MHC class II peptides to CD4<sup>POS</sup> T cells. T-cell activation results in CD40L expression that in turn signals through CD40 on dendritic cells. This enhances dendritic cell maturation and facilitates additional IL-12 production, which leads to high avidity CD8<sup>POS</sup> antitumor T cells and CD4<sup>POS</sup> Th1 cells. The IFN- $\gamma$  production by CD4<sup>POS</sup> T cells can potentially induce immunoglobulin class switching in B cells. **C**, primed DC1 show characteristics of mature dendritic cells. Following maturation with IFN- $\gamma$  and LPS, dendritic cells express high levels of CD80, CD86, and CD83 and low-level expression of CD14. The data are from one vaccinee from one patient and are representative of most vaccinees, although there was some observed variation in levels of CD83 expression. **D**, primed DC1 produce high levels of IL-12p70. DC1 were prepared as described in Materials and Methods. Supernatants from DC1 from the first six subjects were sampled for IL-12p70 production after 16 h following addition of LPS. IL-12 production ranged from 500 to 8,000 pg/mL (blue columns). The DC1 were activated with LPS for 6 h as is done for vaccine administration then cultured in SFM for 16 h, washed, and treated with CD40L trimer. IL-12p70 production was then assessed after additional 14 h. DC1 from all subjects showed the ability to produce an additional burst of IL-12p70 range 10 to 25 ng/mL (red columns). DC1 cultured in the absence of CD40L produced less than 500 pg/mL (data not shown). Results are representative from the DC1 from the first six subjects. The comparison between the amount of IL-12p70 produced by mature DC1 and those additionally activated by CD40L was compared by Wilcoxon signed-rank test for paired data.



**Figure 2.** Evidence for tolerance to HER-2/neu in patients with DCIS. **A**, peripheral blood mononuclear cells were obtained from HLA-A2<sup>POS</sup> healthy donors and HLA-A2<sup>POS</sup> DCIS patients participating in this trial. Cells were stained directly *ex vivo* without stimulation using phycoerythrin (PE)-labeled HLA-A2 tetramer bound to the immunodominant HER-2/neu peptide 369 to 377. The cells were counterstained with FITC anti-CD8. For each subject, the number of cells showing tetramer-positive binding per 100,000 cells was calculated and the two groups were compared by Wilcoxon rank test. **B**, anti-HER-2/neu CD8<sup>POS</sup> T cells before vaccination display enhanced levels of CTLA-4 compared with CD8<sup>POS</sup> T cells after vaccination. Purified CD8<sup>POS</sup> T cells from prevaccine and postvaccine samples were cocultured with autologous immature dendritic cells pulsed with HER-2/neu 369 to 377 peptide for 7 d. The T cells were harvested and stained using 369 to 377 PE-tetramer and counterstained with FITC anti-CTLA-4, anti-CD28, and anti-CD8. The percentage of HER-2/neu tetramer-positive cells expressing CTLA-4 or CD28 was calculated for prevaccine and postvaccine samples. Results are from three subjects. *Columns*, % CTLA-4/% CD28 expression in tetramer HER-2/neu p369 to 377 binding cells; *bars*, SE. **C**, CD8<sup>POS</sup> T cells from healthy donors but rarely DCIS patients before vaccination can be readily sensitized to HER-2/neu 369 to 377. CD8<sup>POS</sup> T cells were prepared from HLA-A2<sup>POS</sup> healthy donors or the prevaccine DCIS patients. The T cells were cocultured with autologous DC1 pulsed with HER-2/neu 369 to 377 peptide for 1 wk. The T cells were harvested and tested for specificity by IFN- $\gamma$  release against T2 cells pulsed with HER-2/neu 369 to 377 or irrelevant HLA-A2-binding peptides. As a control for DC1 activity, the same DC1 were cocultured with postvaccine CD8<sup>POS</sup> T cells and the T cells were harvested and tested as above. The results represent a single experiment from one healthy donor and one DCIS patient. A complete set of all healthy donors sensitized with HER-2/neu-pulsed dendritic cells is shown in Supplementary Fig. S1. A profile of HLA-A2<sup>POS</sup> DCIS prevaccine and postvaccine peptide sensitization data is shown in Supplementary Fig. S2.

but these dual analyses corroborated vaccine responsiveness to at least one MHC class II-restricted HER-2/neu peptide epitope for 11 of 13 (85%) patients (Fig. 3). Furthermore, a consistent absence of detectable IL-4, IL-5, and IL-10 production indicated that such CD4<sup>POS</sup> T-cell responsiveness reflected a strongly polarized TH1 phenotype (data not shown).

**HER-2/neu-pulsed DC1 vaccines induce CD8<sup>POS</sup> T cells that directly recognize HER-2/neu-overexpressing breast cancer lines.** We observed an increase in the numbers of 369 to 377 peptide tetramer<sup>POS</sup> CD8<sup>POS</sup> T cells in most patients following vaccination (Fig. 4A), with further amplification of tetramer-binding frequency,

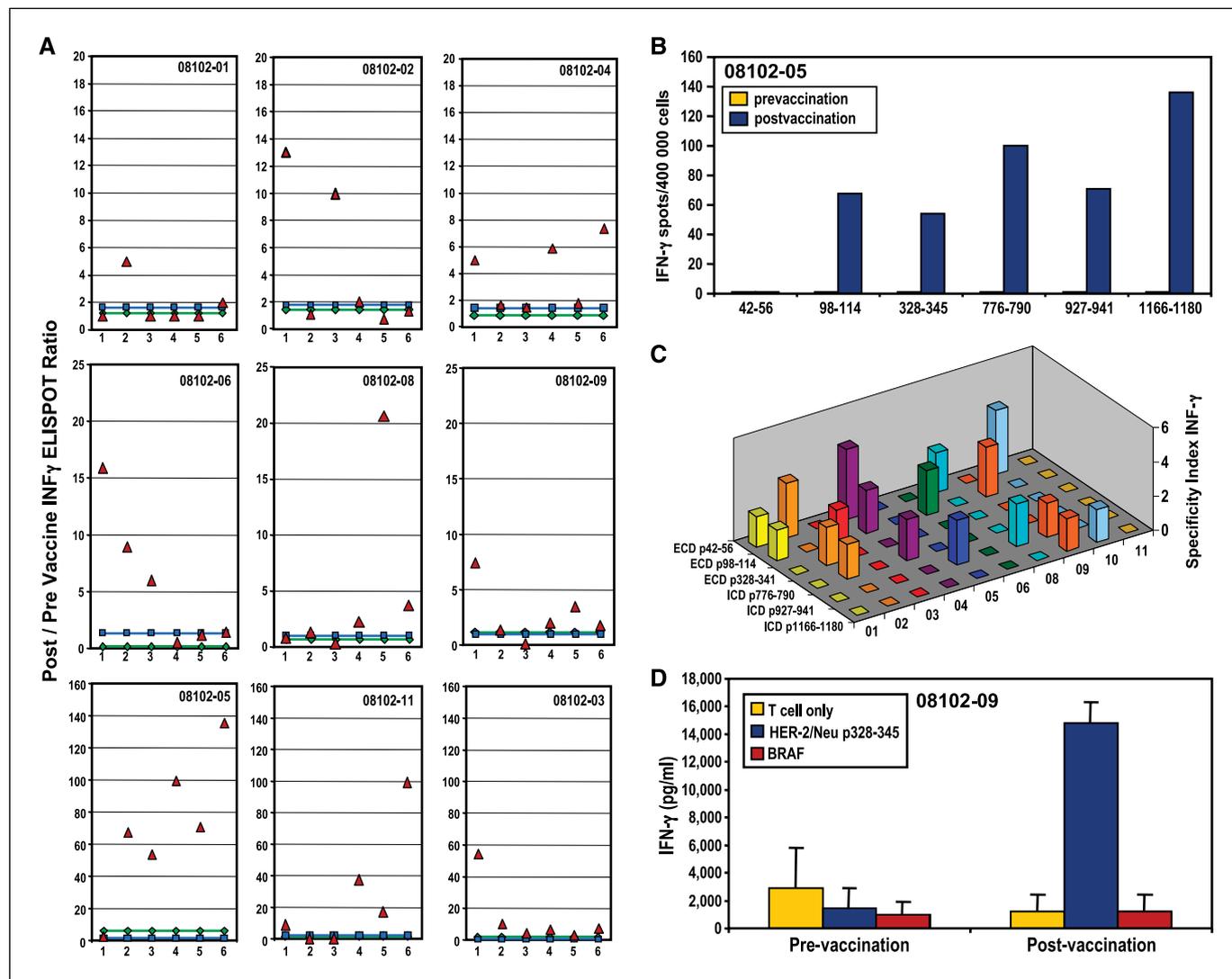
following *in vitro* stimulation with peptide-pulsed dendritic cells. Despite this, one of the ten HLA-A2<sup>POS</sup> subjects displayed prevaccination evidence of CD8<sup>POS</sup> T cells capable of IFN- $\gamma$  secretion in response to HER-2/neu 369 to 377 peptide-pulsed targets (data not shown). However, eight of nine (88%) patients acquired such reactivity to peptide 369 to 377 following vaccination (Fig. 4B; Supplementary Fig. S2), and five of nine patients developed reactivity to peptide 689 to 697 (Supplementary Fig. S2). Perhaps, most importantly, postvaccination CD8<sup>POS</sup> T cells from all subjects that developed peptide recognizing CD8 T cells recognized HLA-A2<sup>POS</sup> HER-2/neu-expressing tumor lines (Fig. 4C). Such T cells did not respond to the HLA-A2<sup>NEG</sup> or HER-2/neu<sup>NEG</sup> control tumor cell

lines, indicating HER-2/neu specificity and HLA-A2 restriction. The acquisition of direct tumor reactivity was not evident before completion of all four vaccinations, although reactivity to peptide-pulsed T2 cells was often already prominent following first vaccination (Fig. 4D). It is worth noting that direct recognition of antigen-expressing tumor cells by peptide-sensitized CD8<sup>pos</sup> T cells has historically been difficult to show and that our previous *in vitro* work predicted high rates of direct tumor recognition when IL-12-secreting dendritic cells were used for vaccination.

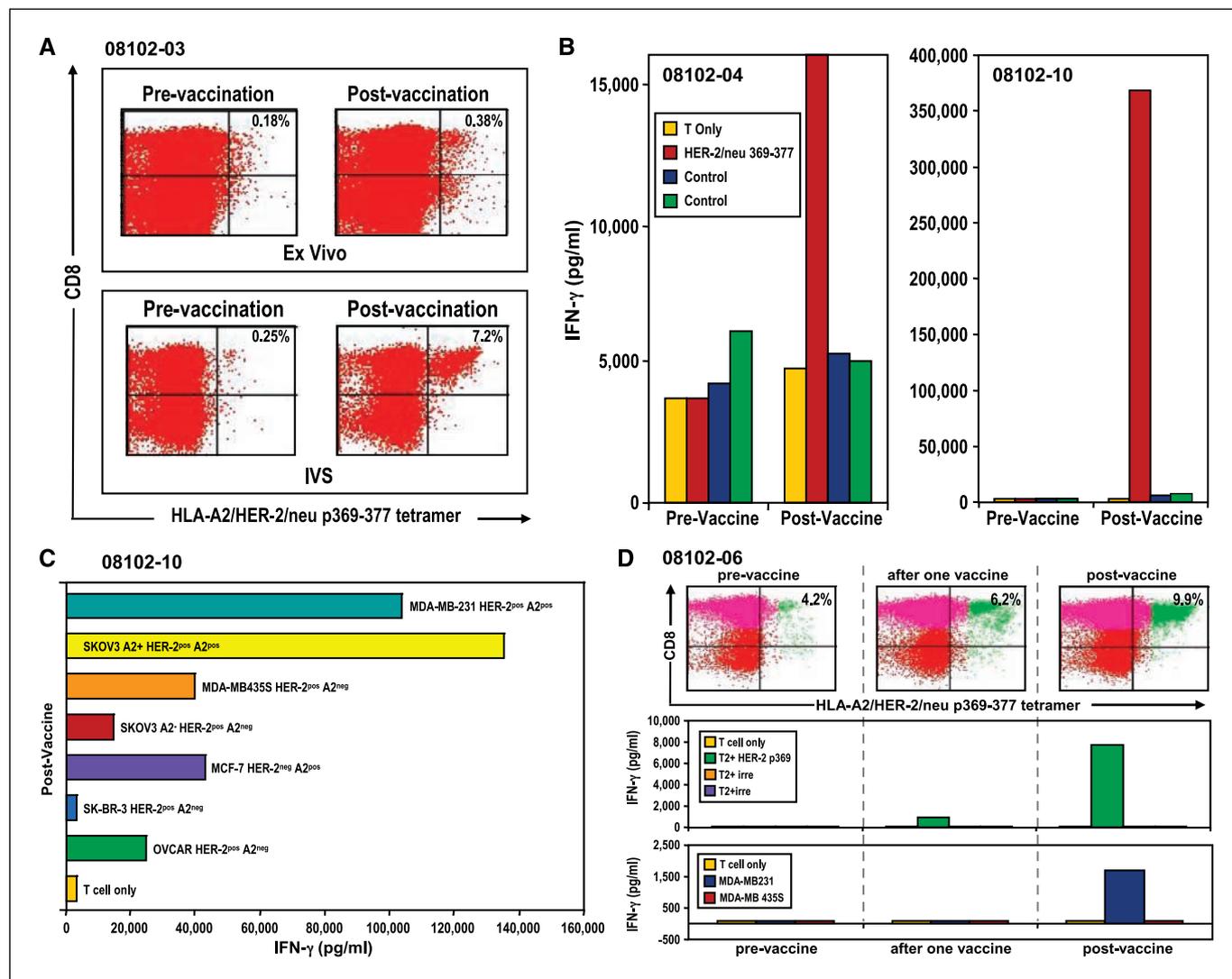
**HER-2/neu–pulsed-DC1 vaccines lead to accumulation of lymphocytes in the breast and changes in residual DCIS. All**

enrolled subjects required conventional definitive surgery after vaccination to remove residual DCIS, based on the initial surgical (subjects 1, 3, 4, 10, 11, and 14) or core (subjects 2, 5, 6, 8, 9, 12, and 13) biopsies. Eleven of 13 subjects showed residual DCIS at the time of postvaccine surgery. The remaining two had either no residual disease (subject 08102-04) or inadequate tissue (subject 08102-01) to do HER-2/neu expression analysis (see below).

Immunohistochemical analysis of prevaccination biopsies showed variable lymphocytic infiltrates in the breast tissue with some patients showing minimal or moderate infiltrates, such as shown in Fig. 5A (including CD4<sup>pos</sup> T cells and B cells). However, in



**Figure 3.** HER-2/neu–pulsed DC1 vaccine induces evidence of IFN- $\gamma$ -secreting CD4<sup>pos</sup> T cells. *A*, CD4<sup>pos</sup> T cells obtained from subjects before and after vaccination were cocultured with dendritic cells pulsed with HER-2/neu peptides, used for immunization, or left unpulsed. Dendritic cells were also pulsed with tetanus toxoid and cocultured with T cells to monitor for nonspecific vaccine-induced changes in immune function. ELISPOT for IFN- $\gamma$  were measured directly without *ex vivo* stimulation. Individual peptide reactivities were measured in quadruplicate. A T-cell ELISPOT required at least 10 spots to be considered positive. Antigen-specific spots were subtracted from groups using unpulsed dendritic cells as stimulators to determine the number of spots, and these are presented as the ratio of postvaccine response to prevaccine response ( $\blacktriangle$ ). Green diamonds and blue squares, the prevaccine and postvaccine ratio of CD4<sup>pos</sup> T-cell reactivity to unpulsed and tetanus-pulsed dendritic cells. X axis, the results for each of the six HER-2/neu peptides: 1, 42 to 56; 2, 98 to 114; 3, 328 to 345; 4, 776 to 790; 5, 927 to 941; 6, 1166 to 1190. Data are presented from 9 of 13 subjects for whom sufficient T cells were available. Patient 08102-10 did not have enough T cells from prevaccine collection to do ELISPOT. The results are from the first 10 patients. *B*, representative data from patient 08102-05 are shown comparing number of IFN- $\gamma$  spots prevaccine and postvaccine. *C*, CD4<sup>pos</sup> T cells purified from prevaccine and postvaccine samples were cocultured with dendritic cells pulsed with HER-2/neu peptides for 10 d constituting a single round of *in vitro* stimulation. The CD4<sup>pos</sup> T cells were harvested and tested for specificity against monocytes pulsed with relevant HER-2/neu peptides or irrelevant control peptides for 24 h. Supernatants were collected and analyzed for IFN- $\gamma$  by ELISA. Results were considered positive if there was at least a 2-fold increase in specific IFN- $\gamma$  compared with control. The ratio of postvaccine to prevaccine IFN- $\gamma$  specificity index is presented for the first nine subjects. There was no evidence of specific secretion of IL-5 or IL-10 (data not shown). *D*, representative IFN- $\gamma$  secretion from prevaccine and postvaccine CD4<sup>pos</sup> T cells from 08102-09 peptide (328–345).



**Figure 4.** HER-2/neu-pulsed DC1 sensitize CD8<sup>pos</sup> T cells to HER-2/neu peptides and HER-2/neu-expressing breast cancer cell lines. *A*, tetramer staining of 369 to 377 HER-2/neu-specific CD8<sup>pos</sup> T cells on prevaccination and postvaccination (unstimulated) peripheral blood mononuclear cells from patient 08102-03 (top). CD8<sup>pos</sup> T cells were purified and cocultured 7 d with 369 to 377 HER-2/neu-pulsed autologous dendritic cells. After 1 wk, CD8<sup>pos</sup> T cells were harvested and stained with labeled tetramer bound to peptide 369 to 377. Comparison of prevaccine and postvaccine T cells showed that postvaccine T cells expanded ~20-fold in 1 wk compared with minimal expansion of tetramer-specific T cells from the prevaccine blood stimulated by dendritic cells (bottom). *B*, CD8<sup>pos</sup> T cells were purified from prevaccine and postvaccine leukapheresis and the CD8<sup>pos</sup> T cells were cocultured with autologous 369 to 377 peptide-pulsed dendritic cells for 10 d. The CD8<sup>pos</sup> T cells were harvested and cocultured overnight with T2 cells (HLA-A2<sup>pos</sup>) pulsed with 369 to 377 or irrelevant peptides, supernatants were harvested, and IFN- $\gamma$  was measured by ELISA. Results are from patients 08102-04 and 08102-10. The results from 8 of the 10 HLA-A2<sup>pos</sup> subjects are shown in Supplementary Fig. S2. The same experiments were carried out using the peptide 688 to 697 and the results for four of the eight patients that showed reactivity to this peptide are shown in Supplementary Fig. S2. *C*, CD8<sup>pos</sup> T cells after vaccination show evidence of recognition of tumor cells endogenously expressing HER-2/neu. CD8<sup>pos</sup> T cells from prevaccine and postvaccine leukapheresis were cocultured with autologous dendritic cells pulsed with HER-2/neu 369 to 377 for 10 d. The CD8<sup>pos</sup> T cells were harvested and cocultured overnight with HER-2/neu<sup>pos</sup> HLA-A2<sup>pos</sup> tumors (MDA-MB-231 and SKOV3) and HER-2/neu<sup>pos</sup> HLA-A2<sup>neg</sup> (MDA-MB-435S, SKOV3, SK-BR-3, and OVCAR) or HER-2/neu<sup>neg</sup> HLA-A2<sup>pos</sup> MCF-7. The results are from patient 08102-10. There was no evidence of reactivity to any of the tumor cell lines in the prevaccine CD8<sup>pos</sup> T cells (data not shown). The results for two cell lines are shown for 8 of 10 HLA-A2<sup>pos</sup> patients in Supplementary Fig. S2. *D*, kinetic development of CD8<sup>pos</sup> T cells following 369 to 377 HER-2/neu-pulsed DC1 vaccination. CD8<sup>pos</sup> T cells were purified from leukapheresis before vaccination, following a single vaccination and after completion of all four vaccines. The CD8<sup>pos</sup> T cells from 08102-06 were cocultured with autologous dendritic cells pulsed with HER-2/neu 369 to 377 for 7 d. The CD8<sup>pos</sup> T cells were then harvested and either stained with 369 to 377 tetramer plus anti-CD8 counterstain, or cocultured with T2 cells pulsed with either 369-377 peptide or irrelevant control peptides, or cocultured with tumor cells that expressed HER-2/neu but were either HLA-A2<sup>pos</sup> or HLA-A2<sup>neg</sup>, with IFN- $\gamma$  secretion read by ELISA as an index of T-cell recognition. Results are from subjects 08102-06 and 08102-10 that had cells available to do this analysis and the results were similar.

most patients, there was a marked postvaccination increase in lymphocytic infiltration with cells congregating at periductal sites surrounding regions of residual DCIS (Fig. 5A). The infiltrate consisted largely of CD4<sup>pos</sup> T cells and CD20<sup>pos</sup> B cells with few CD8<sup>pos</sup> T cells (Fig. 5A; Supplementary Fig. S3) and minimal or no natural killer cells, macrophages, or dendritic cells detected. Some lymphocytes (CD45RO<sup>pos</sup> and CD4<sup>pos</sup>) seemed to enter the

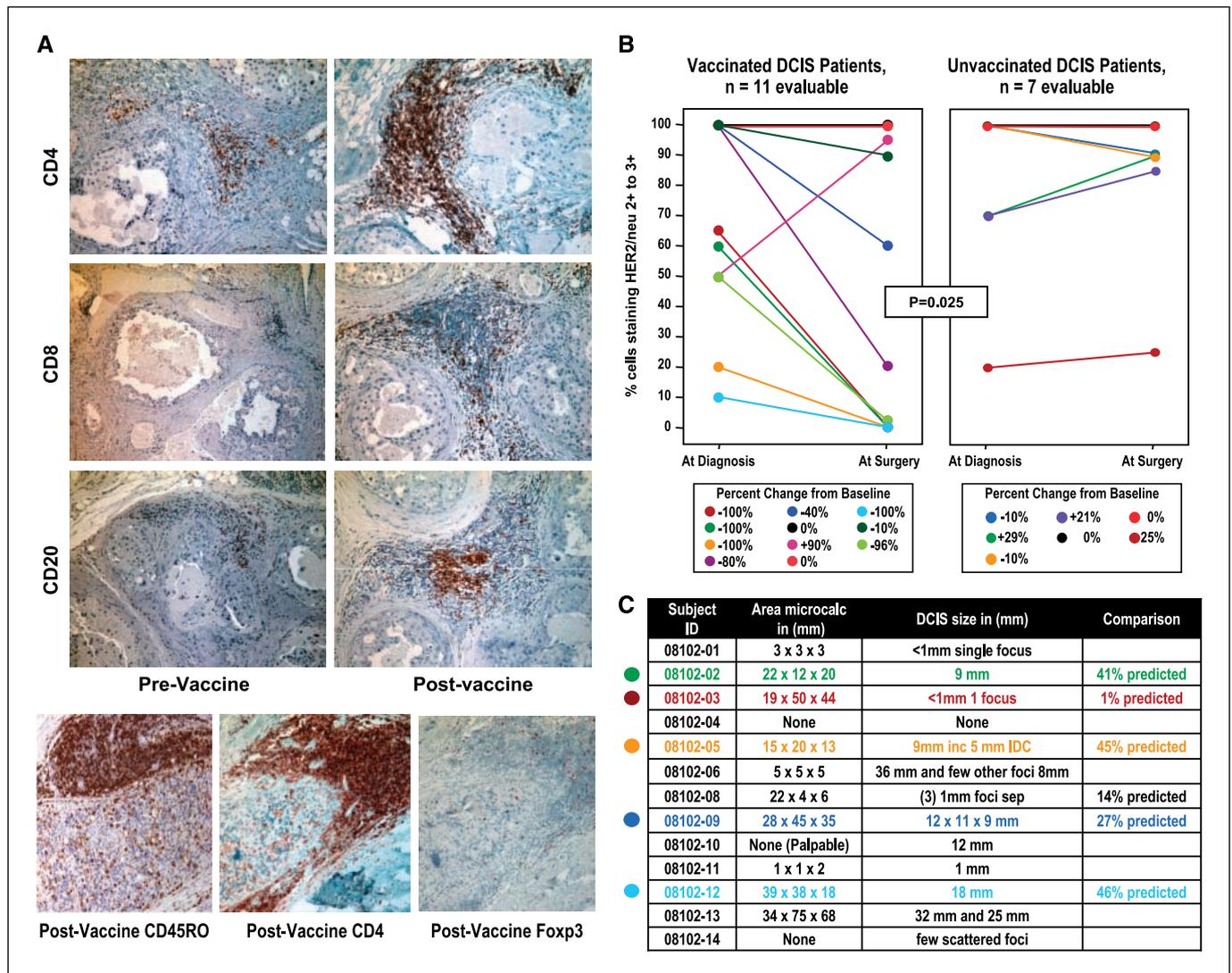
ducts and comingle with the tumor cells (Fig. 5A, bottom). There was little evidence of Foxp3<sup>pos</sup> cells in the breast either prevaccine or postvaccine, suggesting these negative regulatory cells play little role in DCIS (Fig. 5A, bottom).

We then compared HER-2/neu expression before and after vaccination to determine whether apparent immune pressure altered target antigen expression on remaining tumor cells.

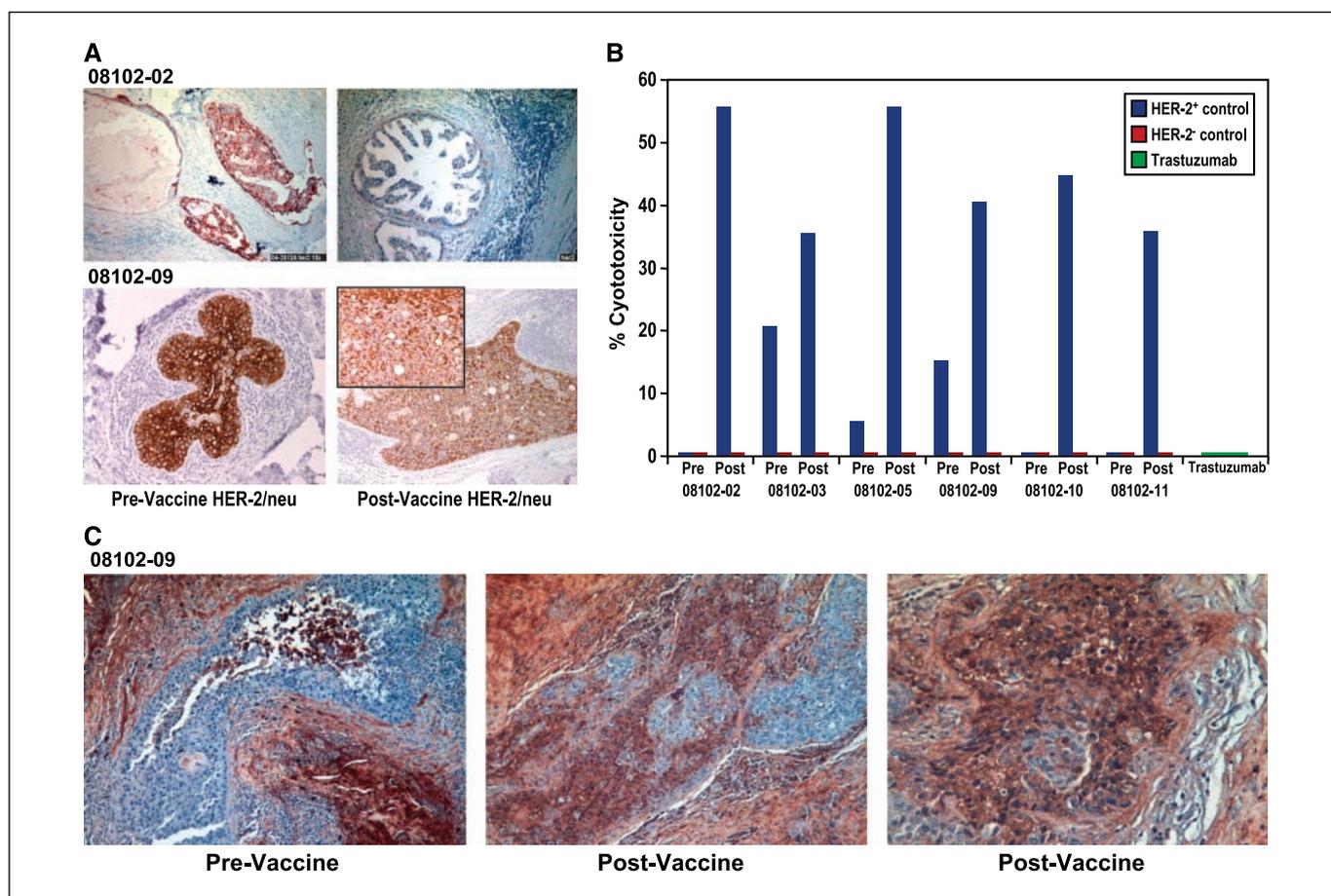
We observed pronounced declines in HER-2/*neu* staining in 7 of 11 subjects compared with no decline in an unvaccinated control group of HER-2/*neu*–overexpressing DCIS patients that also underwent an initial biopsy before definitive surgical resection (Fig. 5B). In three subjects who exhibited loss of HER-2/*neu*, confirmatory fluorescence *in situ* hybridization (FISH) analysis was done to test residual HER-2/*neu* gene amplification. For two of these three, FISH analysis confirmed the loss of HER-2/*neu* initially shown by immunohistochemistry, but the third (08102-06) remained strongly FISH positive (data not shown).

Microcalcifications, formed within areas of high-grade comedo-necrosis DCIS and visualized by mammography, can predict the minimum extent of DCIS (actual tumors often extend beyond

microcalcifications) with a positive predictive value of 0.9 (18–20). We used microcalcifications to estimate the extent of disease and confirmed these findings with MRI. For 6 of the 11 (55%) patients with microcalcifications, the actual tumor removed at the time of definitive surgery was significantly smaller (>50% smaller) than the area predicted by microcalcifications on postbiopsy mammograms (Fig. 5C), suggesting tumor regression. Notably, five of these seven subjects were among the seven shown to have decreased HER-2/*neu* staining on residual tumor cells (exception, 08102-06 no decrease in extent; 08102-14 no calcification). Examples of this loss visualized by immunohistochemical staining are shown for subjects 08102-02 and 08102-09 (Fig. 6A). For subject 08102-09, there was decreased staining intensity plus a postvaccination



**Figure 5.** HER-2/*neu*–pulsed DC1 vaccine induces infiltration of lymphocytes into the breast and changes in the residual DCIS. A, immunohistochemical staining of lymphocytes infiltrating into the breast following DC1 vaccination for DCIS. Results are from 08102-10. Prevacination and postvaccination specimens stained for the presence of CD4<sup>pos</sup>, CD8<sup>pos</sup>, and CD20<sup>pos</sup> cells. In addition, CD45RO and CD4 stains are shown from postvaccination tissues for patient 08102-09. Note the evidence of CD4<sup>pos</sup> T cells surrounding the duct and also infiltrating a duct with residual high-grade DCIS. A table summarizing the lymphocytic infiltrates from the first ten patients is shown in Supplementary Fig. S3. B, expression of HER-2/*neu* on DCIS cells before and after vaccination. HER-2/*neu* staining was done on prevaccination biopsy specimens as well as on the surgical specimen following vaccination. As a control, we stained initial biopsy and definitive surgical specimens from patients with DCIS that were not vaccinated but obtained contemporaneously with this trial. The results are the comparisons of the HER-2/*neu* expression at the time of definitive surgery compared with the HER-2/*neu* staining at the time of diagnosis. C, comparison of the area of microcalcifications on mammography after biopsy with the largest area of DCIS obtained at the time of surgery. Colored dots, correlate with the patient ID from HER-2/*neu* expression in (B). Note patients 08102-02, 08102-03, 08102-05, 08102-09, and 08102-12 showed both decreased HER-2/*neu* expression and a 50% or greater diminishment in the predicted size of the DCIS. The area of microcalcifications was read by single mammographer. Areas of DCIS seen as mammographic microcalcifications were also verified by breast MRI.



**Figure 6.** Vaccination alters HER-2/neu expression and induces complement-fixing antibodies that bind to tumor *in vivo*. **A**, HER-2/neu expression before and after vaccination from patients 08102-02 and 08102-09. Patient 08102-02 shows 60% 2<sup>pos</sup> HER-2/neu staining before vaccination with additional immunohistochemical evidence of estrogen receptor expression. After vaccination, there was no evidence of HER-2/neu staining on any residual DCIS, and the remaining DCIS is cribriform with estrogen receptor staining (data not shown). Patient 08102-09 showed diminished HER-2/neu staining with evidence of altered distribution concentrated in vacuoles. **B**, postvaccination serum from six of nine patients tested shows an increase in complement-fixing antibodies after vaccination. Serum obtained before and after vaccination were stored frozen and then added at dilutions ranging from 1:6 to 1:24 to cultures of either HER-2/neu<sup>pos</sup> or HER-2/neu<sup>neg</sup> breast cancer lines and guinea pig complement was then added for 2 to 3 h. After 4 h, 15  $\mu$ L WST1 was added to the wells. The plates were analyzed by an ELISA reader at 3 and 4.5 h afterwards at a wavelength of 450. Trastuzumab (Herceptin) was also used at 1:6 to 1:240 dilutions. The percentage cytotoxicity was calculated as described in Materials and Methods. **C**, evidence of vaccine-induced IgG bound to tumor *in vivo*. DCIS specimens obtained before and after vaccination from patient 08102-09 were treated with antihuman IgG. In prevaccine samples, there was minimal evidence of antihuman IgG staining (*brown staining*), except in necrotic cells in the duct; however, postvaccine, there was evidence of over 50% of ductal cells staining with antihuman IgG. Cells were counterstained with H&E staining (*blue staining*). Magnifications,  $\times 10$  (first two photos) and  $\times 20$  (the last postvaccine).

redistribution of remaining HER-2/neu staining in cellular vacuoles compared with the uniform 3+ membrane staining in the prevaccine biopsy (Fig. 6A).

**DCI vaccines induce complement-dependent antibody-mediated cell cytotoxicity.** The presence of DCIS trafficking B cells and IFN- $\gamma$ -secreting T cells prompted us to assess the activity of prevaccine and postvaccine sera in a complement-dependent, tumor-lytic assay (Fig. 6B). Postvaccine serum from six of the nine available subjects showed elevated complement-dependent *in vitro* lysis of HER-2/neu<sup>pos</sup> but not HER-2/neu<sup>neg</sup> tumor cell lines. It is notable that four of these six subjects (08102-02, 08102-03, 08102-05, and 08102-09) were among the subjects displaying decreased HER-2/neu staining and smaller areas of DCIS than predicted by areas of microcalcifications. Subject 08102-11 did not develop peritumoral lymphocytic infiltrates despite the presence of serum antibody. In contrast, the anti-HER-2/neu antibody-based therapeutic agent trastuzumab (Herceptin, Genentech, Inc., South San Francisco, CA; refs. 21–30) did not induce complement-mediated lysis of HER-2/neu-expressing breast cancer cells (Fig. 6B).

We next did immunohistochemistry staining to determine whether the endogenously produced antibody actually bound to tumor *in vivo*. Here, prevaccine and postvaccine DCIS samples from one subject (08102-09) with residual high-grade HER-2/neu-expressing DCIS were stained with antihuman IgG. We found minimal evidence of IgG bound to tumor before vaccination, but after vaccination, DCIS cells showed strong anti-IgG staining (Fig. 6C). This shows that endogenous and, apparently, vaccine-induced antibodies bind directly to tumor targets, consistent with a breaking of tolerance. These data further suggest that some of the apparent loss of HER-2/neu staining could be a result of competition from the patients' own antibody. However, these complement-fixing antibodies may also represent a tumor rejection mechanism predicted by HER-2/neu transgenic mouse models (31–36).

## Discussion

Although definitive conclusions cannot yet be drawn, loss of HER-2/neu expression and apparent tumor regression for DCIS

suggests a potential for HER-2/neu DC1 vaccines to improve prognosis and act as adjuncts to breast-conserving surgical strategies. Our method was structured to deliver antigen-pulsed, polarized DC1, such that IL-12p70 bursts coincided with *in vivo* T-cell encounters in the lymph nodes. The initial IL-12 burst was triggered by an exposure to a TLR agonist, which preconditioned dendritic cell preparations for a delayed IL-12 burst if CD40 ligation was done up to 36 h later (Fig. 1D). This delayed IL-12 burst *in vitro* suggests that initial exposure to TLR agonists *in vivo*, followed by later CD40 ligation (from activated CD4<sup>pos</sup> CD40L<sup>pos</sup> helper T cells), may reflect a physiologic signaling sequence that either licenses repetitive IL-12 production from the same population of dendritic cells (thereby postponing dendritic cells exhaustion; ref. 7) or recruits a second population of dendritic cells that did not participate in the initial round of IL-12 secretion. Whatever the precise mechanism, the strategic preconditioning of such clinically desirable DC1 events may contribute substantially to subsequent dendritic cells performance.

In the preliminary evaluation of this trial, our vaccination strategy resulted in induction of CD4<sup>pos</sup> IFN- $\gamma$ <sup>pos</sup> Th1 cells, peritumoral lymphocytic infiltrates (B cells and CD4<sup>pos</sup> and CD8<sup>pos</sup> T cells), complement-dependent tumor-lytic antibodies, and measurable reductions in DCIS. Previous studies have linked lymphocytic tumor infiltrates with better prognosis (37, 38) and HER-2/neu transgenic mouse models have elucidated the roles of B cells (antibody) and CD4<sup>pos</sup> T cells (with less role for CD8<sup>pos</sup> CTL) in tumor rejection (32, 33). It is remarkable, however, that these changes, including clinically apparent reductions in DCIS for half the subjects, were induced in little over 4 weeks by a well-tolerated, weekly DC1 vaccination regimen. Interestingly, antibody bound directly to tumor (indicating an *in vivo* breakdown of tolerance) could be shown after vaccination, although whether the role of tumor-trafficking B cells is to locally increase antibody concentration or to serve as APC is yet to be determined.

For therapeutic cancer vaccines, breaking tolerance is essential, especially because active mechanisms seem to be in place to dampen immunity even at the early DCIS stage. For example, peptide- and tumor-reactive CD8<sup>pos</sup> T cells from healthy donors could be sensitized against HER-2/neu peptides in a single round of *in vitro* stimulation (6), but T cells from prevaccine DCIS patients could not (Fig. 2C). Prevaccine HER-2/neu tetramer<sup>pos</sup> T cells also expressed relatively high levels of CTLA-4 (associated with

inhibitory signaling) and comparatively low levels of CD28 (activation signaling). After vaccination, this ratio inverted, and peptide- and tumor-reactive T cells were easily expanded from peripheral blood (as with healthy donors). Interestingly, we have shown previously that IL-12 production by dendritic cells radically enhances functional avidity and tumor-killing capacity of CD8<sup>pos</sup> cells (6). Therefore, expression ratios for CD28 and CTLA-4 may reflect yet another mechanism by which CD8<sup>pos</sup> T-cell peripheral tolerance is regulated.

The preliminary results of this trial suggest that a focus on combined early disease settings and neoadjuvant therapy may have therapeutic effect, as well as permit meaningful study of modulations in cellular trafficking, tolerance, and changes in tumor antigen expression as a consequence of vaccination. Indeed, the selection of tumor vaccine antigens based on their role in the development and maintenance of disease may be of critical importance (39–45). The immune system has been shown naturally to sculpt characteristics of emerging tumors during carcinogenesis, supplying a natural selective force that eliminates more immunogenic phenotypes. This process, termed “immunoediting” (46, 47), may slow tumor growth initially but, in the long run, probably causes less immunogenic, more aggressive tumors. The approach of DC1 vaccination for HER-2/neu<sup>pos</sup> early breast cancer could have the potential to reverse this course by purposefully and selectively targeting the more aggressive HER-2/neu<sup>pos</sup> phenotype. This strategy may therefore be considered “targeted immunoediting.” Further investigations using the general methods and strategies examined in this study are warranted, both in patients with more advanced breast cancer and in patients with other malignancies.

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## Targeting HER-2/*neu* in Early Breast Cancer Development Using Dendritic Cells with Staged Interleukin-12 Burst Secretion

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