Treatment of Mammary Carcinomas in HER-2 Transgenic Mice through Combination of Genetic Vaccine and an Agonist of Toll-Like Receptor 9

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Abstract

Purpose: Oligodeoxynucleotides containing unmethylated CpG dinucleotides induce innate and adaptive immunity through Toll-like receptor 9 (TLR9). In the present study, we have examined the ability of a novel agonist of TLR9, called immunomodulatory oligonucleotide (IMO), to enhance effects of a HER-2/neu plasmid DNA electroporation/adenovirus (DNA-EP/Ad) vaccine.

Experimental Design: BALB/NeuT mice were treated with DNA-EP vaccine alone, IMO alone, or the combination of two agents starting at week 13, when all mice showed mammary neoplasia. Tumor growth and survival were documented. Antibody and CD8+ T-cell responses were determined. Peptide microarray analysis of sera was carried out to identify immunoreactive epitopes. Additionally, microCT and microPET imaging was carried out in an advanced-stage tumor model starting treatment at week 17 in BALB/NeuT mice.

Results: The combination of DNA-EP and IMO resulted in significant tumor regression or delay to tumor progression. 2-Deoxy-2-[18F]fluoro-D-glucose microPET and microCT imaging of mice showed reduced tumor size in the DNA-EP/IMO combination treatment group. Mice treated with the combination produced greater antibody titers with IgG2a isotype switch and antibody-dependent cellular cytotoxicity activity than did mice treated with DNA-EP vaccine. An immunogenic B-cell linear epitope, r70, within the HER-2 dimerization domain was identified through microarray analysis. Heterologous DNA-EP/Ad vaccination combined with IMO increased mice survival.

Conclusion: The combination of HER-2/neu genetic vaccine and novel agonist of TLR9 had potent antitumor activity associated with antibody isotype switch and antibody-dependent cellular cytotoxicity activities. These results support possible clinical trials of the combination of DNA-EP/Ad-based cancer vaccines and IMO.

Genetic vaccines can elicit immunoresponses against a wide variety of antigens, including tumor-associated antigens (1). In vivo electroporation of plasmid DNA and replication-defective recombinant adenoviruses (DNA-EP/Ad) can induce antibody and cellular antigen-specific immunoresponses in several species (2, 3). Combinations of heterologous modalities of immunization induce stronger immunoresponses than do single-modality vaccines (4–6). The vaccination per se may have limited effect on late-stage patients, however, because tumors can rapidly induce immunosuppression in the host, compromising the response to the vaccine via a variety of complex mechanisms (7). Thus, combination therapies are needed to synergize with cancer vaccines (8).

Toll-like receptors (TLR) recognize microbial pathogens, leading to the activation of protective immunoresponses (9). TLR9, found within endosomal compartments of human B cells and plasmacytoid dendritic cells, detects unmethylated CpG dinucleotides present in viral and prokaryotic genomes; CpG dinucleotides are generally methylated in mammalian DNA (10, 11). TLR9 stimulation first activates innate immunity with a predominantly Th1-type cytokine response. In the second, adaptive phase of the immunoresponse, TLR9 activation enhances antigen-specific humoral and cellular responses following concomitant exposure to a wide variety of antigens in both prophylactic and therapeutic vaccines in animal models (10, 11). TLR9 stimulation first activates innate immunity with a predominantly Th1-type cytokine response. In the second, adaptive phase of the immunoresponse, TLR9 activation enhances antigen-specific humoral and cellular responses following concomitant exposure to a wide variety of antigens in both prophylactic and therapeutic vaccines in animal models (10, 11). Several mechanisms contribute to the strong adjuvant activity of TLR9 agonists, including stimulation of antigen-specific B cells, inhibition of B-cell apoptosis, enhanced IgG class switch, and CTL generation (12). The activation of TLR9 can promote tumor regression either directly, through the antitumor activity of factors such as IFN-α and TRAIL (13), or indirectly, through the activation of natural killer (NK) cell-mediated tumor killing (14).

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Cancer Therapy: Preclinical
Translational Relevance

This study describes a novel combination of a gene-based cancer vaccine and an agonist of Toll-like receptor 9 in a preclinical model that develops spontaneous mammary tumors driven by HER-2/neu overexpression. We show that this combination can impair tumor growth in a therapeutic setting and that this effect is associated with immunologic parameters involving innate and adaptive immunity. As a novel research tool, we characterize the signature of the elicited antibody response by means of a peptide array that led to the identification of a potential efficacy biomarker of the treatment. Additionally, we used microPET and microCT imaging techniques to further refine this therapeutic effect in a more advanced disease stage. Due to its pleiotropic effects, this combination could offer important advantages over anticancer vaccines being explored in current clinical trials. These data may provide new insights for the design of future clinical trials for patients with breast cancer.

Synthetic agonists of TLR9 containing a novel DNA structure and synthetic dinucleotides, called immunomodulatory oligonucleotides (IMOs), induce potent Th1-type immunoresponses in vitro and in vivo through TLR9 activation (15–17). IMOs are resistant to degradation by nucleases because of their structure (15, 18). Previous studies have shown potent antitumor activity of IMOs as monotherapies or in combination with chemotherapeutic agents and monoclonal antibodies (19–21). Currently, a synthetic agonist of TLR9, IMO-2055, is under clinical evaluation in combination with chemotherapy and other agents in cancer patients (22).

HER-2/neu is an optimal target for therapeutic intervention. HER-2/neu oncoprotein is a tyrosine kinase receptor overexpressed in several human tumors and associated with poor prognosis (23, 24). Trastuzumab (Herceptin), a humanized monoclonal antibody directed against HER-2, is an approved product for the treatment of HER-2-expressing breast cancer (25), and pertuzumab (Omnitarg), a novel antibody acting as HER dimerization inhibitor, is being evaluated in clinical trials for different tumor types (26–30).

In this study, we combine a genetic vaccine targeting HER-2/neu with an IMO in BALB/NeuT mice. The combination has a significant therapeutic effect, leading to tumor regression/stabilization. The immunologic variables and biomarkers associated with this effect are characterized. Our data support the integration of TLR9 agonists, such as IMOs, into genetic cancer vaccine clinical trials targeting HER-2/neu.

Materials and Methods

Compounds. IMO, 5'-TCTGACRTICT-X-TCTTRCAGTCT-5' (molecular weight = 7,164; X and R are the glycerol linker and 2'-deoxy-7-deazaguanosine, respectively) was synthesized with phosphorothioate backbone, purified, and analyzed as described previously (16).

Mice. Female BALB/c and BALB/NeuT mice (31) were bred under specific pathogen-free condition by Charles River Breeding Laboratories and screened for the presence of the transgene by duplex PCR as described (32). Mice were treated in accordance with UK Co-ordinating Committee on Cancer Research Guidelines for the Welfare of Animals.

Secreted alkaline phosphatase and interleukin-12 ELISA assays. Secreted alkaline phosphatase in the serum was measured by PhosphoLight System (Applied Biosystems) according to the manufacturer's instructions. Interleukin-12 (IL-12) in mouse sera was measured by mouse IL-12p40/p70 ELISA kit (R&D Systems) according to the manufacturer’s protocol.

Immunogens. Generation of pVII-ratNeuECD.TM plasmid and Ad5-ratNeuECD.TM has been reported previously (33). Plasmid DNA was used to vaccinate mice at various time points by DNA-EP as described previously (34). Ad5-ratNeuECD.TM was diluted in Dulbecco’s PBS and injected into each quadriceps in both limbs in 50 μL to provide each mouse with 1 × 10^7 viral particles.

IFN-γ intracellular staining. IFN-γ production by stimulated T cells was measured as described previously (35). Peripheral blood mononuclear cells were incubated overnight with 5 to 6 μg/mL of a mixture of two immunodominant ratNeu CD8+ epitopes as described previously (33). Cells were analyzed on a FACS Calibur flow cytometer using CellQuest software (BD Biosciences Immunocytometry Systems).

Peptide MicroArray. The rat HER-2 peptide collection has been used to print on Codelink Activated Slides (GE Healthcare) using the Piezorray (Perkin-Elmer). Each peptide was prepared in 1× PBS (pH 7.4), 10% DMSO at a concentration of 3.0 mg/mL. Every spot of the array was obtained by delivering 1 drop of −0.3 nL resulting in a spot diameter of −200 μm. Peptide MicroArray slides were incubated with 1.5% goat serum (Sigma) in PBS for 30 min at room temperature. After a quick rinse in PBS, the arrays were incubated for 2 h at room temperature with 1:30 diluted mouse serum in binding buffer [0.1 mol/L Tris-HCl (pH 8.0), 0.1 mol/L NaCl, 0.02% (v/v) Tween 20, 0.1% (w/v) bovine serum albumin]. The slides were washed in 1× PBS, 0.02% Tween 20 and then incubated with 1:250 diluted goat anti-mouse IgG FITC conjugated (Sigma F4018). After two washes in 1× PBS, 0.02% Tween 20, slides were rinsed in distilled water, dried, and scanned with ScanArray Express (Perkin-Elmer).

Titration of anti-neu and anti-r70 antibodies. Antibodies against neu were measured by ELISA as described previously (33). Alkaline phosphatase-linked antibodies against total IgG, IgG1, or IgG2a (Pharmingen) were used as secondary antibodies. Antibodies against r70 were measured by coating 96-well plates with r70 peptide diluted at 1 μg/mL in coating buffer and left for 3 h at room temperature. Sera were incubated overnight at 4°C at different dilution and revealed by a secondary antibody, alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma; whole molecule) at 1:2,000 in 1% bovine serum albumin-PBS-0.05% Tween 20, and incubated for 30 min at room temperature. Plates were read on an ELISA reader at A 405-620. Serum dilution was plotted versus absorbance values and the Michaelis-Menten curve was fitted to the data using Kaleidagraph (version 3.5, Synergy Software).

NK assay and antibody-dependent cellular cytotoxicity. Activity of NK cells was determined by a classic NK assay against YAC-1 cells. Briefly, 1 × 10^6 YAC-1 cells were labeled with 100 μCi Na^25CrO_4 for 1 h at 37°C. After washing, single-cell suspension splenocytes were added at the indicated concentrations and incubation was carried out for 4 h at 37°C.

Similarly for antibody-dependent cellular cytotoxicity (ADCC), 1 × 10^6 N2C cells (a cell line obtained from BALB/NeuT mammary tumors and expressing rat HER-2/neu) generously provided by Dr. Mario Colombo) were incubated with a 1:20 dilution of pooled sera and 100 μCi Na^25CrO_4 for 1 h at 37°C. Unbound antibodies and excess chromium were removed and the cells were added to mouse splenocytes obtained from naive BALB/c or treated previously with IMO and incubated 4 h at 37°C. Cell-free supernatant was collected and the release of chromium by lysed cells was measured using a scintillation counter. Percent lysis = (experimental - spontaneous lysis) / (maximum lysis - spontaneous lysis).

Histologic and pathologic evaluation. Gross and microscopic analysis of tissues from treated and control mice was done at Research Toxicologic Centre (Pomezia, Rome, Italy). The following tissues were analyzed: bone marrow, brain, heart, injection site (muscle), kidneys,
large intestine (including Peyer’s patches), liver, lungs, lymph nodes (distal and proximal to the injection site), mammary glands, small intestine, spleen, and stomach. After dehydration and embedding in paraffin wax, sections of the tissues were cut at 5 μm thickness and stained with H&E. Sections of each animal were evaluated by the study pathologist in blind fashion.

**In vivo imaging.** BALB/NeuT mice (15–17 weeks old) were initially scanned by microCT to determine tumor burden. Enrollment criteria were set to be a single tumor volume <30 mm³ with total tumor burden <100 mm³ determined by microCT image analysis.

For microCT imaging, mice were given an injection of diluted Omnipaque-300 (1.9 in 0.9% NaCl, 1.5 mL, intraperitoneal injection; GE Healthcare) to assist in delineation of tumor boundaries. microCT images of the entire mouse body were acquired using a GE explore Locus Ultra (GE Healthcare), microCT images were reconstructed to 100 μm cubic voxel dimension, and tumor volumes were obtained by manual segmentation using the Amira 4.1.1 imaging software (Mercury Computer Systems).

microPET imaging studies were done with injections of 2-deoxy-2-[18F]fluoro-D-glucose ([18F]FDG) in 6 h fasted mice (~0.1 mCi, intravenous, tail vein) 60 min before image acquisition. Mice were maintained under isofluorane anesthesia in a plastic “imaging box.” Following maximum *a posteriori* reconstruction of the [18F]FDG acquisitions, regions of interest were drawn using ASIPro (Siemens Microsystems). [18F]FDG uptake is expressed as normalized activity in the tumor, expressed as nCi/mL, and is normalized for injected dose and body weight, which is equivalent to standardized units activity in the tumor, expressed as nCi/mL, and is normalized for injected dose and body weight, which is equivalent to standardized uptake value.

**Statistical analysis.** Log-rank test, two-tailed Student’s *t* test, and Cox proportional hazards model were used where indicated. All analyses were done in JMP version 5.0.1 (SAS Institute).

## Results

**Immunostimulatory activity of IMO in BALB/NeuT mice.** Immunostimulatory activity of IMO in the presence and absence of DNA-EP vaccine was studied in BALB/NeuT mice by measuring serum IL-12 levels and NK-cell activity 2 and 48 h after IMO administration, respectively. Significantly higher levels of IL-12 were found in mice treated with IMO than in untreated or DNA-EP vaccine-treated mice (Fig. 1A). Importantly, DNA-EP vaccine had no or minimal effect on IMO-induced IL-12 levels (Fig. 1A). Splenocytes from DNA-EP vaccine/IMO-treated mice had greater NK-cell activity than did splenocytes from mice treated with DNA-EP vaccine alone (Fig. 1B).

**DNA-EP vaccine/IMO combination therapy in BALB/NeuT mice.** To study the effect of the combination of DNA-EP vaccine/IMO on spontaneous tumor development, groups of BALB/NeuT mice were treated starting at week 13. At this age, all mice were diagnosed with mammary neoplasia, presenting anechoic and/or hypoechoic neoplastic structures in the mammary glands, as revealed by ultrasound imaging evaluation (33). Plasmid DNA vaccine was administered by electroporation intramuscularly at weeks 13, 15, 20, 22, 30, 32, 40, and 42 (Fig. 2). IMO was administered intramuscularly by electroporation 1 week after the vaccine for 8 weeks. By week 15, tumors became palpable and the development of mammary gland lesions was monitored. The control group developed tumors in all 10 mammary glands by week 30 (Fig. 2A). In this setting, the IMO alone had a small but significant antitumor effect (*P* < 0.034, log-rank test of survival). DNA-EP vaccine alone delayed tumor progression in 4 of 10 mice surviving up to week 40, with an average of 6 tumors per animal (*P* < 0.0001). The combination treatment had a significant antitumor effect: 50% of the mice in this group had no evidence of tumor, 40% presented one or two small tumors, and only 10% had more than two palpable lesions at week 40 (Fig. 2A and B). The difference in survival between the DNA-EP vaccine/IMO combination group and either the DNA-EP vaccine-treated group or the IMO alone group was highly significant (*P* < 0.01 or *P* < 0.0001, respectively). This combination effect was more pronounced and led to tumor regression in treated mice immediately following the weeks 40 to 43 of DNA-EP vaccine/IMO treatment.

We compared treated and control groups based on time to reach advanced disease stage, defined as the time required for each mouse to have 20% of the mammary glands affected by the tumor (Fig. 2B). We observed a significant delay in disease progression in mice treated with the DNA-EP vaccine alone and those treated with the DNA-EP vaccine/IMO combination compared with control (*P* < 0.0001 for each comparison). In a separate experiment, similar effects were observed when IMO was administered intramuscularly without electroporation, suggesting that the agonist is able to target its receptor and...
activate the TLR9 pathway with or without electroporation (data not shown).

Immunologic parameters associated with DNA-EP vaccine/IMO combination therapy. To characterize the HER-2/neu-specific immunoresponses, we measured antibody titer, isotype switching of antibodies, cell-mediated responses, and ADCC activity. To assess the antibody responses to the antigen in the absence and presence of IMO, mice were bled at week 24 and the antibody titer was measured by ELISA. The antibody titer was at least 10-fold higher in the group treated with DNA-EP vaccine/IMO combination than in the DNA-EP vaccine-treated group \((P < 0.01; \text{Fig. 3A})\). No anti-HER-2/neu antibodies were detected in the control group or in mice treated with IMO alone. Importantly, the most prevalent anti-HER-2/neu antibody class in DNA-EP vaccine/IMO combination group was IgG2a, suggesting a Th1-type immunoresponse (Fig. 3B).

To test the ability of the elicited antibodies to mediate ADCC, we used effector splenocytes from mice left untreated or injected intramuscularly with IMO. Sera from control and vaccinated groups showed no lysis of autologous mammary tumor cells. Sera from mice that received the DNA-EP vaccine/IMO combination induced ADCC only when effector cells were derived from IMO-treated mice \((P = 0.01, t \text{ test})\), suggesting that IMO activates NK cells (Fig. 3C).

The cell-mediated immunoresponse was measured by IFN-\(\gamma\) intracellular staining of peripheral blood mononuclear cells at week 34. There was no significant difference in the magnitude of CD8+ T-cell responses between the DNA-EP vaccine-treated group and the DNA-EP vaccine/IMO combination-treated group (Fig. 3D). Interestingly, up to 60% of mice in the combination group produced >0.1% CD8+ immunoresponse compared with 30% in DNA-EP vaccine alone group \((P = 0.13, \text{Fisher's exact test}; \text{Fig. 3D})\). There was no significant difference in the survival of mice that showed >0.1% or <0.1% CD8+ responses (data not shown).

Identification of HER-2 dimerization loop as target of antibody response. To characterize the antibody response and to identify the immunoreactive epitopes, a peptide array composed of 15mer peptides overlapping by 11 residues and encompassing the entire rat HER-2 protein was set up. The spots of two overlapping epitopes \((r69, 274TYNTDFESMHNPEG288\) and \(r70, 278DTFESMHNPEGRYTF292\)) were recognized specifically in DNA-EP vaccine/IMO-treated mice (Fig. 4A). The epitope was identified and mapped within the three-dimensional structure of rat HER-2/neu5 and was found to be part of the finger-like domain II (see Fig. 4A), different from the one recognized by trastuzumab (36) but within the region bound by pertuzumab (37).

To extend the analysis to a large number of mice, we set up an ELISA assay for peptide r70, and mice that received DNA-EP vaccine or DNA-EP vaccine/IMO were compared. Most of the mice treated with the combination of DNA-EP vaccine/IMO seroconverted, whereas only a small fraction of mice treated with DNA-EP vaccine alone showed antibodies against this particular epitope \((P = 0.01, t \text{ test}; \text{Fig. 4B})\). Importantly, anti-r70 antibody titers measured at week 24 correlated with the presence of tumors at a later time point (week 33; \text{Fig. 4B}; Spearman’s \(\rho = -0.59; P = 0.03\)). Notably, however, one mouse with r70 antibodies had a tumor. Moreover, anti-r70 titers correlated significantly with mice survival \((P = 0.02)\).

We evaluated the total and r70-specific antibody titers for the entire course of the vaccination and found that the maximal activation of the humoral responses was achieved after the first two DNA-EP vaccine/IMO cycles of vaccination (Fig. 4C).

Tissue histology and toxicology. To characterize the therapeutic potency of the combination and evaluate potential toxicologic effects in treated mice, histologic examination of several tissues was done at week 33. All mice that survived in control and IMO-treated groups had advanced mammary adenocarcinomas in the mammary glands and lung metastases (Fig. 5A and B). Mice treated with DNA-EP vaccine alone had atypical mammary hyperplasia and adenocarcinomas (Fig. 5A). Mice treated with the DNA-EP vaccine/IMO combination had mostly normal mammary glands (Fig. 5A) and atypical hyperplasia. No gross alterations of other organs, lymph nodes and injection sites, or variations of blood variables (hematocrit, glucose, urea, creatinine, bilirubin,
albumin, alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase) were observed. These data indicate that DNA-EP vaccine/IMO treatment is safe and can effectively block tumor initiation/progression in mice.

**DNA-EP vaccine/IMO combination therapy in advanced tumor stages.** To assess whether the DNA-EP vaccine/IMO combination could provide antitumor effects in a more advanced stage, that is, when mice have a well-developed carcinoma in situ, groups of BALB/NeuT mice were vaccinated starting at week 17 with the same protocol described above. We employed microCT and microPET imaging techniques for quantitative measurement of tumor burden in mice. Because BALB/NeuT mice develop many tumors per animal, we tracked one to three tumors per mouse that could be easily discerned by both microCT and microPET image analyses. Two examples of the microCT analysis are shown in Fig. 6A. We observed a significant reduction in tumor growth in mice treated with the DNA-EP vaccine/IMO compared with the vehicle group (Fig. 6A). Tumor volumes were analyzed using fold change from the day before the first vaccination to standardize all tumors to their starting volumes. The fold change in the reduction of tumor growth between treatment and vehicle groups was statistically significant at weeks 23 ($P = 0.005$) and 27 ($P < 0.001$). These microCT data quantify tumor response by tumor volume rather than raw tumor number as was done in the previous study.

A robust antibody (total IgG average titer: $5,976 \pm 7,418$), but not T cell, response was observed in these mice. There was no difference in survival between the two groups (data not shown), suggesting that tumor load inhibits the immune system and that the antibody response is not sufficient to halt tumor progression in this advanced setting.

To examine if heterologous vaccination could further increase time to progression and survival in this advanced therapeutic setting, mice were vaccinated starting at week 17 with a regimen that consisted of five DNA-EP vaccinations with a 2-week interval followed by two adenovirus injections (DNA-EP/Ad). In the combination group, IMO was administered

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**Fig. 3.** DNA-EP and IMO combination increases HER-2/neu-specific immunity. BALB/NeuT mice were treated as described in the text. A, anti-HER-2/neu total IgG antibody titer measured by ELISA. White dot, titer of each mouse; gray dot, group geometric mean. B, anti-HER-2/neu IgG2a/IgG1 ratio in treated groups. White dot, IgG class ratio for each mouse; gray dot, group geometric mean. C, ADCC activity. Mammary tumor N2C cells were labeled with $^{51}C$ and coated with sera from control, vaccinated, and vaccinated/IMO-treated mice. Splenocytes from untreated or IMO-injected (48 h before, intramuscularly) mice were used as effectors at $E:T = 100$. The assay was run in six replicates. Statistical analysis was done with Student’s $t$ test. D, cell-mediated immunoresponse in vaccinated BALB/NeuT mice. Mice were bled at week 34 and peripheral blood mononuclear cells were analyzed by intracellular staining for IFN-$\gamma$. Dot, percentage of CD3$^+$/CD8$^+$/IFN-$\gamma^+$ peripheral blood mononuclear cells per single mouse; triangle, group geometric mean. Percentage of responder mice ($>0.1 \%$ CD3$^+$/CD8$^+$/IFN-$\gamma^+$).
1 week after each DNA-EP/Ad injection. Mice that received DNA-EP/Ad vaccine plus IMO combination produced an enhanced cell-mediated immunoresponse (P = 0.01, t test; Fig. 6B). Vaccine alone and IMO alone were completely ineffective against tumor progression (Fig. 6B), whereas time to advanced disease was delayed and survival was higher in the vaccine/IMO combination group. These data suggest the potential for IMO as an adjuvant in combination with HER-2/neu genetic cancer vaccine in advanced mammary tumors.

Discussion

Although several preclinical studies have shown the anti-tumor activity of various immunologic approaches for the treatment of breast cancer, evidence of clinical activity to date is limited (38). These failures in clinical trials could be as a result of (a) the effect of previous oncolytic treatments (chemotherapy and radiotherapy) on the immune system, (b) the population of metastatic patients treated thus far that is characterized by large tumor burden, (c) the ability of large tumors to escape the immune system, and (d) the difficulty of breaking immunotolerance. If we are to improve the efficacy of cancer vaccines, we must better understand the relationship between innate and adaptive immunoresponses and the imunoescape mechanisms employed by tumor cells.

In this study, we have evaluated the effect of the combination of a HER-2/neu genetic cancer vaccine with a novel agonist of TLR9 (IMO) in BALB/NeuT mice, which spontaneously develop carcinoma in the mammary gland and are extensively used for the evaluation of anticancer immunotherapies (39, 40).

We vaccinated mice at 13 wk of age in the presence of relatively small mammary hyperplasia and in situ carcinomas. Only the group of mice that received the DNA-EP vaccine/IMO combination produced a marked and long-lasting anti-tumor response, disease stabilization, and tumor shrinkage (Fig. 2). Immunohistochemistry analysis showed normal mammary glands or hyperplasia in treated mice and no detectable lung metastases (Fig. 5). Importantly, these mice received the last treatment at week 43 and were subsequently disease-free for their lifespan (>70 weeks), suggesting the induction of durable...
immune memory. The antitumor activity correlated with (a) induction of Th1-type cytokine secretion and activation of NK cells (Fig. 1), (b) an increase in adaptive T-cell responses (Fig. 3), (c) a significant increase in antigen-specific antibody responses with IgG2a isotype switch and induction of ADCC activity against antibody-coated tumor cells (Fig. 3), and (d) development of high-titer antibodies against the HER-2/neu dimerization domain, which may play a relevant role in the inhibition of signaling and tumor growth (Fig. 4).

For the first time in this study, we employed [18F]FDG microPET and microCT imaging as means to characterize treatment efficacy of the HER-2/neu DNA-EP vaccine and IMO in this tumor model. This clinical imaging method provided a more sensitive and accurate measurement of tumor response than palpation. We observed good uptake of the radiotracer in tumors of mice with measurable tumor burden (≈5 mm diameter each, at age ≈17 weeks; data not shown). Similarly, microCT provided an excellent way to determine effects on tumor volumes.

At age 17 weeks, mice treated with DNA-EP/IMO showed a significant delay in tumor growth but no difference in survival rate (Fig. 6A). A significant antibody, but not T-cell immunoresponse, was found. However, the suboptimal T-cell response associated with the lack of antitumor effects could be the result of immunosuppressive activities associated with the tumor burden. For example, myeloid-derived suppressor cells accumulate in blood, lymphoid organs, and tumors in BALB/NeuT mice (41) and inversely correlate with the immunoresponse elicited (33). A more aggressive vaccination program based on four DNA-EP plus two injections of adenovirus combined with IMO resulted in a greater inhibition of tumor progression, increased time to progression, and measurable T-cell response (Fig. 6B), indicating that strong immunogenic vectors are required in addition to pleiotropic activation of the immunoresponse by IMO in the presence of large tumor burdens.

Our results suggest that DNA-EP/Ad vectors induce both cell-mediated and humoral immunoresponses in BALB/NeuT mice and that the DNA-EP/Ad vaccination regimen in combination with IMO can overcome the tolerance to HER-2/neu and increase the survival of mice. The importance of both specific antibodies and IFN-γ-producing T cells in the control of
tumors has been shown previously in prophylactic settings (39, 42, 43).

Several clinical trials have investigated anticancer vaccines using HER-2/neu-derived peptides to induce peptide-specific CTLs (44, 45). The generation of CTLs correlates well with the prevention or eradication of malignant cells in cultures and/or in preclinical tumor models, but the peptides do not control tumor metastases in humans. These peptides may be useful in preventing recurrence in high-risk patients (46). These findings indicate that mere T-cell responses are insufficient to exert a therapeutically effect in patients and that all available weapons of the immune system should be employed and additional combination therapies should be explored. Pretreatment of breast cancer cells with trastuzumab induces turnover of HER-2/neu protein and enhanced killing by HER-2/neu peptide-stimulated CTLs from peptide-vaccinated patients (47), further evidence that a vaccine able to trigger both arms of the adaptive response is highly desirable.

The combination of DNA-EP vaccine and IMO produces both innate and adaptive immunities against the whole protein and not just a single epitope. The humoral response induced leads to ADCC activity on in vivo activation of NK cells by IMO treatment in BALB/NeuT mice (Figs. 1 and 3C). Direct activation of human NK cells through TLR9 significantly
increases cytokine secretion and lytic activity against trastuzumab-coated human HER-2/neu-positive tumor cells (48), further evidence that innate immunoresponses play a role in antitumor activity produced by TLR9 agonists. Similarly, pertuzumab-like antibodies may exert a direct inhibitory activity on HER-2/neu activation, blocking interaction with HER-3, signaling, and tumor growth (49). Of note, the specificity of the DNA-EP-induced antibody population was determined by a novel and relatively simple peptide array technology, which allows rapid identification of antibody-specific determinants, provided that they are linear (not conformational) and in the absence of other modifications (glycosylation, etc.). Additionally, we found that the presence of these specific antibodies predicted the clinical outcome in the mice and could be further evaluated as potential pharmacodynamic marker of the HER-2/neu vaccine/IMO combination therapy (Fig. 4B and C, right). These results support the efficacy of an anti-HER-2/neu monoclonal antibody treatment in HER-2/neu-positive human breast cancer and the finding that vaccines based on a single HER-2/neu B-cell epitope peptide can partially protect HER-2/neu transgenic mice against development of mammary tumors (50, 51).

In summary, our data suggest that a combination treatment using HER-2/neu DNA-EP/Ad vaccine and IMO in BALB/NeuT mice results in tumor stabilization/regression and durable protection against spontaneous mammary carcinoma. We have also characterized the possible immunologic parameters responsible for the antitumor effects of the combination treatment. We used a peptide array to identify the dominant target epitope, r70, for the elicited antibodies. This epitope can be used as a pharmacodynamic biomarker in clinical trials. Our data strongly support the integration of IMO, an agonist of TLR9, into genetic cancer vaccine clinical trials.

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