

# A Combination of Chemoimmunotherapies Can Efficiently Break Self-Tolerance and Induce Antitumor Immunity in a Tolerogenic Murine Tumor Model

Hyun-Jeong Ko,<sup>1</sup> Yeon-Jeong Kim,<sup>1</sup> Yun-Sun Kim,<sup>1</sup> Woo-Sung Chang,<sup>1</sup> Sung-Youl Ko,<sup>1</sup> Sun-Young Chang,<sup>1</sup> Shimon Sakaguchi,<sup>2</sup> and Chang-Yuil Kang<sup>1</sup>

<sup>1</sup>Laboratory of Immunology, Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Seoul, Korea; and

<sup>2</sup>Department of Experimental Pathology, Institute for Frontier Medical Science, Kyoto University, Kyoto, Japan

## Abstract

**Her-2/neu is a well-characterized tumor-associated antigen overexpressed in human carcinomas such as breast cancer. Because Her-2/neu is a self-antigen with poor immunogenicity due to immunologic tolerance, active immunotherapy targeting Her-2/neu should incorporate methods to overcome immunologic tolerance to self-proteins. In this study, we developed a tolerogenic tumor model in mice using mouse Her-2/neu as self-antigen and investigated whether genetic vaccination with DNA plasmid and/or adenoviral vector expressing the extracellular and transmembrane domain of syngeneic mouse Her-2/neu or xenogenic human Her-2/neu could induce mouse Her-2/neu-specific CTL responses. Interestingly, adenoviral vectors expressing xenogenic human Her-2/neu (AdhHM) proved capable of breaking immune tolerance and of thereby inducing self-reactive CTL and antibodies, but not to the degree required to induce therapeutic antitumor immunity. In attempting to generate therapeutic antitumor immunity against established tumors, we adopted several approaches. Treatment with agonistic anti-glucocorticoid-induced TNFR family-related receptor (GITR) antibody plus AdhHM immunization significantly increased self-reactive CTL responses, and  $\alpha$ -galactosylceramide ( $\alpha$ GalCer)-loaded dendritic cells (DC) transduced with AdhHM were shown to break self-tolerance in a tolerogenic murine tumor model. Furthermore, gemcitabine treatment together with either AdhHM plus agonistic anti-GITR antibody administration or  $\alpha$ GalCer-loaded DC transduced with AdhHM showed potent therapeutic antitumor immunity and perfect protection against preexisting tumors. Gemcitabine treatment attenuated the tumor-suppressive environment by eliminating CD11b<sup>+</sup>/Gr-1<sup>+</sup> myeloid-derived suppressor cells. When combined with immunotherapies, gemcitabine offers a promising strategy for the Ag-specific treatment of human cancer. [Cancer Res 2007;67(15):7477–86]**

## Introduction

Her-2/neu is a well-characterized tumor-associated antigen overexpressed in human carcinomas such as breast cancer (1, 2). It has been shown that Her-2/neu-specific CD8<sup>+</sup> CTL played an

important role in antitumor immunity in both murine tumor models and in cancer patients (3–5). However, the efficacy of genetic vaccination might be limited by the fact that Her-2/neu is a self-antigen with poor immunogenicity due to immunologic tolerance. Therefore, the vaccine strategies directed against self-Her-2/neu and other self-tumor antigens may have to incorporate methods to overcome immunologic tolerance to self-proteins (6, 7).

Aside from the question of immunologic tolerance against self-tumor Ag, it has been reported that even antigenic tumors having a strong antigen are prone to escape host immune surveillance in a tumor environment (8, 9), perhaps because the tumor microenvironment enhances the activation and expansion of regulatory T cells as well as the secretion of immunosuppressive factors such as transforming growth factor- $\beta$ , vascular endothelial growth factor, and interleukin-10 (10, 11). Naturally occurring or tumor-driven regulatory CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells act as cellular suppressors of antitumor immunity (9, 12). Furthermore, immature myeloid or dendritic cells (DCs) take part in the formation of an immunosuppressive environment in tumor-bearing hosts (10, 13). A number of recent studies have shown that the elimination of regulatory T cells or myeloid-derived suppressor cells (MDSCs) in the tumor-bearing host unmasked tumor immunogenicity in the established tumor (14, 15). Such tumor-suppressive mechanisms must be overcome if an effective antitumor effect is to be generated and preexisting tumors is to be annihilated.

Although considerable success has been achieved using a variety of approaches to break self-tolerance and induce effective antitumor immunity in Her-2/neu-transgenic mice, those results obtained from transgenic mice may not represent natural conditions of self-tolerance against self-tumor antigens in normal mice (16–19). Thus, to develop effective immunotherapeutics against Her-2/neu, it is necessary to establish a tolerogenic murine tumor model expressing a syngeneic Her-2/neu mimicking the human cancer scenario.

The current study seeks to determine whether novel vaccination strategies could circumvent tolerance to self-tumor antigen Her-2/neu to the degree necessary to establish successful therapeutic antitumor immunity. To assess vaccine strategies capable of reversing T cell tolerance, we set up a syngeneic tolerance model in mice using murine Her-2/neu as a self-antigen. Although the self-reactive CTL could be generated by the administration of adenoviral vector expressing xenogenic human Her-2/neu (AdhHM) and enhanced by the administration of agonistic anti-glucocorticoid-induced TNFR family-related receptor (GITR) antibody (Ab), it did not evoke sufficient antitumor immunity against preexisting tumors. However, AdhHM-transduced and  $\alpha$ -GalCer-loaded DC-based vaccines did significantly reduce tumor growth with the help of NKT cells and anti-GITR Ab treatment. In addition, the

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

**Requests for reprints:** Chang-Yuil Kang, Laboratory of Immunology, College of Pharmacy, Seoul National University, Shillim-9-dong, Kwanak-gu, Seoul 151-742, Korea. Phone: 82-2-880-7860; Fax: 82-2-885-1373; E-mail: cykang@snu.ac.kr.

©2007 American Association for Cancer Research.

doi:10.1158/0008-5472.CAN-06-4639

elimination of MDSCs by gemcitabine treatment before genetic immunization generated significantly higher levels of therapeutic antitumor immunity against established tumors than did a single treatment of chemo- or immunotherapy. Taken together, these results suggest that both the generation of tumor Ag-specific effectors at the cellular level and the attenuation of the tumor-suppressive environment are required for the induction of effective antitumor immunity against preexisting tumors.

## Materials and Methods

**Mice.** Female BALB/c mice were purchased from Charles River and used at the age of 6 to 8 weeks. All mice were kept under specific pathogen-free conditions in the Animal Center for Pharmaceutical Research at Seoul National University. All of the experiments were approved by the Institutional Animal Care and Use Committee of Seoul National University.

**Cell lines.** The Her-2/*neu*-expressing human breast carcinoma SK-BR-3 cell line and murine colon adenocarcinoma cell line CT26 were obtained from the American Type Culture Collection (ATCC). Human or murine Her-2/*neu*-expressing transfectoma hHer2/CT26 or mHer2/CT26 cells were developed by transduction of CT26 using a retroviral vector system. Tumor cells were maintained in DMEM (Bio Whittaker) supplemented with 10% heat-inactivated fetal bovine serum (Bio Whittaker) and 1% penicillin-streptomycin (Bio Whittaker).

**Antibodies and reagents.** The anti-GITR (DTA-1) and anti-CD25 (PC61) monoclonal antibodies (mAb) were obtained from ascites as described previously (15). Hybridoma secreting anti-CTLA-4 mAb (UC10-4F10-11) was a gift from J. Bluestone (University of California, San Francisco, CA), and hybridomas producing depleting anti-CD4 (GK1.5) and anti-CD8 (2.43) mAb were obtained from the ATCC.

For the  $\alpha$ GITR Ab treatment, mice were i.p. injected with 500  $\mu$ g of purified mAb. Rat immunoglobulin G (IgG; Sigma) was used as the control antibody. The following FITC-, PE-, or APC-conjugated mAbs to mouse cell surface molecules were purchased from BD Biosciences Pharmingen: CD4, CD8, B220, IFN- $\gamma$ , CD11b, Gr-1, and F4/80. Goat anti-mouse IgG-Fc Ab was purchased from Pierce. For the isolation of CD11b<sup>+</sup> cells from splenocytes, anti-CD11b microbeads (Miltenyi) were used.

Doxorubicin (Dong-A Pharmaceuticals), gemcitabine (Dong-A Pharm), cyclophosphamide (Choongwae Pharma), and docetaxel (Aventis Pharma) were used for chemotherapy of established mHer2/CT26 tumors.

**Construction of recombinant adenoviruses.** Recombinant adenoviruses encoding the extracellular and transmembrane domains of hHer-2 (hHM) or mHer2 (mHM) were obtained from Viomed. Titers of infective adenovirus particles were determined using the standard plaque-forming assay with 293 cells.

**In vivo cytotoxicity assay.** The *in vivo* cytolytic response was measured by flow cytometry as described previously (20), using CTL epitope peptides, hP63 (TYLPTNASL) or mP63 (TYLPANASL).

**Tumor challenge and evaluation of tumor growth.** In the prophylactic model, naive mice were i.m. injected with genetic vaccines according to the immunization schedule. Fourteen days after the last immunization, mice were s.c. challenged with  $3 \times 10^5$  of mHer2/CT26 cells in the right flank for tumorigenic growth of tumor cells.

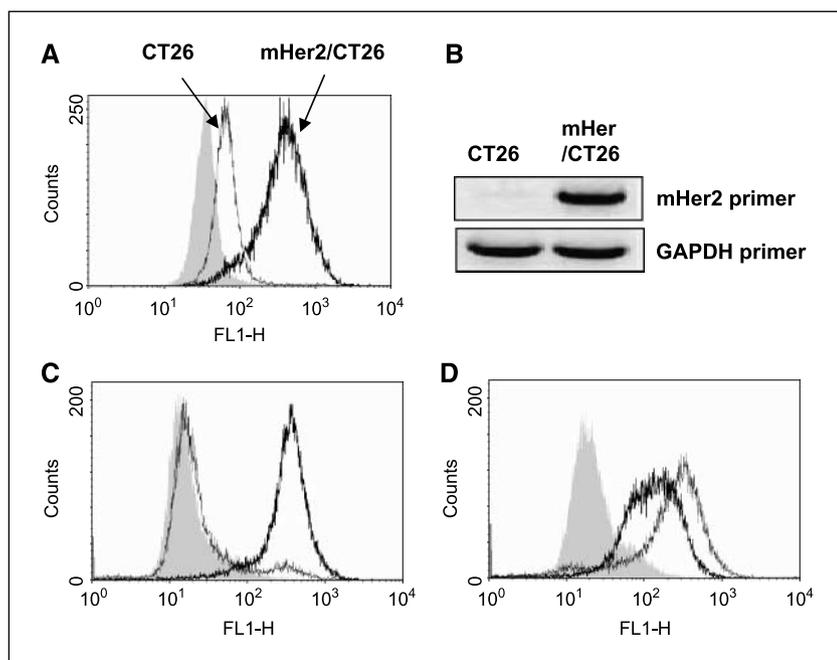
To test the therapeutic vaccine effect,  $1 \times 10^5$  or  $2 \times 10^5$  of mHer2/CT26 cells were administered on day 0, and treatment was started at least 12 days later. Calipers were used to attain a three-dimensional measurement of the tumor (21). Mice were sacrificed for humane reasons when the tumor exceeded a three-dimensional volume of 2,000 mm<sup>3</sup>. In some experiments, tumor-free mice were further inoculated s.c. with the same tumor, and then tumor growth was monitored.

**Preparation of DC and DC vaccines.** Bone marrow-derived DC and splenic DC were prepared as described previously (22, 23). Bone marrow-derived DC were transduced with 100 multiplicity of infection of AdhHM for 90 min and then loaded with 1  $\mu$ g/mL of  $\alpha$ -galactosylceramide ( $\alpha$ GalCer) for additional 18 to 22 h.

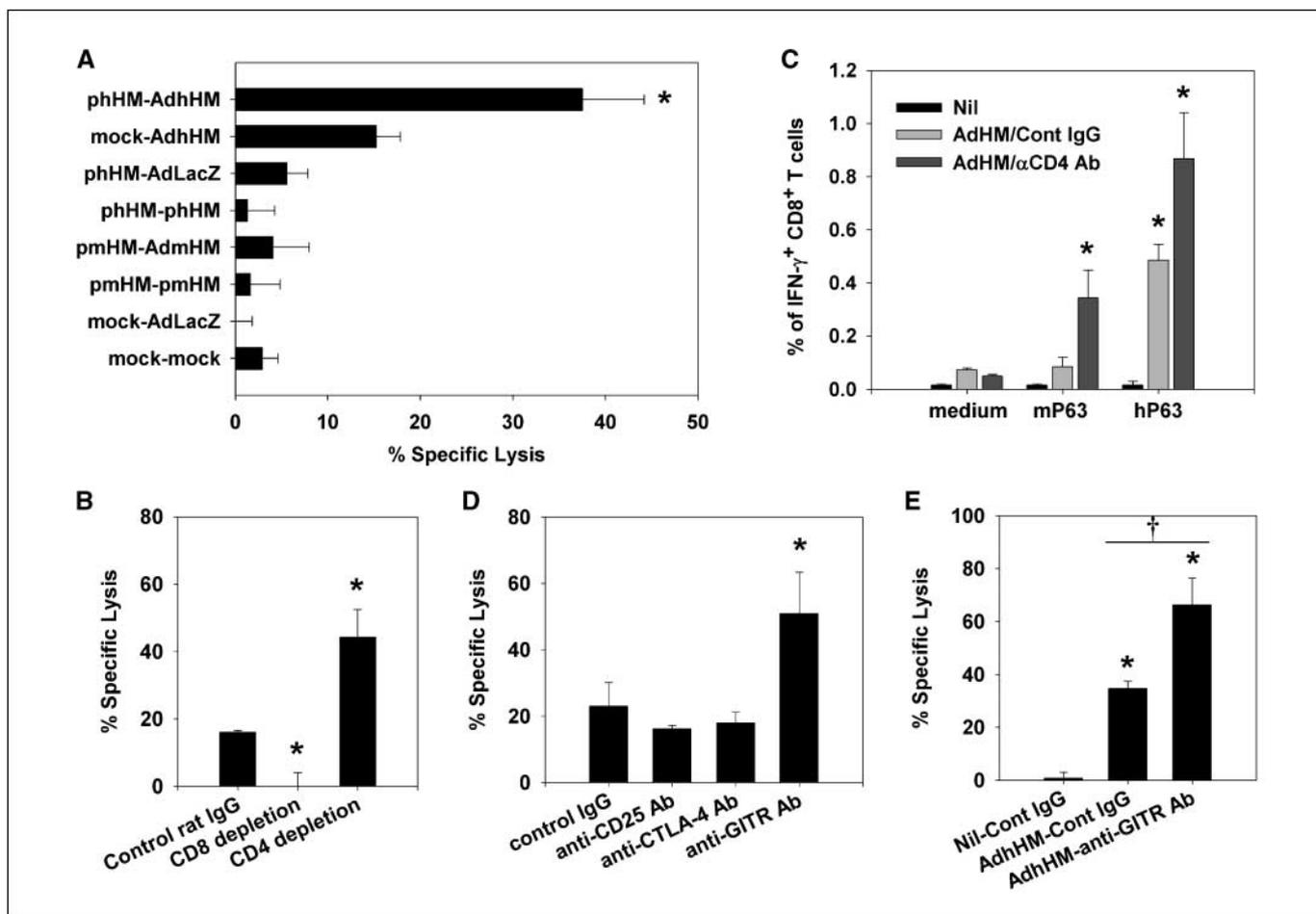
**Functional studies of CD11b<sup>+</sup> MDSCs.** To examine the suppressive role of CD11b<sup>+</sup> cells on CTL activity and proliferation, we prepared splenocytes from cured mice that had undergone gemcitabine-AdhHM- $\alpha$ GITR Ab treatment. These effectors were restimulated *in vitro* with mitomycin C (MMC; Sigma)-treated mHer2/CT26 cells and then incubated together with CD11b<sup>+</sup> cells from naive or tumor-bearing mice. Six days later, cells were harvested, and cytolytic activity against mHer2/CT26 target cells was measured using a standard Cr-51 release assay as described previously (21).

For the effect on the proliferation of CD11b<sup>+</sup> MDSCs, effectors were plated at  $5 \times 10^5$  spleen cells per well in 96-well round-bottom microtiter plates (Nunc) and cultured for 4 days with  $2.5 \times 10^4$  of MMC-treated mHer2/CT26 and diluted CD11b<sup>+</sup> cells. After 96 h of incubation, including a final 24-h pulse with [<sup>3</sup>H]-thymidine (1  $\mu$ Ci per well), the [<sup>3</sup>H]-thymidine incorporation was measured as described previously (24).

**Statistics.** Results are expressed as the means  $\pm$  SE. To statistically compare multiple groups, we employed one-way ANOVA, followed by the



**Figure 1.** Construction of a murine cell line expressing the full length of murine Her-2/*neu* on the cell surface. **A**, the FACS binding assay of rat anti-mHer2-specific polyclonal serum to mHer2/CT26 cells. Rat anti-mHer2-specific polyclonal serum was obtained from a rat that had been immunized thrice with plasmid DNA expressing the extracellular and transmembrane domains of mHer2 (pmHM) at 3-week intervals. Anti-mHer2 polyclonal serum was added to CT26 or mHer2/CT26 cells and then stained with goat anti-rat IgG-FITC. **B**, RT-PCR analysis of mHer2 mRNA transcription in mHer2/CT26 cells. Total RNA was extracted and analyzed for relative levels of mHer2 mRNA by RT-PCR. **C** and **D**, binding activities of HRO G1 and HRT G1 to murine (**C**) or human Her-2/*neu* (**D**) were measured by flow cytometry. Antihuman Her-2/*neu* mAb HRO G1 (bold line) and HRT G1 (narrow line) or control antibody (filled area) were added to Her-2/*neu*-expressing tumor cells and then stained with goat anti-mouse IgG-FITC.



**Figure 2.** Tolerance against self-tumor antigen can be broken by a xenogenic immunization with DNA vaccine and with an adenovirus vector expressing human Her-2/neu. **A**, BALB/c mice ( $n = 3-5$ /group) were injected i.m. with 100  $\mu$ g of each DNA and boosted 2 wks later with  $2 \times 10^8$  plaque-forming units (pfu) of adenovirus vectors expressing hHM, mHM, or LacZ. After 7 d, *in vivo* cytotoxicity against peptide mP63 was measured. \*,  $P < 0.001$  versus mock-mock, mock-AdLacZ, pmHM-pmHM, pmHM-AdmHM, phHM-phHM, and phHM-AdLacZ (ANOVA-Tukey). **B**, BALB/c mice ( $n = 3-4$  per group) were i.m. injected with 100  $\mu$ g of phHM at day 0 and boosted with  $10^8$  pfu of AdhHM at day 21. The recipient mice received anti-CD4, anti-CD8-depleting antibodies, or rat IgG as a control on day 27. These mice were tested for *in vivo* cytotoxicity against peptide mP63 on day 28. \*,  $P < 0.05$ , in comparison with phHM-AdhHM immunization plus control rat IgG-treated group (ANOVA-Tukey). **C**, BALB/c mice ( $n = 4$  per group) were i.m. injected with  $10^8$  pfu of AdhHM at day 0. The recipient mice received anti-CD4 or rat IgG as a control 4 d after immunization. The percentage of IFN- $\gamma$ -secreting CD8<sup>+</sup> T cells was determined after *in vitro* stimulation with 10  $\mu$ g/mL of mP63 at day 7. \*,  $P < 0.05$ , in comparison with the group receiving AdhHM immunization together with anti-CD4-depleting antibody without peptide restimulation (ANOVA-Tukey). **D**, BALB/c mice ( $n = 3$  per group) were i.m. injected with 100  $\mu$ g of phHM at day 0 and boosted with  $10^8$  pfu of AdhHM at day 21. One week after the final immunization, spleens from the immunized mice were obtained, and specific lysis of mP63-loaded target cells was estimated by *in vivo* CTL. About 250  $\mu$ g of each antibody was i.p. injected at days -2, +3, +8, +13, +17, and +23. \*,  $P < 0.05$ , compared with phHM-AdhHM (ANOVA-Tukey). **E**, BALB/c mice ( $n = 3$ /group) were i.m. injected with  $10^8$  pfu of AdhHM at day 0 and i.p. injected with 500  $\mu$ g of anti-GITR Ab 2 d later. These mice were tested for *in vivo* cytotoxicity against peptide mP63 on day 10. \*, the percentages of specific lysis of AdhHM and AdhHM- $\alpha$ GITR Ab-treated mice were significantly different (ANOVA,  $P < 0.05$ ) from those of control IgG-treated group. †,  $P < 0.05$ , as compared with groups treated with AdhHM and AdhHM- $\alpha$ GITR Ab (ANOVA-Tukey).

Tukey post hoc test using SPSS. When appropriate, the Student's *t* test was used, and the means  $\pm$  SD were reported. For the statistical analysis of the study that did not show normal distribution, a Wilcoxon two-sample rank-sum test (Mann-Whitney *U* test) was used. Values of  $P < 0.05$  were considered significant at 95% confidence interval. Each experiment was repeated at least twice.

## Results

**Establishment of a murine syngeneic tumor model.** To assess vaccine strategies capable of breaking self-tolerance and inducing therapeutic antitumor immunity against preexisting tumors in a tolerogenic tumor model, a syngeneic tumor cell line abundantly expressing murine Her-2/neu (mHer2) on the cell surface was constructed by transducing a retroviral vector expressing the full length of murine Her-2/neu. Using flow cytometry with anti-

murine Her-2/neu polyclonal serum obtained from rats immunized with plasmid DNA expressing the extracellular and transmembrane domains of mHer2 (pmHM), we detected the surface expression of murine Her-2/neu on the CT26 transfectant (Fig. 1A), and using reverse transcription-PCR (RT-PCR), we confirmed the presence of mHer2 mRNA transcripts in mHer2-transfectant cells (Fig. 1B).

To check that the monoclonal antibodies, which were known to specifically bind to human Her-2/neu (hHer2), could cross-react to murine Her-2/neu, anti-human Her-2/neu mAb HRO G1 and HRT G1 were added to mHer2/CT26 cells and then assayed by fluorescence-activated cell sorting (FACS; ref. 25). HRO was shown to specifically bind to mHer2/CT26, whereas HRT did not (Fig. 1C and D). Furthermore, mHer2/neu overexpressed on mHer2/CT26 cells could function as signal tyrosine kinase (Supplementary Fig. S1). Thus, the constructed syngeneic tumor mHer2/CT26 was

deemed suitable as a tolerogenic tumor overexpressing self-tumor antigen on the cell surface.

**AdhHM immunization induces murine Her-2/*neu*-specific CTL responses.** To evaluate the induction of self-reactive CTL responses by genetic vaccination, we prepared two kinds of synthetic CTL epitope peptides, mP63 (TYLPANASL) and hP63 (TYLPTNASL), based on the previously suggested epitopes (26, 27). The nanomer sequence of the major H2-K<sup>d</sup>-restricted CD8<sup>+</sup> T cell epitope in human Her-2/*neu* differs by only one amino acid from the mouse peptide mP63. Because mice are naturally tolerant to murine Her-2/*neu*, wild-type BALB/c mice were used as a natural model of peripheral tolerance to a self-tumor-associated antigen, mouse Her-2/*neu*. To check the self-reactive CTL against murine Her-2/*neu* under tolerance conditions, the specific lysis of mP63-pulsed target cells *in vivo* was assessed. Prime-boost immunization with plasmid DNA (phHM) and adenovirus vectors (AdhHM) expressing extracellular and transmembrane domains of human Her-2/*neu* induced significant levels of CTL responses against mP63, whereas phHM immunization alone or boosting with an irrelevant adenovirus expressing  $\beta$ -galactosidase (AdLacZ) could not generate significant specific lysis (Fig. 2A). Furthermore, AdhHM immunization alone could lyse the target cells significantly (Fig. 2E). None of the immunizations with genetic vaccines expressing murine Her-2/*neu* or control vectors produced self-reactive CTL (Fig. 2A). In addition, analysis of sera from phHM- and/or AdhHM-vaccinated mice revealed the existence of self-reactive antibodies, albeit at low levels, as well as high titers of anti-human Her-2/*neu* antibodies (Supplementary Fig. S2).

Collectively, these findings clearly show that xenogenic adenoviral genetic vaccination (AdhHM) breaks immune tolerance against self-tumor antigen by inducing self-reactive CTL and antibodies.

**Controlling of regulatory T cell functions can enhance the generation of self-reactive CD8<sup>+</sup> CTL by AdhHM immunization.** We next used anti-CD4 or anti-CD8 mAb to deplete CD4<sup>+</sup> T or CD8<sup>+</sup> T cells at the effector phase of immune responses so that the relative importance of those cells in the generation of self-reactive CTL responses could be determined. As expected, CD8 depletion led to the depletion of self-reactive CTL, completely blocking the killing of target cells (Fig. 2B). Surprisingly, the depletion of CD4<sup>+</sup> T cells instead enhanced the CTL responses of AdhHM-immunized mice (Fig. 2B), indicating that CD4<sup>+</sup> T cells are dispensable or suppressive for the generation of self-antigen-specific CTL immunity.

Next, we checked the effectiveness of CTL after AdhHM immunization by examining mP63-specific IFN- $\gamma$  production of CD8<sup>+</sup> T cells. Although AdhHM induced mP63-specific lysis, it provoked only a minimal number of IFN- $\gamma$ -secreting CD8<sup>+</sup> T cells

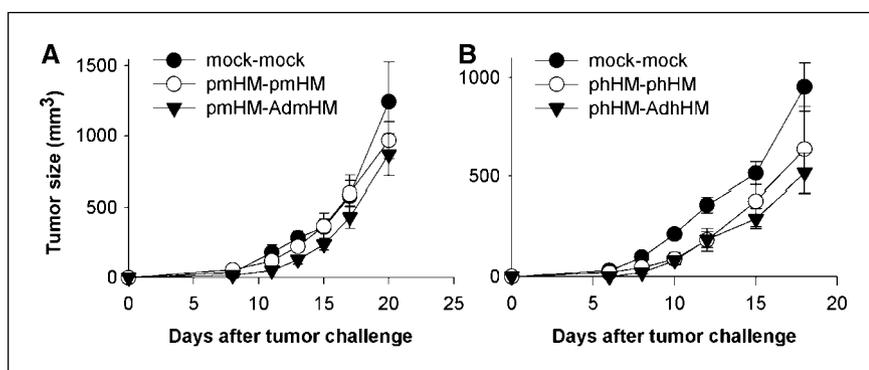
(Fig. 2C). Furthermore, we could not obtain mP63-specific IFN- $\gamma$ -producing cells after restimulation with MMC-treated CT26 or mHer2/CT26 cells for 3 days (data not shown). On the contrary, a remarkable number of IFN- $\gamma$ -producing cells were detected after administration of hP63 (Fig. 2C) or MMC-treated hHer2/CT26 cells (data not shown), implying that xenogenic vaccination efficiently induces xenogenic Ag-specific, IFN- $\gamma$ -secreting CD8<sup>+</sup> T cells. Interestingly, depletion of CD4<sup>+</sup> T cells at the effector phase significantly increased the number of mP63-specific IFN- $\gamma$ -secreting CD8<sup>+</sup> T cells (Fig. 2C).

Because depletion of CD4<sup>+</sup> T cells subsequently resulted in the depletion of CD4<sup>+</sup>CD25<sup>+</sup> T cells and other CD4<sup>+</sup> regulatory T cells, the role of CD4<sup>+</sup> regulatory T cells in the generation of self-antigen-reactive CTL was examined by using blocking agents such as anti-GITR agonistic Ab, anti-CD25-depleting Ab, and anti-CTLA-4-blocking Ab to suppress T cell regulatory function. After treatment with agonistic antibody against GITR during phHM and AdhHM immunization, splenocytes of immunized mice showed significantly increased mP63-specific CTL levels (Fig. 2D). Furthermore, a single administration of 500  $\mu$ g of anti-GITR Ab significantly increased the mP63-specific CTL responses induced by AdhHM immunization (Fig. 2E). However, anti-CD25 Ab or anti-CTLA-4 Ab treatment did not affect mP63-specific target lysis of immunized mice, suggesting that self-reactive CTL responses by CD4-depleting Ab might be augmented by the elimination of non-CD25<sup>+</sup> regulatory T cells rather than by CD25<sup>+</sup> conventional Treg. Alternatively, the ineffectiveness of anti-CD25 mAb treatment may be ascribed to the depletion of activated CD25<sup>+</sup> effector T cells as depicted previously (15).

Taken together, these results show that cytotoxicity elicited by xenogenic vaccination can be enhanced by modifying the suppressive immunologic environment via depletion of regulatory T cells or administration of anti-GITR agonistic Ab.

**Xenogenic immunization with AdhHM induced protective antitumor immunity in a murine tolerance model, but failed to eradicate preexisting tumor.** To check whether immune responses elicited by genetic vaccines could result in prophylactic antitumor effects in a tolerogenic tumor model, we immunized mice with either syngeneic or xenogenic vaccines. All of the mice injected with pmHM-pmHM or pmHM-AdmHM developed palpable tumors within 10 days, and no visible inhibition was observed, indicating that syngeneic immunization did not have any protective effect against self-tumor-Ag-expressing tumor (Fig. 3A).

Next, we investigated whether any protective antitumor effects against mHer2/CT26 cells could be elicited by xenogenic immunization of mice with phHM and/or AdhHM. Discordant



**Figure 3.** Induction of protective antitumor activity against murine Her-2/*neu*-expressing tumor by syngeneic or xenogenic vaccination. BALB/c mice were vaccinated with 100  $\mu$ g of plasmid DNA and  $2 \times 10^8$  pfu of adenovirus i.m. twice at 2-wk intervals. Two weeks after the last immunization, mice ( $n = 5$  per group) were challenged s.c. with  $3 \times 10^5$  of mHer2/CT26 cells, and their tumor growth was measured.

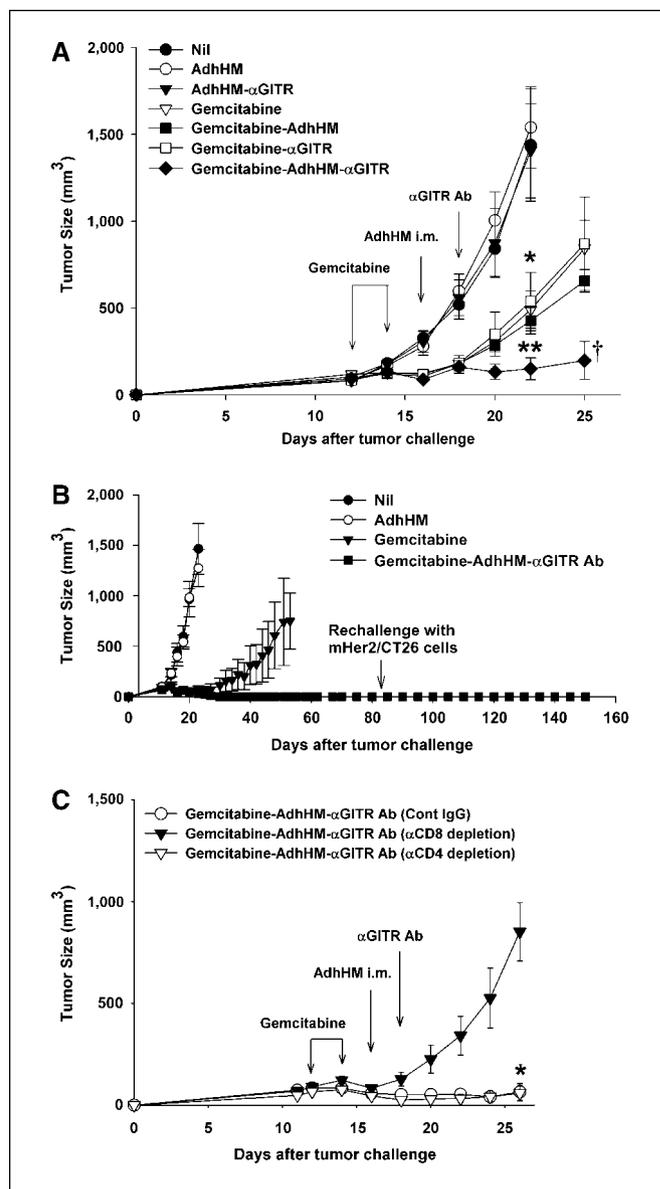
with our findings regarding CTL activity (Fig. 2A), mHer2/CT26 tumor growth was not significantly lessened by vaccination with phHM alone or by prime-boost vaccination with phHM and AdhHM, compared with controls ( $P = 0.342$  and  $P = 0.147$ , respectively, by ANOVA-Tukey).

Furthermore, adenoviral vaccination could not induce therapeutic antitumor immunity when given as a xenogenic immunization (data not shown), leaving us to look for other methods to do so.

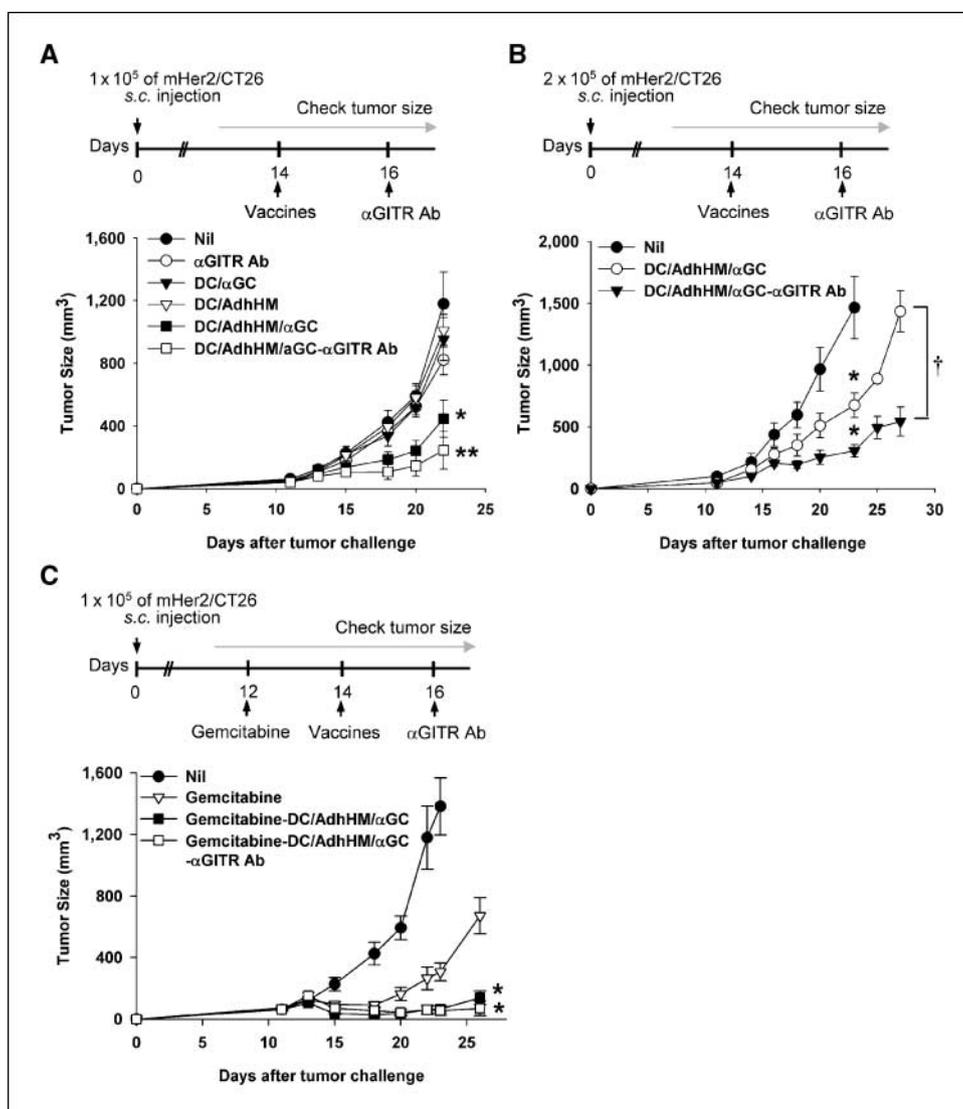
**Gemcitabine treatment together with AdhHM plus  $\alpha$ GITR Ab can efficiently break self-tolerance and induce antitumor immunity.** First, we attempted to induce therapeutic antitumor immunity by a combined regimen of AdhHM immunization followed by  $\alpha$ GITR agonistic Ab treatment. However, the enhanced CTL activity induced by AdhHM- $\alpha$ GITR Ab treatment could not eradicate pre-established tumor (data not shown). Therefore, we next set out to determine whether a combination of chemotherapy and genetic immunization could induce antitumor immunity against preexisting tumor. Because chemotherapy cannot only trigger tumor Ag cross-presentation by inducing apoptosis of tumor cells but also reduce tumor burden and attenuate the tumor-suppressive microenvironment (28), several antineoplastic agents were tested in combination with AdhHM immunization, with each chemotherapeutic agent administered at day 7 followed by AdhHM at day 9 (Supplementary Fig. S3). Of the chemotherapeutic agents tested, gemcitabine most successfully inhibited the tumor growth, and so we next tested whether combining xenogenic vaccination with gemcitabine treatment could enhance antitumor immunity against preexisting tumor.

Mice were given 60 mg/kg of gemcitabine at days 12 and 14 after mHer2/CT26 tumor inoculation when the tumor size reached 100 mm<sup>3</sup>. The gemcitabine-treated group had smaller tumors and a slower growth rate than did the control group (Fig. 4A). Unexpectedly, and inconsistent with the day 7-gemcitabine treatment model, no additional antitumor effect for AdhHM was seen in mice receiving gemcitabine treatment followed by AdhHM immunization (Supplementary Fig. S3). This discrepancy between the day 7 and day 12/14 gemcitabine treatment model may stem from the relative size of the tumor at the time of vaccination. However, mice treated with gemcitabine-AdhHM and  $\alpha$ GITR Ab at day 18 showed significantly less tumor growth than did those receiving gemcitabine alone, gemcitabine-AdhHM, or gemcitabine- $\alpha$ GITR Ab treatment ( $P < 0.05$ , ANOVA-Tukey; Fig. 4A).

To investigate whether tumor-free mice of the gemcitabine-AdhHM- $\alpha$ GITR Ab treatment group established long-lasting memory against syngeneic tumor expressing mHer2, tumor-bearing mice were treated when the tumor size reached 50 mm<sup>3</sup>. Ninety days after the first challenge, all tumor-free mice received a s.c. injection of  $5 \times 10^5$  cells of mHer2/CT26 on the side opposite that of the first injection. As of the end of the study, i.e., after at least 150 days, all of the tumor-free mice remained tumor free, indicating that chemoimmunotherapy set up memory immune responses against the challenged syngeneic tumor (Fig. 4B). Furthermore, depletion of CD8<sup>+</sup> cells in gemcitabine-AdhHM- $\alpha$ GITR Ab-treated mice abrogated the antitumor effect, showing that the therapeutic antitumor effect was dependent on CD8<sup>+</sup> CTL (Fig. 4C). Because gemcitabine treatment alone can directly kill tumor cells and induce tumor-specific CD8<sup>+</sup> T cells responses, we checked whether CD8 depletion would abrogate the efficacy of gemcitabine against established tumor. Our results suggest that the efficacy of gemcitabine is not CD8 dependent (Supplementary Fig. S4). In contrast, depletion of CD4<sup>+</sup> cells did not affect the



**Figure 4.** Combined chemoimmunotherapy with gemcitabine-AdhHM- $\alpha$ GITR Ab could break self-tolerance and significantly enhance therapeutic antitumor effects against established tumors. **A**, groups of BALB/c mice (5–6 mice per group) were s.c. inoculated with  $2 \times 10^5$  cells of mHer2/CT26. When the tumor size reached 100 mm<sup>3</sup>, mice were given the following sequential treatment: 60 mg/kg (1.2 mg per mouse) of gemcitabine on days 12 and 14, followed by AdhHM treatment on day 16. Two days later, 500  $\mu$ g of agonistic  $\alpha$ GITR Ab (DTA-1) or control IgG were administered. Data are representative of three separate experiments. \*,  $P < 0.05$ ; \*\*,  $P = 0.001$ , as compared with groups treated with Nil, AdhHM, and AdhHM- $\alpha$ GITR Ab (ANOVA-Tukey). †,  $P < 0.05$ , as compared with groups treated with gemcitabine, gemcitabine-AdhHM, and gemcitabine- $\alpha$ GITR Ab (ANOVA-Tukey). **B**, combined chemoimmunotherapy generated long-lasting memory responses. Groups of BALB/c mice (5–6 mice per group) were s.c. injected with  $2 \times 10^5$  cells of mHer2/CT26 on day 0. When the tumor size reached 50 mm<sup>3</sup>, sequential treatments were started. Ninety days after the first challenge, tumor-free mice from the gemcitabine-AdhHM- $\alpha$ GITR Ab-treated groups (5/5) were reinoculated with  $5 \times 10^5$  cells of mHer2/CT26 in a site opposite that of the first s.c. injection. Tumor recurrence was monitored by palpation for at least 150 d. **C**, CD8<sup>+</sup> T cells were essential for effective antitumor immunity. Groups of BALB/c mice (5–6 mice per group) were s.c. injected with  $2 \times 10^5$  cells of mHer2/CT26 on day 0. Mice were given an i.p. injection of 1.2 mg per mouse of gemcitabine when the tumor size reached  $\sim 100$  mm<sup>3</sup> (days 12 and 14), and AdhHM was injected at day 16. Two days later, 500  $\mu$ g of  $\alpha$ GITR Ab or control IgG were administered. CD4<sup>+</sup> and CD8<sup>+</sup> cells were depleted using 200  $\mu$ L of ascites (GK1.5 and 2.43, respectively) given on days 11, 15, 19, and 23. \*, ANOVA-Tukey,  $P < 0.001$ , as compared with the group treated with gemcitabine-AdhHM- $\alpha$ GITR Ab ( $\alpha$ CD8 depletion).



**Figure 5.** AdhHM-transduced DC vaccine could offer therapeutic antitumor immunity against mHer2/CT26 tumor with the help of NKT cells and  $\alpha$ GITR ligation, combined with gemcitabine treatment. Groups of BALB/c mice (5–6 mice per group) were vaccinated with the indicated DC vaccines on day 14. Fourteen days before treatment,  $1 \times 10^5$  cells (A and C) or  $2 \times 10^5$  cells (B) of mHer2/CT26 cells were s.c. injected into the mice. Two days later, 500  $\mu$ g of  $\alpha$ GITR Ab or control IgG were administered. Tumor mass was measured thrice a week, and the mean  $\pm$  SE was expressed. A, \*,  $P < 0.05$  and \*\*,  $P < 0.01$ , as compared with the Nil,  $\alpha$ GITR Ab, DC/ $\alpha$ GC, and DC/AdhHM groups. Nil, no treatment after tumor inoculation (ANOVA-Tukey). B, \*,  $P < 0.001$ , as compared with the Nil group (ANOVA-Tukey). †,  $P = 0.0018$ , as compared between the DC/AdhHM/ $\alpha$ GC and DC/AdhHM/ $\alpha$ GC- $\alpha$ GITR Ab groups (Student's *t* test). C, gemcitabine (60 mg/kg) was given on day 12, and 2 d later, DC vaccines were i.v. injected. On day 16,  $\alpha$ GITR Ab (500  $\mu$ g) or control IgG were i.p. injected. \*,  $P < 0.001$ , compared with the gemcitabine-treated group (ANOVA-Tukey).

antitumor effect of chemoimmunotherapy when depletion preceded gemcitabine treatment (Fig. 4C).

Collectively, these findings suggest that gemcitabine therapy followed by AdhHM- $\alpha$ GITR Ab treatment enhanced CD8<sup>+</sup> T cell-dependent antitumor effects and established long-lasting memory responses against syngeneic tumor expressing mHer2.

**$\alpha$ GalCer-loaded DCs transduced with AdhHM broke self-tolerance in a tolerogenic murine tumor model.** Previously, we showed that  $\alpha$ GalCer-loaded DCs (DC/ $\alpha$ GC), when pulsed with antigenic peptide or transduced with adenovirus expressing Her-2, can establish long-lasting antitumor immunity (20).<sup>3</sup> Here, we asked whether DC/AdhHM/ $\alpha$ GC could enhance such therapeutic antitumor activity in a tolerogenic tumor model. To test therapeutic antitumor activity in an established tumor model, groups of mice were vaccinated at day 14 after s.c. transplant, when the tumor had reached a size of 100 mm<sup>3</sup>. No inhibition of tumor growth was observed in mice receiving control Ab,  $\alpha$ GITR Ab, DC/ $\alpha$ GC, or DC/AdhHM (Fig. 5A). Although tumor growth could not be

completely inhibited by DC-mediated vaccination after  $1 \times 10^5$  or  $2 \times 10^5$  tumor challenge, it was restrained with the help of iNKT cells by  $\alpha$ GalCer loading and  $\alpha$ GITR Ab treatment (Fig. 5A and B). Furthermore, a single treatment of gemcitabine at day 12 after  $1 \times 10^5$  mHer2/CT26 challenge significantly reduced the tumor burden in both DC/AdhHM/ $\alpha$ GC and DC/AdhHM/ $\alpha$ GC- $\alpha$ GITR Ab treatment groups (Fig. 5C). However, additional  $\alpha$ GITR Ab treatment did not significantly increase the therapeutic antitumor effect elicited by an  $\alpha$ GalCer-loaded DC vaccine combined with gemcitabine treatment (Fig. 5C).

Taken together, these data show that a DC-based vaccine transduced with AdhHM could not alone break self-tolerance but required supplementary methods to enhance antitumor immunity and to weaken the tumor-suppressive environment, methods which included the use of  $\alpha$ GalCer or  $\alpha$ GITR Ab as cellular adjuvants and gemcitabine treatment.

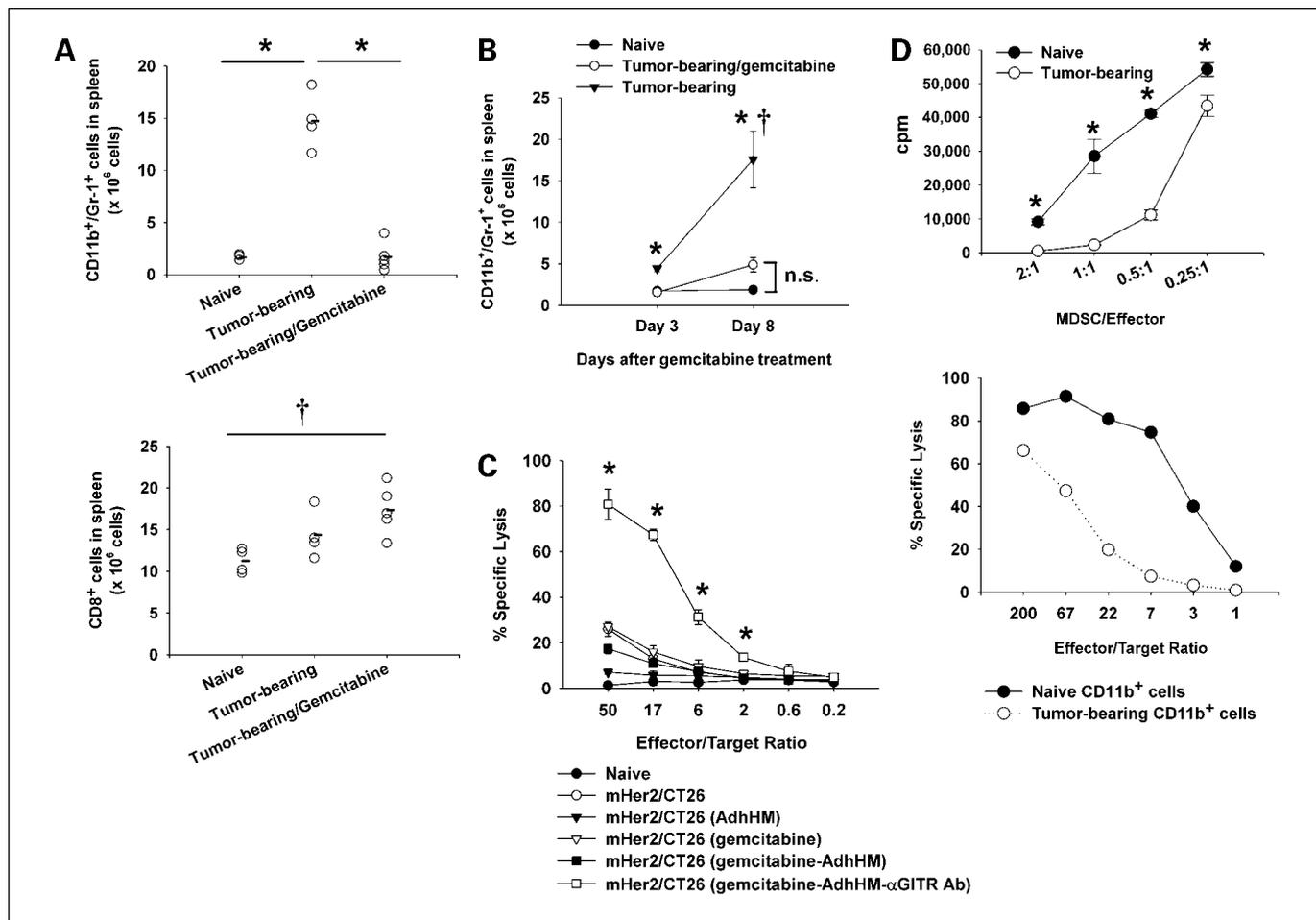
**Gemcitabine treatment significantly reduced CD11b<sup>+</sup>/Gr-1<sup>+</sup> myeloid suppressive cells in the spleens of tumor-bearing mice.** Given the efficacy of gemcitabine, when combined with other immunotherapeutic approaches, at eliciting antitumor immunity against self-tumor antigen-expressing syngeneic tumor and at inducing long-term memory, we next set out to explain its

<sup>3</sup> Y.-J. Kim, H.-J. Ko, Y.-S. Kim, D.-H. Kim, S. Kang, J.-M. Kim, Y. Chung, and C.-Y. Kang, unpublished data.

mechanism of action. Another study reported that gemcitabine selectively reduces the CD11b<sup>+</sup>/Gr-1<sup>+</sup> MDSCs, which are significantly increased in the spleens of tumor-bearing mice, thereby inhibiting CTL activity (29). Based on this finding, we began by examining the CD11b<sup>+</sup>/Gr-1<sup>+</sup> cell population in tumor-bearing mice, finding that gemcitabine treatment reduced the number of CD11b<sup>+</sup>/Gr-1<sup>+</sup> MDSCs in the spleens of tumor-bearing mice to the levels of those in naive mice (Fig. 6A). Furthermore, in our therapeutic model, the number of CD11b<sup>+</sup>/Gr-1<sup>+</sup> cells gradually increased as the tumor grew, with gemcitabine treatment significantly reducing the number and the percentage of CD11b<sup>+</sup>/Gr-1<sup>+</sup> cells in the spleen without altering the total number of CD8<sup>+</sup> T cells (Fig. 6A and B).

When an *in vitro* CTL assay was run on purified CD8<sup>+</sup> T cells from the mHer2/CT26 (nil) or mHer2/CT26 (gemcitabine-treated) groups, CD8<sup>+</sup> T cells from both groups of mice elicited only low levels of CTL activity against mHer2/CT26 cells after 6 days of restimulation with splenic DCs pulsed with MMC-treated mHer2/CT26 cells (Fig. 6C). However, gemcitabine-AdhHM- $\alpha$ GITR Ab treatment of mHer2/CT26-bearing mice greatly increased the tumor-specific cytotoxicity of purified CD8<sup>+</sup> T cells against mHer2/CT26 over levels seen in tumor-bearing mice treated with gemcitabine-AdhHM or gemcitabine alone (Fig. 6C).

To determine whether the increased numbers of MDSCs helped suppress tumor-specific T cells, we examined T cell proliferation and CTL activity using the splenocytes of cured mice that had



**Figure 6.** Gemcitabine treatment specifically eliminated tumor-induced CD11b<sup>+</sup> MDSCs. **A**, BALB/c mice were s.c. inoculated with mHer2/CT26 on day 0 and i.p. injected with 2.4 mg of gemcitabine on day 20. Two days after gemcitabine treatment, the number of CD11b<sup>+</sup>/Gr-1<sup>+</sup> cells (*top*) or CD8<sup>+</sup>/B220<sup>-</sup> cells (*bottom*) in splenocytes was assessed. \*, *P* < 0.0001, ANOVA-Tukey; †, *P* < 0.05, as compared between the naive and tumor-bearing/gemcitabine groups (ANOVA-Tukey). **B**, BALB/c mice (*n* = 3–4 per group) were s.c. inoculated with 2 × 10<sup>5</sup> cells of mHer2/CT26 and received an injection of 1.2 mg of gemcitabine 12 and 14 d after tumor challenge, when the tumor reached 100 mm<sup>3</sup>. The number of CD11b<sup>+</sup>/Gr-1<sup>+</sup> cells in splenocytes was determined by flow cytometry at days 3 and 8 after final gemcitabine treatment, and the mean ± SD was expressed. \*, *P* < 0.05, as compared with naive mice (ANOVA-Tukey); †, *P* < 0.05, as compared with tumor-bearing/gemcitabine-treated group (ANOVA-Tukey). *n.s.*, not significant (ANOVA-Tukey, *P* = 0.871). **C**, groups of BALB/c mice (2–3 mice per group) were s.c. injected with 2 × 10<sup>5</sup> cells of mHer2/CT26 on day 0. Mice were given an i.p. injection of 1.2 mg/mouse of gemcitabine once the tumor size reached ~100 mm<sup>3</sup> (days 12 and 14), and AdhHM was injected at day 16. Two days later, 500 μg of  $\alpha$ GITR Ab or control IgG was administered. On day 28, CD8<sup>+</sup> cells were isolated with antibody-coated magnetic beads from the pooled splenocytes of respective groups. Purified CD8<sup>+</sup> cells (2 × 10<sup>7</sup> cells) were restimulated *in vitro* with splenic DCs (2 × 10<sup>6</sup> cells) pulsed with MMC-treated mHer2/CT26 cells (1 × 10<sup>6</sup> cells), and cytotoxicity against mHer2/CT26 was measured using a standard Cr-51 release assay after 6 d of culture. Data are representative of two separate experiments. \*, *P* < 0.001, ANOVA-Tukey. **D**, to examine the suppressive role of CD11b<sup>+</sup> cells on proliferation (*top*), splenocytes from tumor-free mice of the gemcitabine-AdhHM- $\alpha$ GITR Ab treatment group were used as effectors and restimulated *in vitro* with MMC-mHer2/CT26 at 1:20 ratio. Splenic CD11b<sup>+</sup> cells from indicated groups were coinoculated with effectors and mHer2/CT26 for 96 h. Thymidine incorporation during the final 24 h was measured and expressed as counts per minute of mean ± SD. \*, *P* < 0.05, as compared with the naive group (Mann-Whitney *U* test). To examine the suppressive role of CD11b<sup>+</sup> cells on CTL activity (*bottom*), CD11b<sup>+</sup> cells from naive or mHer2/CT26 tumor-bearing mice were added to *in vitro* cultures of effector and stimulator cells. Splenocytes from tumor-free mice of the gemcitabine-AdhHM- $\alpha$ GITR Ab treatment group were used as effectors, and the initial number of effector cells was used for the *E/T* ratio calculation. Cytotoxicity against mHer2/CT26 was measured using a standard Cr-51 release assay after 6 d of culture. Data are representative of two separate experiments.

been challenged with mHer2/CT26 cells and treated with gemcitabine-AdhHM- $\alpha$ GITR Ab. As expected, CD11b<sup>+</sup> cells isolated from mice bearing large tumors both significantly reduced the proliferation of splenocytes under the stimulation of MMC-treated mHer2/CT26 cells and inhibited the specific killing of target cells (Fig. 6D).

These findings lead us to conclude that gemcitabine contributes to therapeutic antitumor immunity by eliminating CD11b<sup>+</sup> MDSCs, thereby attenuating the tumor-suppressive environment.

## Discussion

To destroy established tumors, with their vascularized structure extending into the stroma, tumor Ag-specific immune responses must be elicited, and tumor-mediated immune suppressive mechanisms must be inhibited (30). In attempting to establish therapeutic antitumor vaccines against preexisting tumor in the tolerogenic tumor model, we adopted several strategies to break immune tolerance and overcome the tumor-suppressive environment. First, xenogenic adenoviral vaccination induced cross-reactive immune responses and antitumor immunity in the tolerogenic model. Second, self-reactive CTL responses were significantly increased by treatment with agonistic anti-GITR antibody. Third,  $\alpha$ GalCer-loaded DCs transduced with AdhHM were able to break self-tolerance in a tolerogenic murine tumor model. Fourth, gemcitabine treatment before genetic vaccination significantly enhanced therapeutic antitumor effects by reducing the number of MDSCs.

In terms of protecting autoimmunity, negative selection in the thymus is capable of deleting almost all of self-reactive T cells (31, 32). However, because this process was not complete, several peripheral tolerance mechanisms persisted (32). Because most known tumor antigens, including Her-2/*neu*, come from self-protein, these incompletely removed self-reactive T cells must be activated before being applied to tumor immunotherapy. Recently, several reports have shown that xenogenic vaccination could break self-tolerance and inhibit the progression of established tumor in syngeneic or transgenic mouse models (5, 17, 27). In our study, xenogenic immunization with adenovirus induced cross-reactive CTL against murine Her-2/*neu*, whereas vaccination with mP63 or murine Her-2/*neu* could not induce mP63-specific CTL responses (data not shown and Fig. 2A). Those T cells that specifically recognize the mP63-MHC I complex might be high-avidity T cells. Such high-avidity T cells are eliminated in the thymus during the negative selection process due to autoreactivity or exist in the periphery in an anergic state. In contrast, low-avidity T cells induced by AdhHM seem to recognize the altered peptide sequences of mP63, allowing the T cells to escape central tolerance and to remain intact in the periphery. Recently, it was shown that peripheral CD8<sup>+</sup> T cell tolerance to self-protein was maintained by blocking expanded responses to antigen stimulation, not by defects in induction of effector functions such as cytolytic activity and effector cytokine secretion (33). Thus, we sought to test whether this autoreactive CTL could be expanded *in vivo* to induce antitumor immunity against syngeneic tumor expressing self-tumor antigen.

Although GITR was first known as a marker of regulatory T cells (34), administration of agonistic anti-GITR Abs to mice led to costimulation of previously activated T cells without any involvement of regulatory T cells (12, 35). In addition, GITR stimulation was shown to be able to break tolerance to self-antigens (35).

Likewise, in the current study, GITR stimulation provided strong costimulation to AdhHM-induced self-reactive CTL responses, but failed to induce significant therapeutic antitumor effects in the established tumor model.

Cellular vaccines using antigen-presenting cells such as DCs are known to reliably generate effective T cell immunity (30). Recently, DC genetically engineered to express Her-2/*neu* have been reported to induce stronger Her-2/*neu*-specific immune responses than did DNA vaccination (36). Furthermore, several reports have shown that mature DC can break self-tolerance against tumor-associated antigen, thereby inducing activated self-antigen-specific CTLs (30, 37). Although a peptide-loaded DC immunization can assault self-tolerance at the cellular level, i.e., by activating autoreactive CTLs, host levels of antitumor responses are governed by a diverse regulatory mechanism established between the host and the tumor environment (14, 38). Recently, we and others have developed  $\alpha$ GalCer as an adjuvant of cell-mediated vaccines (20, 39). In the current study, we have explored the possibility that NKT cells assist in tolerance breaking. In our study,  $\alpha$ GalCer-loaded DC, when transduced with AdhHM, significantly inhibited tumor growth by converting tolerogenic into immunogenic conditions. Although the exact mechanism underlying this induction of therapeutic antitumor immunity remains unclear, the cytokine milieu created by activated NKT cells or natural killer (NK) cells in addition to costimulation by mature DC certainly seems to play a role in the reversal of self- and tumor tolerance (40).

Gemcitabine has been the subject of several clinical trials. For instance, it was evaluated in breast cancer as a single agent or in combination with cisplatin or paclitaxel. A combined therapy with gemcitabine plus docetaxel with trastuzumab was shown to be efficacious as a first-line therapy for metastatic breast cancer overexpressing Her-2 (41). In addition, it has been reported that gemcitabine increased the antitumor activity of CD8<sup>+</sup> T cells and activated NK cells by selectively reducing the CD11b<sup>+</sup>/Gr-1<sup>+</sup> MDSCs, known to be significantly increased in the spleen and tumor of tumor-bearing mice; by inhibiting the function of CTL; and by promoting tumor angiogenesis (13, 29). In any case, treatment of an established tumor with gemcitabine could induce apoptosis of tumor cells and, hence, prime antitumor immunity by increasing the amount of Ag cross-presentation (28). Based on these findings, we attempted to combine gemcitabine with immunotherapy. To minimize the adverse effect of antineoplastics on the activated tumor-specific CTL, gemcitabine was administered in minimal doses and before genetic immunization. Together, gemcitabine treatment and immunotherapy significantly augmented therapeutic antitumor immunity against preexisting tumor.

MDSCs in mice are heterogeneous myeloid cells primarily comprised of CD11b<sup>+</sup>/Gr-1<sup>+</sup> cells (42). Tumor-mediated generation of MDSCs and their suppressive role against tumor-specific T cells have been well described in many tumor models (43–45). Recently, drugs including N<sup>G</sup>-monomethyl L-arginine, Nor-NOHA, NO-aspirin (45), Vitamin D3 (46), or Sildenafil (47) have been used to ameliorate the MDSC-mediated suppressive environment. We here found that gemcitabine treatment in synergy with immunotherapy delays tumor growth. Our tumor model was different from previous ones in that we adopted a syngeneic tumor cell line overexpressing putative tumor antigen on the cell surface to mimic Her-2/*neu* overexpression. Because it was not easy to develop a syngeneic tumor tolerance model using an appropriate tumor antigen, we have not yet been able to test our strategy using other tumor

antigens. However, it is conceivable that gemcitabine-attenuated MDSCs could maximize the effect of immunotherapy, at least in cases where MDSCs are increased by tumor.

The size of tumor and the timing of treatment have long been subjects of controversy in the development of antitumor immunotherapy. Recently, several strategies have been proposed for eradicating established tumors by targeting the tumor stroma together with the tumor (48, 49). Using our own strategies in our tolerogenic tumor model, we treated mice bearing tumors of 100 mm<sup>3</sup> (>7 mm in average diameter), obtaining significant tumor regression. For the complete cure of preexisting tumors, we have to treat tumor-bearing mice while the tumor is still quite small, yet easily palpable (50 mm<sup>3</sup> or ~5 mm in average diameter). When large tumors (400 mm<sup>3</sup> or >12 mm in average diameter) were treated, none of the strategies we tested resulted in significant tumor regression. Thus, although gemcitabine treatment can attenuate the MDSC-mediated tumor-suppressive environment, it should be accompanied by other methods such as surgery or irradiation when treating large tumors.

In summary, to determine which vaccine strategies are capable of reversing T cell tolerance, we set up a syngeneic tolerance

model in mice using mouse Her-2/*neu* as self-antigen and established and used a combination of genetic immunizations followed by  $\alpha$ GITR Ab treatment and chemotherapy with gemcitabine to efficiently break self-tolerance and induce long-lasting antitumor immunity. Furthermore, the synergistic effect of this combined chemoimmunotherapy using gemcitabine and subsequent immunotherapy seems to be dependent primarily on CD8<sup>+</sup> T cells. Gemcitabine succeeded in breaking self-tolerance by eliminating MDSCs and thereby attenuating the tumor-suppressive environment. Taken together, these data suggest that a two-pronged approach, the generation of effector cells against self-tumor Ag and the attenuation of the tumor-suppressive environment, is required for the induction of successful antitumor immunity.

## Acknowledgments

Received 12/21/2006; revised 3/21/2007; accepted 5/16/2007.

**Grant support:** National R&D Program for Cancer Control, Ministry of Health & Welfare, Republic of Korea (0420090-3, to C-Y. Kang).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

## References

- Gullick WJ, Love SB, Wright C, et al. c-erbB-2 protein overexpression in breast cancer is a risk factor in patients with involved and uninvolved lymph nodes. *Br J Cancer* 1991;63:434-8.
- Disis ML, Calenoff E, McLaughlin G, et al. Existent T-cell and antibody immunity to HER-2/*neu* protein in patients with breast cancer. *Cancer Res* 1994;54:16-20.
- Fisk B, Blevins TL, Wharton JT, Ioannides CG. Identification of an immunodominant peptide of HER-2/*neu* protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. *J Exp Med* 1995;181:2109-17.
- Peoples GE, Goedegebuure PS, Smith R, Linehan DC, Yoshino I, Eberlein TJ. Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/*neu*-derived peptide. *Proc Natl Acad Sci U S A* 1995;92:432-6.
- Lin CC, Chou CW, Shiau AL, et al. Therapeutic HER2/*Neu* DNA vaccine inhibits mouse tumor naturally overexpressing endogenous *neu*. *Mol Ther* 2004;10:290-301.
- Pupa SM, Invernizzi AM, Forti S, et al. Prevention of spontaneous *neu*-expressing mammary tumor development in mice transgenic for rat proto-*neu* by DNA vaccination. *Gene Ther* 2001;8:75-9.
- Lu Y, Wei YQ, Tian L, et al. Immunogene therapy of tumors with vaccine based on xenogeneic epidermal growth factor receptor. *J Immunol* 2003;170:3162-70.
- Yu P, Lee Y, Liu W, et al. Intratumor depletion of CD4<sup>+</sup> cells unmasks tumor immunogenicity leading to the rejection of late-stage tumors. *J Exp Med* 2005;201:779-91.
- Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004;10:942-9.
- Muller AJ, Scherle PA. Targeting the mechanisms of tumoral immune tolerance with small-molecule inhibitors. *Nat Rev Cancer* 2006;6:613-25.
- Zou W. Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol* 2006;6:295-307.
- Stephens GL, McHugh RS, Whitters MJ, et al. Engagement of glucocorticoid-induced TNFR family-related receptor on effector T cells by its ligand mediates resistance to suppression by CD4<sup>+</sup>CD25<sup>+</sup> T cells. *J Immunol* 2004;173:5008-20.
- Gallina G, Dolcetti L, Serafini P, et al. Tumors induce a subset of inflammatory monocytes with immunosuppressive activity on CD8<sup>+</sup> T cells. *J Clin Invest* 2006;116:2777-90.
- Huang B, Pan PY, Li Q, et al. Gr-1<sup>+</sup>CD115<sup>+</sup> immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. *Cancer Res* 2006;66:1123-31.
- Ko K, Yamazaki S, Nakamura K, et al. Treatment of advanced tumors with agonistic anti-GITR mAb and its effects on tumor-infiltrating Foxp3<sup>+</sup>CD25<sup>+</sup>CD4<sup>+</sup> regulatory T cells. *J Exp Med* 2005;202:885-91.
- Rovero S, Amici A, Carlo ED, et al. DNA vaccination against rat her-2/*Neu* p185 more effectively inhibits carcinogenesis than transplantable carcinomas in transgenic BALB/c mice. *J Immunol* 2000;165:5133-42.
- Pupa SM, Iezzi M, Di Carlo E, et al. Inhibition of mammary carcinoma development in HER-2/*neu* transgenic mice through induction of autoimmunity by xenogeneic DNA vaccination. *Cancer Res* 2005;65:1071-8.
- Ercolini AM, Machiels JP, Chen YC, et al. Identification and characterization of the immunodominant rat HER-2/*neu* MHC class I epitope presented by spontaneous mammary tumors from HER-2/*neu*-transgenic mice. *J Immunol* 2003;170:4273-80.
- Reilly RT, Gottlieb MB, Ercolini AM, et al. HER-2/*neu* is a tumor rejection target in tolerized HER-2/*neu* transgenic mice. *Cancer Res* 2000;60:3569-76.
- Chung Y, Kim BS, Kim YJ, et al. CD1d-restricted T cells license B cells to generate long-lasting cytotoxic antitumor immunity *in vivo*. *Cancer Res* 2006;66:6843-50.
- Chang SY, Lee KC, Ko SY, Ko HJ, Kang CY. Enhanced efficacy of DNA vaccination against Her-2/*neu* tumor antigen by genetic adjuvants. *Int J Cancer* 2004;111:86-95.
- Chung Y, Chang JH, Kwon MN, Rennert PD, Kang CY. CD8-11b<sup>+</sup> dendritic cells but not CD8 $\alpha$ <sup>+</sup> dendritic cells mediate cross-tolerance toward intestinal antigens. *Blood* 2005;106:201-6.
- Wu X, Hou W, Sun S, et al. Novel function of IFN- $\gamma$ : negative regulation of dendritic cell migration and T cell priming. *J Immunol* 2006;177:934-43.
- Ko HJ, Ko SY, Kim YJ, Lee EG, Cho SN, Kang CY. Optimization of codon usage enhances the immunogenicity of a DNA vaccine encoding mycobacterial antigen Ag85B. *Infect Immun* 2005;73:5666-74.
- Kim KM, Shin EY, Moon JH, et al. Both the epitope specificity and isotype are important in the antitumor effect of monoclonal antibodies against Her-2/*neu* antigen. *Int J Cancer* 2002;102:428-34.
- Nagata Y, Furugen R, Hiasa A, et al. Peptides derived from a wild-type murine proto-oncogene c-erbB-2/*HER2/neu* can induce CTL and tumor suppression in syngeneic hosts. *J Immunol* 1997;159:1336-43.
- Gallo P, Dharmapuri S, Nuzzo M, et al. Xenogeneic immunization in mice using HER2 DNA delivered by an adenoviral vector. *Int J Cancer* 2005;113:67-77.
- Nowak AK, Lake RA, Marzo AL, et al. Induction of tumor cell apoptosis *in vivo* increases tumor antigen cross-presentation, cross-priming rather than cross-tolerizing host tumor-specific CD8 T cells. *J Immunol* 2003;170:4905-13.
- Suzuki E, Kapoor V, Jassar AS, Kaiser LR, Albelda SM. Gemcitabine selectively eliminates splenic Gr-1<sup>+</sup>/CD11b<sup>+</sup> myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity. *Clin Cancer Res* 2005;11:6713-21.
- Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. *Nat Med* 2004;10:909-15.
- Palmer E. Negative selection-clearing out the bad apples from the T-cell repertoire. *Nat Rev Immunol* 2003;3:383-91.
- Li L, Boussiotis VA. Physiologic regulation of central and peripheral T cell tolerance: lessons for therapeutic applications. *J Mol Med* 2006;84:887-99.
- Ohlen C, Kalos M, Cheng LE, et al. CD8(+) T cell tolerance to a tumor-associated antigen is maintained at the level of expansion rather than effector function. *J Exp Med* 2002;195:1407-18.
- Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol* 2002;3:135-42.
- Ramirez-Montagut T, Chow A, Hirschhorn-Cymerman D, et al. Glucocorticoid-induced TNF receptor family related gene activation overcomes tolerance/ignorance to melanoma differentiation antigens and enhances antitumor immunity. *J Immunol* 2006;176:6434-42.
- Chan T, Sami A, El-Gayed A, Guo X, Xiang J. HER-2/*neu*-gene engineered dendritic cell vaccine stimulates stronger HER-2/*neu*-specific immune responses compared to DNA vaccination. *Gene Ther* 2006;13:1391-402.

37. Evel-Kabler K, Song XT, Aldrich M, Huang XF, Chen SY. SOCS1 restricts dendritic cells' ability to break self tolerance and induce antitumor immunity by regulating IL-12 production and signaling. *J Clin Invest* 2006;116:90-100.
38. Sakaguchi S. Naturally arising CD4<sup>+</sup> regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 2004;22:531-62.
39. Fujii S, Liu K, Smith C, Bonito AJ, Steinman RM. The linkage of innate to adaptive immunity via maturing dendritic cells *in vivo* requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *J Exp Med* 2004;199:1607-18.
40. Chung Y, Chang WS, Kim S, Kang CY. NKT cell ligand  $\alpha$ -galactosylceramide blocks the induction of oral tolerance by triggering dendritic cell maturation. *Eur J Immunol* 2004;34:2471-9.
41. Wirk B, Perez E. Role of gemcitabine in breast cancer management: an update. *Semin Oncol* 2006;33:S6-14.
42. Rabinovich GA, Gabrilovich D, Sotomayor EM. Immunosuppressive strategies that are mediated by tumor cells. *Annu Rev Immunol* 2007;25:267-96.
43. Gabrilovich DI, Velders MP, Sotomayor EM, Kast WM. Mechanism of immune dysfunction in cancer mediated by immature Gr-1<sup>+</sup> myeloid cells. *J Immunol* 2001;166:5398-406.
44. Melani C, Chiodoni C, Forni G, Colombo MP. Myeloid cell expansion elicited by the progression of spontaneous mammary carcinomas in c-erbB-2 transgenic BALB/c mice suppresses immune reactivity. *Blood* 2003;102:2138-45.
45. Serafini P, Borrello I, Bronte V. Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression. *Semin Cancer Biol* 2006;16:53-65.
46. Kusmartsev S, Cheng F, Yu B, et al. All-*trans*-retinoic acid eliminates immature myeloid cells from tumor-bearing mice and improves the effect of vaccination. *Cancer Res* 2003;63:4441-9.
47. Serafini P, Meckel K, Kelso M, et al. Phosphodiesterase-5 inhibition augments endogenous antitumor immunity by reducing myeloid-derived suppressor cell function. *J Exp Med* 2006;203:2691-702.
48. Yu P, Rowley DA, Fu YX, Schreiber H. The role of stroma in immune recognition and destruction of well-established solid tumors. *Curr Opin Immunol* 2006;18:226-31.
49. Zhang B, Bowerman NA, Salama JK, et al. Induced sensitization of tumor stroma leads to eradication of established cancer by T cells. *J Exp Med* 2007;204:49-55.

# Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

## A Combination of Chemoimmunotherapies Can Efficiently Break Self-Tolerance and Induce Antitumor Immunity in a Tolerogenic Murine Tumor Model

Hyun-Jeong Ko, Yeon-Jeong Kim, Yun-Sun Kim, et al.

*Cancer Res* 2007;67:7477-7486.

<b>Updated version</b>	Access the most recent version of this article at: <a href="http://cancerres.aacrjournals.org/content/67/15/7477">http://cancerres.aacrjournals.org/content/67/15/7477</a>
<b>Supplementary Material</b>	Access the most recent supplemental material at: <a href="http://cancerres.aacrjournals.org/content/suppl/2007/07/30/67.15.7477.DC1">http://cancerres.aacrjournals.org/content/suppl/2007/07/30/67.15.7477.DC1</a>

<b>Cited articles</b>	This article cites 49 articles, 27 of which you can access for free at: <a href="http://cancerres.aacrjournals.org/content/67/15/7477.full#ref-list-1">http://cancerres.aacrjournals.org/content/67/15/7477.full#ref-list-1</a>
<b>Citing articles</b>	This article has been cited by 24 HighWire-hosted articles. Access the articles at: <a href="http://cancerres.aacrjournals.org/content/67/15/7477.full#related-urls">http://cancerres.aacrjournals.org/content/67/15/7477.full#related-urls</a>

<b>E-mail alerts</b>	<a href="#">Sign up to receive free email-alerts</a> related to this article or journal.
<b>Reprints and Subscriptions</b>	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at <a href="mailto:pubs@aacr.org">pubs@aacr.org</a> .
<b>Permissions</b>	To request permission to re-use all or part of this article, use this link <a href="http://cancerres.aacrjournals.org/content/67/15/7477">http://cancerres.aacrjournals.org/content/67/15/7477</a> . Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.