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

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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 α-galactosylceramide (αGalCer)–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 αGalCer-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 $CD11b^+/Gr\text{-}1^+$ 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 $CD8^+$ 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-β, vascular endothelial growth factor, and interleukin-10 (10, 11). Naturally occurring or tumor-driven regulatory $CD4^+CD25^+FoxP3^+$ 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 α-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/).

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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 αGITR Ab treatment, mice were i.p. injected with 500 μg 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-γ, CD11b, Gr-1, and F4/80. Goat anti-mouse IgG-Fc Ab was purchased form Pierce. For the isolation of $CD11b^+$ 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 Viromed. 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 3×10^5 of mHer2/CT26 cells in the right flank for tumorigenic growth of tumor cells.

To test the therapeutic vaccine effect, 1×10^5 or 2×10^5 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^3. 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 μg/mL of α-galactosylceramide (αGalCer) for additional 18 to 22 h.

Functional studies of $CD11b^+$ MDSCs. To examine the suppressive role of $CD11b^+$ cells on CTL activity and proliferation, we prepared splenocytes from cured mice that had undergone gemcitabine-AdhHM-αGITR Ab treatment. These effectors were restimulated *in vitro* with mitomycin C (MMC; Sigma)–treated mHer2/CT26 cells and then incubated together with $CD11b^+$ 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 $CD11b^+$ MDSCs, effectors were plated at 5×10^5 spleen cells per well in 96-well round-bottom microtiter plates (Nunc) and cultured for 4 days with 2.5×10^4 of MMC-treated mHer2/CT26 and diluted $CD11b^+$ cells. After 96 h of incubation, including a final 24-h pulse with $[^3H]$-thymidine (1 μCi per well), the $[^3H]$-thymidine incorporation was measured as described previously (24).

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

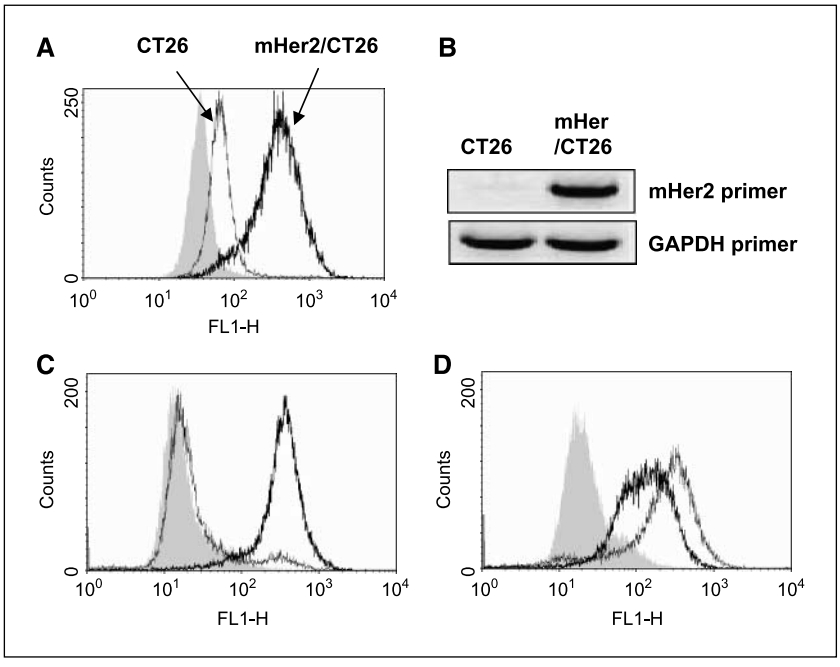

Figure 1. Construction of a murine cell line expressing the full length of murine Her-2/*neu* on the cell surface. *A,* the FACS binding assay of rat anti-mHer2–specific polyclonal serum to mHer2/CT26 cells. Rat anti-mHer2–specific polyclonal serum was obtained from a rat that had been immunized thrice 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.

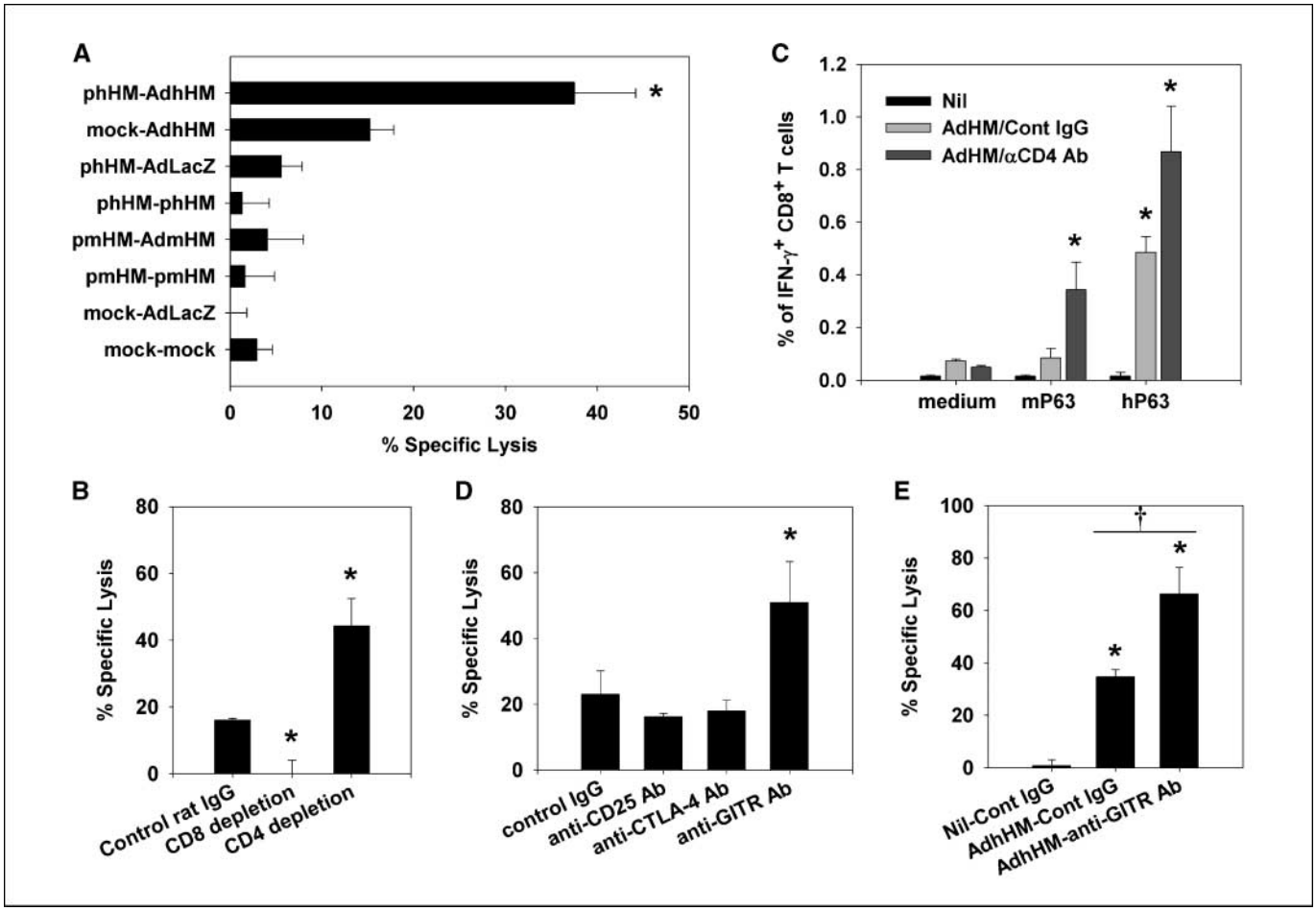

Figure 2. Tolerance against self-tumor antigen can be broken by a xenogenic immunization with DNA vaccine and with an adenovirus vector expressing human Her-2/*neu*. *A,* BALB/c mice (n = 3–5/group) were injected i.m. with 100 μg of each DNA and boosted 2 wks later with 2×10^8 plaque-forming units (pfu) 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 μg 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-γ–secreting $CD8^+$ T cells was determined after *in vitro* stimulation with 10 μg/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 μg 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 μg 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 μg 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-αGITR 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-αGITR Ab (ANOVA-Tukey).

Tukey post hoc test using SPSS. When appropriate, the Student's t test was used, and the means ± 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. 1*A*), and using reverse transcription-PCR (RT-PCR), we confirmed the presence of mHer2 mRNA transcripts in mHer2-transfectant cells (Fig. 1*B*).

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. 1*C* 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^d-restricted $CD8^+$ 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 β-galactosidase (AdLacZ) could not generate significant specific lysis (Fig. 2*A*). Furthermore, AdhHM immunization alone could lyse the target cells significantly (Fig. 2*E*). None of the immunizations with genetic vaccines expressing murine Her-2/*neu* or control vectors produced self-reactive CTL (Fig. 2*A*). 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 $CD8^+$ CTL by AdhHM immunization. We next used anti-CD4 or anti-CD8 mAb to deplete $CD4^+$ T or $CD8^+$ 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. 2*B*). Surprisingly, the depletion of $CD4^+$ T cells instead enhanced the CTL responses of AdhHM-immunized mice (Fig. 2*B*), indicating that $CD4^+$ 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-γ production of $CD8^+$ T cells. Although AdhHM induced mP63-specific lysis, it provoked only a minimal number of IFN-γ–secreting $CD8^+$ T cells (Fig. 2*C*). Furthermore, we could not obtain mP63-specific IFN-γ–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-γ–producing cells were detected after administration of hP63 (Fig. 2*C*) or MMC-treated hHer2/CT26 cells (data not shown), implying that xenogenic vaccination efficiently induces xenogenic Ag-specific, IFN-γ–secreting $CD8^+$ T cells. Interestingly, depletion of $CD4^+$ T cells at the effector phase significantly increased the number of mP63-specific IFN-γ–secreting $CD8^+$ T cells (Fig. 2*C*).

Because depletion of $CD4^+$ T cells subsequently resulted in the depletion of $CD4^+CD25^+$ T cells and other $CD4^+$ regulatory T cells, the role of $CD4^+$ 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. 2*D*). Furthermore, a single administration of 500 μg of anti-GITR Ab significantly increased the mP63-specific CTL responses induced by AdhHM immunization (Fig. 2*E*). 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-$CD25^+$ regulatory T cells rather than by $CD25^+$ conventional Treg. Alternatively, the ineffectiveness of anti-CD25 mAb treatment may be ascribed to the depletion of activated $CD25^+$ 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. 3*A*).

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

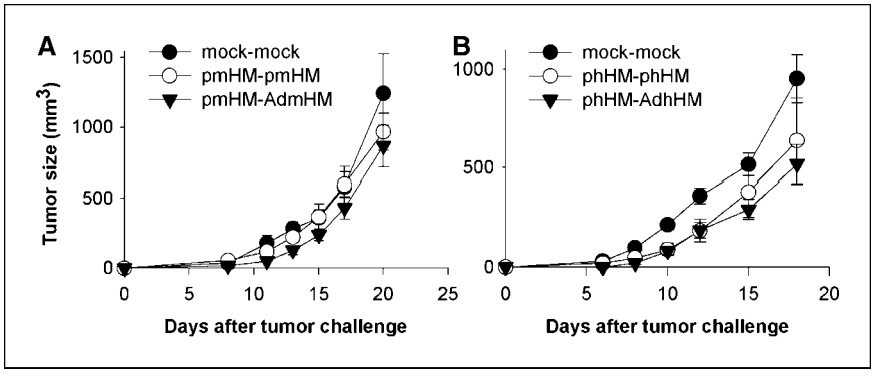

Figure 3. Induction of protective antitumor activity against murine Her-2/*neu*–expressing tumor by syngeneic or xenogenic vaccination. BALB/c mice were vaccinated with 100 μg of plasmid DNA and 2×10^8 pfu of adenovirus i.m. twice at 2-wk intervals. Two weeks after the last immunization, mice ($n = 5$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

with our findings regarding CTL activity (Fig. 2*A*), 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 αGITR 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 αGITR agonistic Ab treatment. However, the enhanced CTL activity induced by AdhHM-αGITR 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^3. The gemcitabine-treated group had smaller tumors and a slower growth rate than did the control group (Fig. 4*A*). 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 αGITR Ab at day 18 showed significantly less tumor growth than did those receiving gemcitabine alone, gemcitabine-AdhHM, or gemcitabine-αGITR Ab treatment (P < 0.05, ANOVA-Tukey; Fig. 4*A*).

To investigate whether tumor-free mice of the gemcitabine–AdhHM-αGITR Ab treatment group established long-lasting memory against syngeneic tumor expressing mHer2, tumor-bearing mice were treated when the tumor size reached 50 mm^3. Ninety days after the first challenge, all tumor-free mice received a s.c. injection of 5×10^5 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. 4*B*). Furthermore, depletion of $CD8^+$ cells in gemcitabine-AdhHM-αGITR Ab-treated mice abrogated the antitumor effect, showing that the therapeutic antitumor effect was dependent on $CD8^+$ CTL (Fig. 4*C*). Because gemcitabine treatment alone can directly kill tumor cells and induce tumor-specific $CD8^+$ 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 $CD4^+$ cells did not affect the

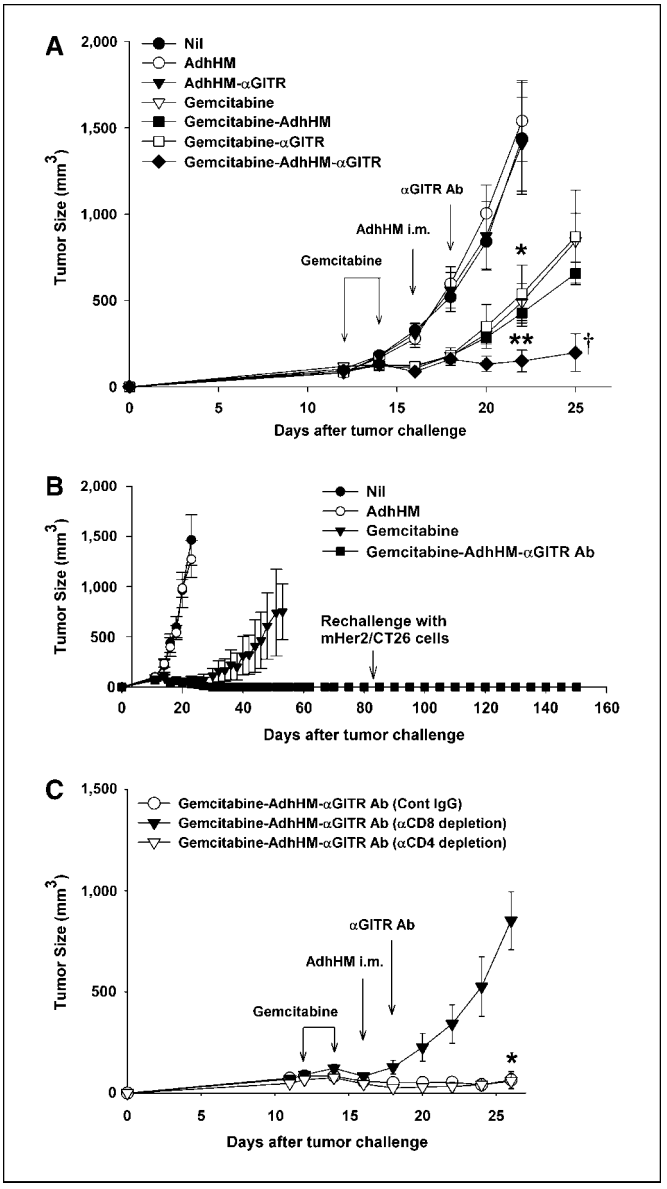

Figure 4. Combined chemoimmunotherapy with gemcitabine-AdhHM-αGITR Ab could break self-tolerance and significantly enhance therapeutic antitumor effects against established tumors. *A,* groups of BALB/c mice (5–6 mice per group) were s.c. inoculated with 2×10^5 cells of mHer2/CT26. When the tumor size reached 100 mm^3, 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 μg of agonistic αGITR 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-αGITR Ab (ANOVA-Tukey). †, P < 0.05, as compared with groups treated with gemcitabine, gemcitabine-AdhHM, and gemcitabine-αGITR 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 2×10^5 cells of mHer2/CT26 on day 0. When the tumor size reached 50 mm^3, sequential treatments were started. Ninety days after the first challenge, tumor-free mice from the gemcitabine-AdhHM-αGITR Ab-treated groups (5/5) were reinoculated with 5×10^5 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,* $CD8^+$ T cells were essential for effective antitumor immunity. Groups of BALB/c mice (5–6 mice per group) were s.c. injected with 2×10^5 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 ~100 mm^3 (days 12 and 14), and AdhHM was injected at day 16. Two days later, 500 μg of αGITR Ab or control IgG were administered. $CD4^+$ and $CD8^+$ cells were depleted using 200 μL 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-αGITR Ab (αCD8 depletion).

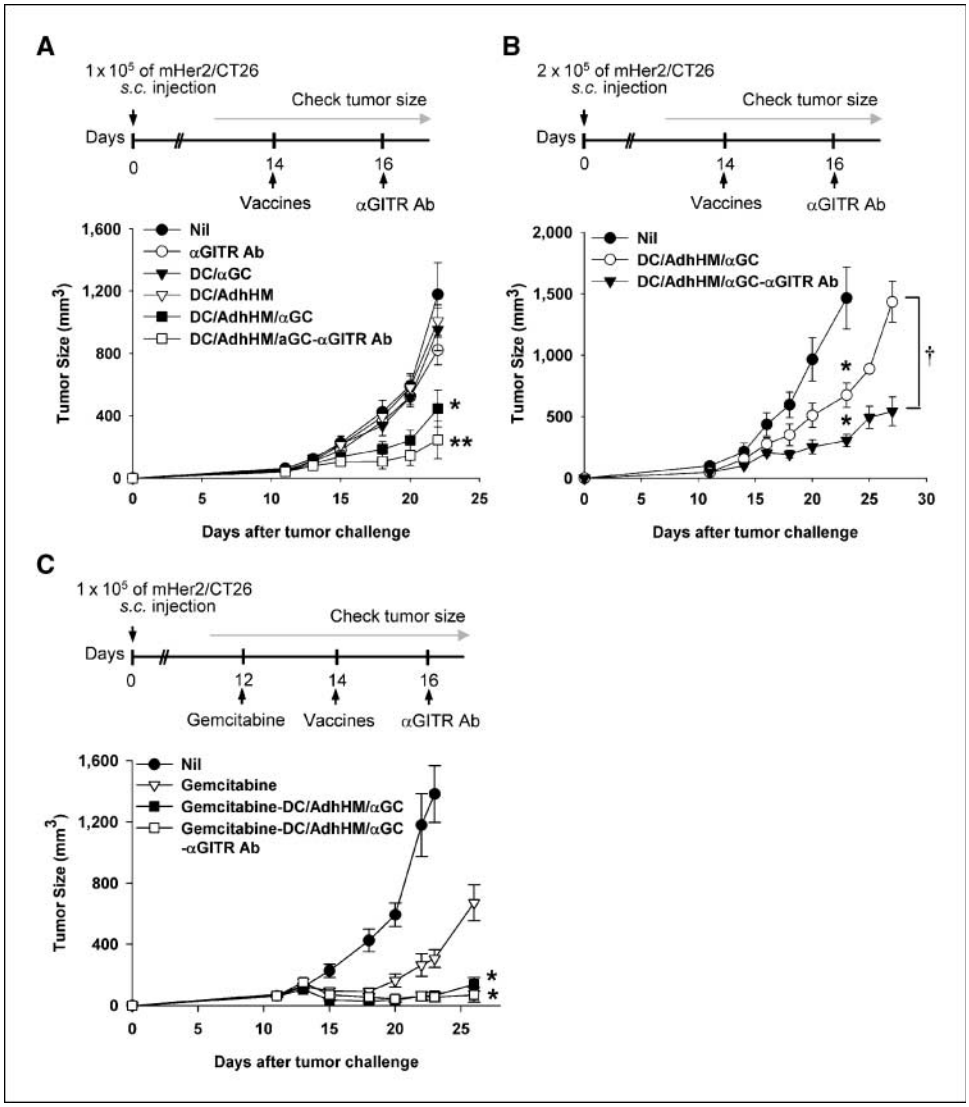

Figure 5. AdhHM-transduced DC vaccine could offer therapeutic antitumor immunity against mHer2/CT26 tumor with the help of NKT cells and αGITR ligation, combined with gemcitabine treatment. Groups of BALB/c mice (5–6 mice per group) were vaccinated with the indicated DC vaccines on day 14. Fourteen days before treatment, 1×10^5 cells (*A* and *C*) or 2×10^5 cells (*B*) of mHer2/CT26 cells were s.c. injected into the mice. Two days later, 500 μg of αGITR Ab or control IgG were administered. Tumor mass was measured thrice a week, and the mean ± SE was expressed. *A*, *, $P < 0.05$ and **, $P < 0.01$, as compared with the Nil, αGITR Ab, DC/αGC, 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/αGC and DC/AdhHM/αGC-αGITR 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, αGITR Ab (500 μg) 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. 4*C*).

Collectively, these findings suggest that gemcitabine therapy followed by AdhHM-αGITR Ab treatment enhanced $CD8^+$ T cell–dependent antitumor effects and established long-lasting memory responses against syngeneic tumor expressing mHer2.

αGalCer-loaded DCs transduced with AdhHM broke self-tolerance in a tolerogenic murine tumor model. Previously, we showed that αGalCer-loaded DCs (DC/αGC), when pulsed with antigenic peptide or transduced with adenovirus expressing Her-2, can establish long-lasting antitumor immunity (20).[3] Here, we asked whether DC/AdhHM/αGC 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^3. No inhibition of tumor growth was observed in mice receiving control Ab, αGITR Ab, DC/αGC, or DC/AdhHM (Fig. 5*A*). Although tumor growth could not be completely inhibited by DC-mediated vaccination after 1×10^5 or 2×10^5 tumor challenge, it was restrained with the help of iNKT cells by αGalCer loading and αGITR Ab treatment (Fig. 5*A* and *B*). Furthermore, a single treatment of gemcitabine at day 12 after 1×10^5 mHer2/CT26 challenge significantly reduced the tumor burden in both DC/AdhHM/αGC and DC/AdhHM/αGC-αGITR Ab treatment groups (Fig. 5*C*). However, additional αGITR Ab treatment did not significantly increase the therapeutic antitumor effect elicited by an αGalCer-loaded DC vaccine combined with gemcitabine treatment (Fig. 5*C*).

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 αGalCer or αGITR Ab as cellular adjuvants and gemcitabine treatment.

Gemcitabine treatment significantly reduced $CD11b^+/Gr\text{-}1^+$ 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

[3] 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 $CD11b^+/Gr\text{-}1^+$ 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 $CD11b^+/Gr\text{-}1^+$ cell population in tumor-bearing mice, finding that gemcitabine treatment reduced the number of $CD11b^+/Gr\text{-}1^+$ MDSCs in the spleens of tumor-bearing mice to the levels of those in naive mice (Fig. 6*A*). Furthermore, in our therapeutic model, the number of $CD11b^+/Gr\text{-}1^+$ cells gradually increased as the tumor grew, with gemcitabine treatment significantly reducing the number and the percentage of $CD11b^+/Gr\text{-}1^+$ cells in the spleen without altering the total number of $CD8^+$ T cells (Fig. 6*A* and *B*).

When an *in vitro* CTL assay was run on purified $CD8^+$ T cells from the mHer2/CT26 (nil) or mHer2/CT26 (gemcitabine-treated) groups, $CD8^+$ 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. 6*C*). However, gemcitabine-AdhHM-αGITR Ab treatment of mHer2/CT26-bearing mice greatly increased the tumor-specific cytotoxicity of purified $CD8^+$ T cells against mHer2/CT26 over levels seen in tumor-bearing mice treated with gemcitabine-AdhHM or gemcitabine alone (Fig. 6*C*).

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

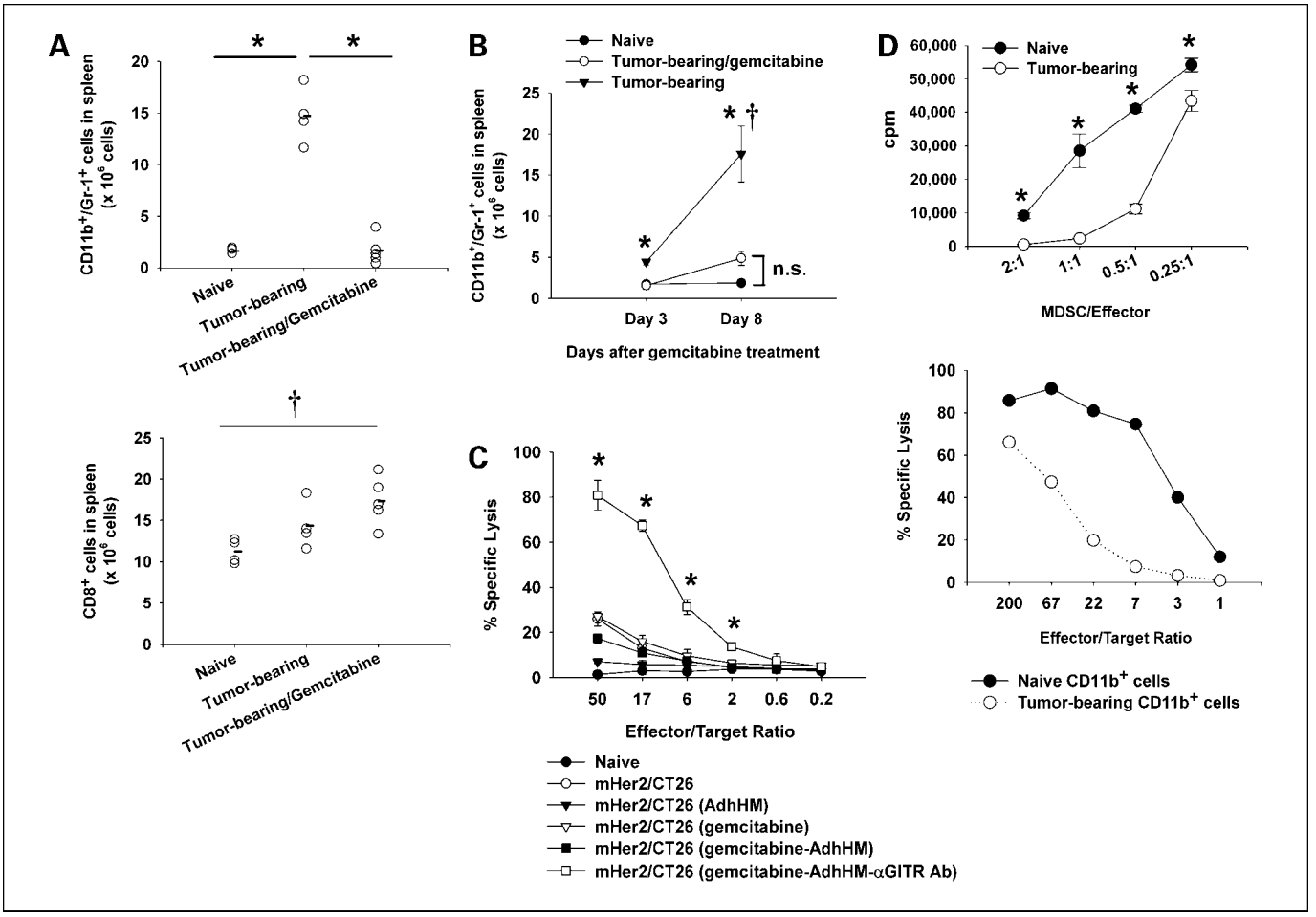

Figure 6. Gemcitabine treatment specifically eliminated tumor-induced $CD11b^+$ MDSCs. *A,* BALB/c mice were s.c. inoculated with mHer2/CT26 on day 0 and i.p. injected with 2.4 mg of gemcitabine on day 20. Two days after gemcitabine treatment, the number of $CD11b^+/Gr\text{-}1^+$ cells (*top*) or $CD8^+/B220^-$ 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 2×10^5 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^3. The number of $CD11b^+/Gr\text{-}1^+$ 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 2×10^5 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^3 (days 12 and 14), and AdhHM was injected at day 16. Two days later, 500 μg of αGITR Ab or control IgG was administered. On day 28, $CD8^+$ cells were isolated with antibody-coated magnetic beads from the pooled splenocytes of respective groups. Purified $CD8^+$ cells (2×10^7 cells) were restimulated *in vitro* with splenic DCs (2×10^6 cells) pulsed with MMC-treated mHer2/CT26 cells (1×10^6 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 $CD11b^+$ cells on proliferation (*top*), splenocytes from tumor-free mice of the gemcitabine-AdhHM-αGITR Ab treatment group were used as effectors and restimulated *in vitro* with MMC-mHer2/CT26 at 1:20 ratio. Splenic $CD11b^+$ cells from indicated groups were coincubated 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 $CD11b^+$ cells on CTL activity (*bottom*), $CD11b^+$ 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-αGITR 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-αGITR Ab. As expected, $CD11b^+$ 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. 6*D*).

These findings lead us to conclude that gemcitabine contributes to therapeutic antitumor immunity by eliminating $CD11b^+$ 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, αGalCer-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. 2*A*). 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 $CD8^+$ 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 αGalCer 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, αGalCer-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 $CD8^+$ T cells and activated NK cells by selectively reducing the $CD11b^+/Gr\text{-}1^+$ 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 $CD11b^+/Gr\text{-}1^+$ 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^G-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^3 (>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^3 or $\sim$5 mm in average diameter). When large tumors (400 mm^3 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 αGITR 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 $CD8^+$ 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.

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