Adjuvant effect of HER-2/neu-specific adenoviral vector stimulating CD8\(^+\) T and natural killer cell responses on anti-HER-2/neu antibody therapy for well-established breast tumors in HER-2/neu transgenic mice

Y Chen\(^1,4\), Y Xie\(^1,4\), T Chan\(^1\), A Sami\(^2\), S Ahmed\(^2\), Q Liu\(^3\) and J Xiang\(^1,2\)

\(^1\)Cancer Research Unit, Research Division, Saskatchewan Cancer Agency, Saskatoon, Saskatchewan, Canada; \(^2\)Department of Oncology, Saskatoon Cancer Center, University of Saskatchewan, Saskatoon, Saskatchewan, Canada and \(^3\)Vaccine and Infectious Disease Organization, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Approximately one third of patients with advanced human epidermal growth factor receptor 2 (HER-2/neu-positive breast cancer respond to trastuzumab monotherapy, a humanized anti-HER-2/neu antibody. However, de novo and acquired antibody resistance is one of the major limitations of trastuzumab therapy warranting the search for other therapeutic strategies. One of the most remarkable features of adenovirus (AdV)-based vaccine is its ability to induce exceptionally high and sustained frequencies of transgene product-specific CD8\(^+\) T-cell responses. In this study, we constructed two recombinant AdVs (AdV\(_{OVA}\) and AdV\(_{HER-2}\)) expressing ovalbumin (OVA) and HER-2/neu, and assessed AdV-induced antigen-specific cellular immune responses and preventive/therapeutic antitumor immunity. We demonstrate that AdV\(_{OVA}\) stimulates efficient OVA-specific CD8\(^+\) cytotoxic T lymphocyte (CTL) and natural killer responses, leading to preventive long-term immunity against OVA-expressing BL6-10ova melanoma in wild-type C56BL/6 mice. We further demonstrate that AdV\(_{HER-2}\) stimulates HER-2/neu-specific CD8\(^+\) CTL responses, leading to a significant reduction in breast carcinogenesis in transgenic FVBneuN mice (\(P<0.05\)), but has little therapeutic effect on pre-existing Tg1-1 tumor even at early stage (15 mm\(^3\)). In contrast, the anti-HER-2/neu antibody therapy is capable of completely inhibiting Tg1-1 tumor growth at early stage, but fails to eradicate well-established Tg1-1 breast tumor (100 mm\(^3\)). Interestingly, a combinational immunotherapy of anti-HER-2/neu antibody with AdV\(_{HER-2}\) vaccine was capable of curing 4 of 10 studied mice bearing well-established Tg1-1 breast tumors and significantly delaying in death of the remaining six tumor-bearing mice (\(P<0.05\)). Taken together, our results suggest an adjuvant effect of AdV\(_{HER-2}\) on anti-HER-2/neu antibody therapy for well-established breast tumor in transgenic FVBneuN mice, and this combinational immunotherapy of trastuzumab with AdV\(_{HER-2}\) vaccine may be used as a new therapeutic strategy for treatment of advanced HER-2/neu-positive breast cancer.

Cancer Gene Therapy (2011) 18, 489–499; doi:10.1038/cgt.2011.18; published online 13 May 2011

Keywords: adenoviral vaccine; antibody therapy; HER-2/neu; breast cancer; CD8\(^+\) CTL; NK
CD8\(^+\) cytotoxic T lymphocytes (CTLs) have an important role in host defense against viruses, intracellular bacteria and tumors.\(^{12-16}\) For this reason, a vaccine capable of inducing a strong CD8\(^+\) CTL response becomes a major goal in the field of tumor immunity. Replication-deficient adenoviruses (AdVs) have been found to be very effective vaccine vectors, as they mimic a natural infection and stimulate the innate immune responses, leading to development of an effective CD4\(^+\) and CD8\(^+\) T-cell responses to the vaccine-encoded Ag.\(^{17-20}\) Therefore, genetic vaccines based upon recombinant AdVs has been used to immunize against infectious diseases such as Ebola,\(^{21}\) SARS\(^{22}\) and human immunodeficiency virus.\(^{23,24}\) Vaccination with ovalbumin (OVA)-expressing recombinant AdVs stimulates efficient OVA-specific CTL responses,\(^{25-27}\) leading to protection against virus challenge.\(^{28}\) In addition, recombinant AdVs have also been applied for induction of antitumor immunity. In most of the studies, recombinant AdV vaccines have been shown to induce efficient prophylactic antitumor immunity. For example, vaccination with tyrosinase-related protein-expressing AdVs induced Gp100- and tyrosinase-related protein-expressing melanoma cell line BL6-10ova was generated in our laboratory.\(^{39}\) The mouse breast cancer cell line Tg1-I (H-2K\(^b\)) derived from a spontaneous HER-2/neu-expressing breast cancer tumor was obtained from Dr T Kipps, University of California, San Diego, CA. The natural killer (NK)-sensitive tumor cell line Yac-1 was obtained from American Tissue Cell Collection (Rockville, MD). Female wild-type C57BL/6 (B6, H-2K\(^b\)) mice and FVB/NJ TgN(MMTVneu)202Mul (FVBneuN) (H-2K\(^b\)) Tg mice expressing the rat neu under the control of a mouse mammary tumor virus promoter were obtained from Jackson Laboratories (Bar Harbor, ME). All mice were housed in the animal facility at the Saskatoon Cancer Center; with all animal experiments carried out in accordance to the Canadian Council for Animal Care guidelines.

**Materials and methods**

**Reagents, cell lines and animals**

Monoclonal Ab 7,16,4, a mouse IgG2a Ab reactive with the rat HER-2/neu oncogen-encoded p185 molecule was obtained from American Tissue Type Collection (Rockville, MD).\(^{38}\) The biotin-labeled anti-CD69 Ab was obtained from Pharmingen Canada (Mississauga, Ontario, Canada). The fluorescein isothiocyanate (FITC)-conjugated Abs specific for CD4 and CD8 and phycoerythrin (PE)-labeled H-2K\(^b\)/OVA\(^{257-264}\) tetramer and PE-labeled H-2D\(^b\)/HER-2 peptide (PDSLRLDSVF) tetramer were obtained from Beckman Coulter (San Diego, CA) and NIH Tetramer Facility (Bethesda, MD), respectively. Major histocompatibility complex class I (H-2K\(^b\))-restricted OVA1 (OVA\(^{257-264}\), SIINFEKL) peptide and irrelevant Mut1 peptide (FEQNTAQW) were synthesized by Multiple Peptide Systems (San Diego, CA). The highly lung metastatic OVA-transfected B16 melanoma cell line BL6-10ova was generated in our laboratory.\(^{39}\) The mouse breast cancer cell line Tg1-I (H-2K\(^b\)) derived from a spontaneous HER-2/neu-expressing breast cancer tumor was obtained from Dr T Kipps, University of California, San Diego, CA. The natural killer (NK)-sensitive tumor cell line Yac-1 was obtained from American Tissue Cell Collection (Rockville, MD). Female wild-type C57BL/6 (B6, H-2K\(^b\)) mice and FVB/NJ TgN(MMTVneu)202Mul (FVBneuN) (H-2K\(^b\)) Tg mice expressing the rat neu under the control of a mouse mammary tumor virus promoter were obtained from Jackson Laboratories (Bar Harbor, ME). All mice were housed in the animal facility at the Saskatoon Cancer Center; with all animal experiments carried out in accordance to the Canadian Council for Animal Care guidelines.

**Construction of recombinant adenovirus AdV\(_{OVA}\)**

Construction of recombinant AdV-expressing OVA (AdV\(_{OVA}\)) was performed by insertion of OVA gene cloned from pAc-OVA vector obtained from Dr M Bevan, University of Washington (Seattle, Washington) into pShuttle vector (Stratagene, La Jolla, CA) and NIH Tetramer Facility (Bethesda, MD), to form pLP-OVA-expressing OVA gene (Figure 1). The Pmel-digested shuttle vector was then cotransformed into BJ5183 Escherichia coli cells already containing the backbone vector for increased efficiency of homologous recombination to form the recombinant AdV\(_{OVA}\). The recombinant AdV\(_{OVA}\) vector was then linearized by PacI digestion, and then transfected into 293 cells using Lipofectamine (Gibco/BRL, Burlington, Ontario, Canada) to generate AdV\(_{OVA}\). Recombinant AdV\(_{HER-2}\)

![Figure 1](image)

**Figure 1** Schematic representation of adenovirus (AdV) vectors. The E1/E3-deleted replication-deficient AdV vectors are under the regulation of the cytomegalovirus (CMV) early/immediate promoter/enhancer. The AdV vectors include AdVnull without any transgene expression, AdV\(_{OVA}\)-expressing transgene ovalbumin (OVA) and AdV\(_{HER-2}\)-expressing transgene HER-2/neu. HER, human epidermal growth factor receptor; ITR, inverted terminal repeat.
expressing the rat neu gene and the control AdVNull without any inserted transgene (Figure 1) were previously constructed in our laboratory. All recombinant AdVs were amplified in 293 cells, purified by a series of cesium chloride ultracentrifugation gradients, and stored at −80°C until use.

Characterization of NK cell activity
Lymphocytes of the drainage lymph nodes of C57BL/6 mice with s.c. immunization of AdVova (1 × 10⁷ p.f.u. per mouse) were harvested 2 days after the immunization, stained with FITC-anti-CD8 Ab and PE-anti-NK1.1 Ab and then analyzed by flow cytometry. To assess their killing activity, we performed chromium 51-release assay, in which ⁵¹Cr-Yac-1 and lymphocytes derived from immunized mouse drainage lymph nodes were used as target and effector cells, respectively. The target cells were radiolabeled by culturing these cells for 1 h in the culture medium in the presence of 50 μl of sodium [⁵¹Cr]-chromate (36 mCi ml⁻¹; Amersham, Arlington Heights, IL), then washed twice with phosphate-buffered saline. Approximately 1 × 10⁵ labeled target cells per triplicate wells were mixed with effector cells at various effector (E): target (T) cell ratios, and then incubated for 6 h. The percentage of specific lysis was calculated as: 100 × [Experimental c.p.m.—spontaneous c.p.m.]/(maximal c.p.m.—spontaneous c.p.m.). Spontaneous c.p.m. release in the absence of effector cells was <10% of specific lysis. The maximal c.p.m. release was determined by lysis of the target cells with 0.25% Triton X-100.

Cytotoxicity assay
In vivo cytotoxicity assay, C57BL/6 mouse spleen cells pulsed with OVA1 peptide were strongly labeled with carboxyl-fluorescein succinimidyl ester (CFSE) (3.0 μM, CFSE<br>36> and served as OVA-specific target cells, whereas spleen cells pulsed with irrelevant Mut1 peptide were weakly labeled with CFSE (0.6 μM, CFSE<sub>36></sub>) and served as nonspecific control target cells, respectively. Eleven days following the immunization with AdVova, the immunized mice were then i.v. injected with a 1:1 (CFSE<sub>36>·CFSE<sub>36></sub>) mixture of splenocytes targets. Sixteen hours after target cell delivery, spleens of the recipient mice were removed, and the relative proportions of CFSE<sub>36> and CFSE<sub>36></sub> target cells remaining in the spleens were analyzed by flow cytometry.

Animal studies
Three types of animal studies were conducted. The first type of animal study was performed for evaluation of the preventive antitumor immunity. Wild-type C57BL/6 mice (10 mice per group) were s.c. vaccinated with AdVova (1 × 10⁷ p.f.u. per mouse). Eleven days after the immunization, C57BL/6 mice were s.c. injected in their right thighs with BL6-10<sub>0</sub>ova tumor cells (0.3 × 10⁶ cells per mouse). To assess the cellular mechanism of AdVova-induced antitumor immunity, C57BL/6 mice were i.p. injected with anti-CD8 (53.6.7) or anti-NK1.1 (PK136) Ab (0.3 mg per mouse) to deplete CD8<sup>+</sup> T cells or NK cells 10 days after AdVova immunization. One day after the Ab treatment, C57BL/6 mice were challenged by s.c. injection of BL6-10<sub>0</sub>ova (0.3 × 10⁶ cells per mouse). The Ab treatment was repeated once every 3 days for a total five times. To assess the long-term immunity, C57BL/6 mice were also s.c. inoculated with BL6-10<sub>0</sub>ova (0.3 × 10⁶ cells per mouse) 60 days after the immunization. The second type of animal study was performed to evaluate the prevention of breast carcinogenesis, the Tg FVBneuN mice (10 mice per group) at age of 2 months were vaccinated s.c. with AdVHER-2 (1 × 10⁷ p.f.u. per mouse) at 1-month interval for a total of five vaccinations. Spontaneous breast tumor development was monitored weekly for up to 12 months. The third type of animal study was designed to evaluate therapeutic antitumor immunity. FVBneuN mice (10 mice per group) were s.c. injected with Tg1-1 (1 × 10⁶ cells per mouse) cells. Each mouse was monitored weekly for tumor growth measured in two perpendicular diameters using a caliper. Tumor volume (mm<sup>3</sup>) was calculated using the formula V = a × b<sup>2</sup>/2, where a is the largest and b is the smallest diameter, and represented as mean ± s.d. When Tg1-1 tumors grew to a size of ~15 mm<sup>3</sup> (early stage, around 6 days after tumor cell injection) or ~100 mm<sup>3</sup> (well established, around 12 days after tumor cell injection), tumor-bearing FVBneuN Tg mice were s.c. injected with AdVHER-2 (1 × 10⁷ p.f.u. per mouse) at 5-day interval for a total of three times. To mimic the clinical administration of trastuzumab in patients with a loading dose of 4 mg (trastuzumab) per kg (patient body weight), Tg1-1 tumor-bearing mice were also i.p. injected with the anti-HER-2/neu Ab at a dose of 4 mg kg<sup>−1</sup> (mouse body weight) once every 3 days for a total of five times. In another Tg1-1 tumor-bearing mouse group, mice were treated with both AdVHER-2 and anti-HER-2 Ab. Tumor growth was monitored daily for 30 days; for ethical reason, all mice with tumors that achieved a size of 1000 mm<sup>3</sup> in volume were killed.

Statistical analyses
Statistical analyses were conducted using Prism software (GraphPad Software, San Diego, CA) to perform log-rank test for comparing mouse survival between groups. To determine the significance of differences between groups, Student’s t-tests were performed. P-values < 0.05 were considered statistically significant.
Results

**AdV<sub>OVA</sub> stimulates OVA-specific functional CD8<sup>+</sup> CTL and NK responses in wild-type C57BL/6 mice**

To assess the cellular immune responses, we i.v. immunized wild-type C57BL/6 mice with a recombinant OVA-expressing adenovirus AdV<sub>OVA</sub> and then evaluated OVA-specific CD8<sup>+</sup> T-cell responses in mouse peripheral blood using FITC-anti-CD8 Ab and PE-H-2K<sup>b</sup>OVA<sub>257-264</sub> tetramer staining by flow cytometry. We found that AdV<sub>OVA</sub> vaccine stimulated a sustained OVA-specific CD8<sup>+</sup> T-cell response accounting for 18.6% of the total CD8<sup>+</sup> T-cell population (Figure 2a), which is significantly larger than 0.07% in mice immunized with the control AdV<sub>Null</sub> (*P* < 0.05). The OVA-specific CD8<sup>+</sup> T-cell responses had a peak on day 11 after the immunization and then declined slowly. To assess the functional effect of CD8<sup>+</sup> T cells, we performed *in vivo* cytotoxicity assay. We adoptively transferred OVA<sub>I</sub> peptide-pulsed splenocytes that had been strongly labeled with CFSE (CFSE<sup>high</sup>), as well as the control peptide Mut1-pulsed splenocytes that had been weakly labeled with CFSE (CFSE<sup>low</sup>), into recipient mice that had been vaccinated with AdV<sub>OVA</sub>. As expected, there was a substantial loss (85%) of the CFSE<sup>high</sup> (OVA<sub>I</sub> peptide-pulsed) cells in the AdV<sub>OVA</sub>-immunized mice, whereas little cytotoxicity (8%) was induced in mice immunized with the control AdV<sub>Null</sub> (Figure 2b) (*P* < 0.05), indicating that AdV<sub>OVA</sub> vaccine efficiently stimulates CD8<sup>+</sup> T-cell differentiation into functional OVA-specific CTL effectors.

To assess the potential AdV-stimulated NK responses, lymphocytes of C57BL/6 mice with s.c. immunization of AdV<sub>OVA</sub> were harvested from the drainage lymph nodes 2 days after the immunization and analyzed for NK activation and killing activity by flow cytometry and *in vitro* cytotoxicity assay, respectively. As shown in Figure 3a, the proportion of active CD69<sup>+</sup>NK1.1<sup>+</sup> T cells was significantly greater in AdV<sub>OVA</sub>-treated mice (~60%) compared with the control mice (~10%) (*P* < 0.05). In addition, NK cells derived from AdV (AdV<sub>Null</sub> and AdV<sub>OVA</sub>)-treated mice displayed stronger killing activity against Yac-1 tumor cells than NK cells from the control wild-type C57BL/6 mice (*P* < 0.05) (Figure 3b), indicating that AdV<sub>OVA</sub> stimulates nonspecific NK cell responses.

AdV<sub>OVA</sub> stimulates CD8<sup>+</sup> CTL-mediated antitumor immunity and long-term T-cell memory in wild-type C57BL/6 mice

To assess preventive antitumor immunity, the above-immunized mice were s.c. challenged with OVA-expressing B16 melanoma BL6-10ova on day 11 subsequent to the immunization. We found that all (10/10) mice immunized with the control AdV<sub>Null</sub> died of tumor within 21 days subsequent to tumor cell challenge, whereas all (10/10) mice immunized with AdV<sub>OVA</sub> were tumor free (Figure 4a), indicating that AdV<sub>OVA</sub> stimulates a preventive antitumor immunity in C57BL/6 mice. To assess the cellular mechanism of the antitumor immunity, we treated immunized mice with anti-CD8 and anti-NK1.1 Ab to deplete CD8<sup>+</sup> T and NK cells, respectively, before tumor cell challenge. We demonstrated that all (10/10) mice with treatment of anti-CD8 Ab, but not anti-NK Ab lost their antitumor protection, indicating that...
AdVVOA-induced antitumor immunity is mainly mediated by CD8\(^+\) CTLs. To assess the long-term immunity, AdVVOA-immunized C57BL/6 mice were challenged by s.c. inoculation of BL6-10OVA tumor cells 60 days after the immunization. We found that none of the immunized mice (0/10) grew tumor (Figure 4b), indicating that AdVVOA vaccination can also induce a long-term antitumor immunity.

AdVHER-2 stimulates functional CD8\(^+\) CTL responses leading to reduction in breast carcinogenesis in Tg FVBneuN mice

Tg FVBneuN mice that have neu-specific self-immune tolerance\(^{44,45}\) spontaneously develop multiple HER-2/neu-expressing breast cancers with different sizes at different ages (Figures 5a and b).\(^{46}\) These Tg mice have been extensively used for evaluation of HER-2/neu-specific immunotherapeutics.\(^{47-49}\) To assess CD8\(^+\) T-cell responses, the peripheral blood samples of the mice immunized with AdVneu were harvested on day 11 after immunization, stained with FITC-anti-CD8 Ab and PE-anti-H-2K\(^d\)/HER-2/neu peptide tetramer, and analyzed by flow cytometry. We found that AdV\(_{HER-2}\) stimulated HER-2/neu-specific CD8\(^+\) T-cell responses accounted for 0.8% of the total CD8\(^+\) T-cell population (Figure 5c), indicating that AdV\(_{HER-2}\) stimulates HER-2/neu-specific CD8\(^+\) T-cell responses in Tg FVBneuN mice with self-HER-2/neu-specific immune tolerance. To determine whether AdV\(_{HER-2}\)-induced cellular immune responses could reduce breast carcinogenesis, the Tg FVBneuN mice at the age of 2 months were vaccinated s.c. with AdV\(_{HER-2}\) at 1-month interval, for a total of four vaccinations. As shown in Figure 5d, AdV\(_{HER-2}\) vaccination protected 3/10 of the mice from breast carcinogenesis and induced a significant delay in tumor formation in 7/10 of the mice compared with the control AdV\(_{Null}\) vaccination (\(P<0.05\)), indicating that AdV\(_{HER-2}\) vaccination can partly overcome self-HER-2/neu-specific immune tolerance and reduce breast carcinogenesis in Tg FVBneuN mice.

AdV\(_{HER-2}\) is adjuvant to Ab therapy of well-established tumors in Tg FVBneuN mice

To assess the potential therapeutic effect of HER-2/neu-expressing AdVHER-2 in Tg mice, we repeated the above experiments using AdV\(_{HER-2}\) vaccination in HER-2/...
neu-expressing Tg1-1 tumor-bearing Tg FVBneuN mice with HER-2/neu-specific self-immune tolerance. We found that all (10/10) of the vaccinated mice bearing early stage (15 mm³) Tg1-1 breast cancer died of cancer within 17 days after the initial vaccination though mouse survival was prolonged ($P < 0.05$) (Figure 5a). It has been demonstrated that the anti-HER-2/neu Ab had potent therapeutic effect on established HER-2/neu-expressing tumors in mice.\textsuperscript{50,51} To assess its therapeutic efficiency, we repeated the above experiments using anti-HER-2/neu Ab in HER-2/neu-expressing Tg1-1 tumor-bearing Tg FVBneuN mice. Tg1-1 tumor cells grew aggressively (Figure 6a). We found that all (10/10) of the vaccinated mice bearing early stage (15 mm³) Tg1-1 breast cancer became tumor free (Figure 6b), whereas 0/10 of the mice bearing well-established (100 mm³) tumors survived though these mice had a longer survival ($P < 0.05$) (Figure 6c). Interestingly, we found that 4/10 of the mice bearing well-established tumor were free of tumor when these mice were treated with AdV\textsubscript{HER-2} vaccination in combination with anti-HER-2/neu Ab therapy, whereas the remaining 6/10 tumor-bearing mice had a longer survival ($P < 0.05$), indicating that vaccination with recombinant HER-2/neu-expressing adenoviral vector can not only reduce breast carcinogenesis, but also provide adjuvant effect on anti-HER-2/neu Ab therapy for eradication of well-established breast cancers in Tg FVBneuN mice with self-HER-2/neu-specific immune tolerance.

**Figure 5** AdV\textsubscript{HER-2} stimulates HER-2/neu-specific CD8$^+$ cytotoxic T lymphocyte (CTL) responses and reduces breast carcinogenesis in transgenic FVBneuN mice. (a) Photographs of representative samples of spontaneous breast tumors taken from FVBneuN transgenic mice at various time points ranging from 4 to 10 months of age and ordered in progressing size and time frame. (b) Histologic photomicrographs of breast tumors at (A) 4 and (B) 10 months of age. Magnifications are $\times$ 100. (c) The tail blood samples of AdV\textsubscript{HER-2}-immunized FVBneuN mice were harvested on day 11 after the immunization and stained with PE-H-2Kq/HER-2 peptide tetramer (PE-tetramer) and fluorescein isothiocyanate (FITC)-anti-CD8 anti-body (Ab) (FITC-CD8), and then analyzed by flow cytometry. The value in each panel represents the percentage of HER-2/neu-specific (tetramer-positive) CD8$^+$ T cells vs the total CD8$^+$ T-cell population. The value in parenthesis represents the s.d. $^*P < 0.05$ vs cohorts of the control AdVnull group (Student’s t-test). (d) The transgenic FVBneuN mice at the age of 2 months were vaccinated s.c. with AdV\textsubscript{HER-2} at 1-month interval, for a total of four vaccinations. Spontaneous formation of breast tumors was monitored weekly. $^*P < 0.05$ vs cohorts of the control groups (phosphate-buffered saline (PBS) and AdVnull) (log-rank test). One representative experiment of two is shown. AdV, adenovirus; HER, human epidermal growth factor receptor.

**Discussion**

Conventional cancer therapies including surgery, radiation therapy and chemotherapy have demonstrated a considerable clinical success over the past years. However, tumor-free survival is not always accomplished. For example, surgery and radiation therapy are quite effective in treatment of localized tumors, but they often have only a palliative role in treatment of disseminated diseases. Chemotherapy remains the treatment modality of choice, but severe toxic side-effects often limit its use. The identification of tumor-associated Ags and tumor-specific T-cell responses in cancer patients led to the development of immunotherapies aimed at augmenting antitumor immune responses. Antitumor immunotherapies include the active immunotherapy such as the use of various antitumor vaccines\textsuperscript{52} to stimulate the patients’ antitumor CD8$^+$ CTL responses and the adoptive immunotherapy such as infusion of antitumor monoclonal Ab trastuzumab\textsuperscript{53} or tumor-specific tumor-infiltrating lymphocytes.\textsuperscript{53}

The original anti-HER-2/neu murine monoclonal antibody inhibited HER-2/neu-positive tumor growth in vivo.\textsuperscript{50,51,54} Trastuzumab is a humanized monoclonal Ab directed against the extracellular domain of HER-2/neu and its use, in combination with chemotherapy, was approved by the FDA in 1998 for metastatic HER-2/neu overexpressing breast cancer.\textsuperscript{43} Preclinical studies demonstrated interesting properties of trastuzumab, including\textsuperscript{55} internalization and degradation of the HER-2 protein.
inhibition of cell-cycle progression via inhibition of the mitogen-activated protein kinase pathway, suppression of the antiapoptotic phosphatidylinositol 3-kinase and Akt pathway and Ab-dependent cellular cytotoxicity. Clinical studies have shown that approximately one third of patients with advanced HER-2/neu-positive breast cancer will respond to trastuzumab monotherapy. Trastuzumab-based therapy has also been shown to be effective in both adjuvant and neoadjuvant setting in the management of early stage HER-2/neu-positive breast cancer. However, one of the major limitations of trastuzumab immunotherapy is the development of Ab resistance usually within 1 year from the beginning of the treatment in the metastatic setting. Schematically, the resistance to trastuzumab may be derived from (i) a truncated and active form of receptor that lacks the trastuzumab-binding extracellular domain, (ii) constitutive activation of downstream elements, making activation of the pathway independent of the HER-2/neu receptor and/or (iii) bypassing the HER-2/neu receptor through activation of another transmembrane receptor. Additionally, the risk of cardiac toxicity, especially in patients previously treated with anthracyclines, may also limit the use of trastuzumab.

Since 1993, immune responses to HER-2/neu have been frequently found in patients with HER-2/neu-positive breast cancer. As shown in preclinical model, such immune responses are associated with slower tumor development at early stages of the disease. These observations, together with reports about the efficacy and the resistance of passive trastuzumab therapy, motivated the development of various combinatorial immunotherapies. Among them, the combining therapeutic monoclonal antibodies (that is trastuzumab) with various antitumor vaccines (tumor cells, dendritic cells, DNA, peptides, proteins and viral vectors) is a promising avenue for combination immunotherapy.

One of the most remarkable features of AdV-based vaccines is their ability to induce exceptionally high and sustained frequencies of transgene product-specific CD8+ T-cell responses, which, unlike those induced by other subunit vaccine carriers such as DNA vaccines or poxvirus vectors, do not contract after the initial CTL activation. In this study, we also demonstrated that AdVHER-2 vaccination induces a sustained CD8+ CTL responses due to persistent Ag stimulation, which is consistent with some previous reports by others. It has been elucidated that replication-defective AdV vector genomes similar to those of wild-type AdV vectors acquired by natural infections persist. These replication-defective AdV vectors have been found in the muscle at the site of inoculation, in the liver and in the lymphatic tissues of experimental animals. It has been demonstrated that AdVHER-2 vaccination can stimulate
both HER-2/neu-specific Ab and CD8\(^+\) CTL responses and preventive antitumor immunity in wild-type mice.\(^{31-36}\) However, it was not able to reduce breast carcinogenesis in Tg mice with self-immune tolerance though their survival was prolonged.\(^{26,32}\) In this study, we have demonstrated that AdV\(_{HER-2}\) not only induce both HER-2/neu-specific functional CD8\(^+\) T cell, but also NK responses, leading to protective antitumor immunity. However, CD8\(^+\) T cells, but not NK cells have a major role in the immunity, which is consistent with a recent report by Wan and colleagues.\(^{80}\) We previously have demonstrated that vaccination with dendritic cells engineered to express HER-2/nu, resulted in a significant delay in tumor formation, however, despite stronger HER-2/neu-specific immune responses than DNA vaccine, it did not reduce breast carcinogenesis.\(^{81}\) In the present study, we demonstrated that AdV\(_{HER-2}\) vaccination significantly reduced breast carcinogenesis in Tg FVBneuN mice with self-immune tolerance, which is consistent with another recent report by Berzofsky \textit{et al.}.\(^{33}\)

The therapeutic efficacies of AdV in cancer remain controversial. For example, AdV vaccine was found to be ineffective when it was administered as early as only one\(^3\) or two days\(^3\) after seeding the tumor cells in wild-type mice. Conversely, it has also been demonstrated that recombinant AdV\(_{HER-2}\) vaccine efficiently eradicated advanced established murine breast cancer in wild-type mice.\(^{37}\) In this study, we showed that AdV\(_{HER-2}\) vaccine had little therapeutic effect on pre-existing tumors. AdV\(_{HER-2}\) only slightly delayed HER-2/neu-expressing Tg1-1 breast tumor growth, but did not cure any of Tg1-1 tumors in Tg FVBneuN mice even though they were in early stage (15 mm\(^3\)). The difference in AdV\(_{HER-2}\)-mediated therapeutic effects seen in our study and a previous report\(^3\) may be derived from the use of different types of mice. In this study, we used Tg FVBneuN mice with self-HER-2/neu-specific immune tolerance, whereas Myun \textit{et al.}\ used wild-type mice.\(^{37}\) In comparison, however, the anti-HER-2/neu Ab therapy was much effective since it cured all early stage (15 mm\(^3\)) Tg1-1 tumors in Tg FVBneuN mice, but failed in eradication of well-established Tg1-1 tumors (100 mm\(^3\)). Bocangel \textit{et al.}\(^{82}\) have demonstrated that a combinatorial synergy can be induced by AdVmda-7 vaccine expressing a tumor suppressor gene (melanoma differentiation-associated gene-7) and trastuzumab in delaying growth of HER-2/ neu-expressing human breast cancer in nude mice. Interestingly, for the first time, we demonstrated that a combinatorial immunotherapy of anti-HER-2/neu Ab and HER-2/neu-specific AdV\(_{HER-2}\) vaccine was capable of eradicating 4/10 Tg FVBneuN mice bearing well-established HER-2/neu-expressing breast cancer Tg1-1 (100 mm\(^3\)) and significantly prolonging the survival of the remaining 6/10 tumor-bearing mice, indicating that the synergistic effect of AdV\(_{HER-2}\) vaccine is capable of stimulating both HER-2/neu-specific CD8\(^+\) CTL and nonspecific NK cell responses on anti-HER-2/neu Ab therapy for well-established tumors in Tg FVBneuN mice with self-HER-2/neu-specific immune tolerance.

Taken together, we demonstrate that adenoviral vector vaccine can stimulate both CD8\(^+\) CTL and NK cell responses and provide an adjuvant effect on anti-HER-2/ neu Ab therapy. Our results suggest an adjuvant effect of AdV\(_{HER-2}\) on anti-HER-2/neu Ab therapy, and this combinatorial immunotherapy with trastuzumab and AdV\(_{HER-2}\) vaccine may be used as a new therapeutic strategy for treatment of advanced HER-2/neu-positive breast cancer.

\section*{Conflict of interest}

The authors declare no conflict of interest.

\section*{Acknowledgements}

This study was supported by a research grant (406991) from Canadian Breast Cancer Foundation.

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