Antibody-Dependent Natural Killer Cell–Mediated Cytotoxicity Engendered by a Kinase-Inactive Human HER2 Adenovirus-Based Vaccination Mediates Resistance to Breast Tumors

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Abstract
Cancer vaccines may have applications in the therapy and prevention of mammary carcinoma. To investigate such applications, we constructed a recombinant adenoviral vaccine expressing a kinase-inactive mutant form of human HER2 and introduced this into BALB/c wild-type (WT) or HER2 transgenic mice. Here, we report contributions by antibody responses and natural killer (NK) cells in tumor protection in this model. One i.p. vaccination protected WT mice from the HER2-expressing mouse carcinoma D2F2/E2. Half of the HER2 transgenic mice were protected fully and long term after preventive vaccination. Tumor growth in mice that eventually developed neoplastic lesions was delayed. Protection in WT and HER2 transgenic mice was associated with high or low levels of IgG2a antibodies, respectively, whereas CTLs were observed in WT but not in HER2 transgenic mice. Depleting CD4+ or CD8+ cells in vaccinated WT mice had limited effects, suggesting that protection was largely independent of CD4+ or CD8+ T cells. In contrast, antibody-mediated tumor rejection seemed to contribute significantly based on a loss of protection in mice deficient for Fc-γ RI/III or B cells. Further, a role for antibody-dependent cellular cytotoxicity (ADCC) mediated by NK cells was indicated by evidence that vaccine protection could be abolished by in vivo depletion of NK cells. Lastly, NK cells and immune sera purified from WT or HER2 transgenic mice exhibited efficient ADCC of HER2-expressing tumor cells in vitro. Our findings define a critical requirement for NK cells in vaccine-induced protection against HER2-expressing tumors. Cancer Res; 70(19); 7431–41. ©2010 AACR.

Introduction
The development of antitumor vaccines is generally focused on strategies to efficiently activate CTLs, although such approaches are constrained by many factors, including difficulties in generating high-avidity tumor-specific CTLs that can eradicate the tumor, and by defects in MHC class I antigen processing and presentation manifested by a large proportion of human tumors (1). In contrast, recognition of cell surface tumor antigens does not rely on MHC class I–restricted CTLs alone and can be based on an integrated immune response where antibodies acting in concert with the innate immune system are also of critical importance. HER2 (also termed ErbB-2), a 185-kDa transmembrane glycoprotein encoded by the ERBB-2 proto-oncogene, can be regarded as a “prototype” for this type of tumor antigen. HER2 belongs to the epidermal growth factor receptor (EGFR) family, with four closely related surface receptors (HER-1 to HER-4; refs. 2, 3). Both in vitro and animal studies showed that HER2 plays a key role in oncogenic transformation and tumorigenesis, tumor progression, and metastatic potential through interaction with other members of the EGFR family, leading to more potent intracellular signaling. Amplification and/or overexpression of HER2 have been shown at a relatively high frequency in both cell lines and biopsies derived from a wide range of human cancers of distinct histology (4). Clinical efficacy of the HER2-specific humanized monoclonal antibody (mAb) trastuzumab in combination with chemotherapy or as a single drug (5) has recently been heralded as a “triumph” for targeted cancer therapy. This has spurred efforts to actively induce a protective immunity against HER2-expressing tumors with vaccines based on peptides, plasmid DNA (pDNA), or recombinant proteins tested in models of spontaneous or transplantable mammary carcinomas (6–9). HER2 tumor vaccines have now also reached the clinic with several phase I and II studies based on different approaches (10–15). These studies show that HER2 can be immunogenic and also suggest an improved clinical outcome as the result of the vaccination (16, 17).
Recombinant adenoviral vectors are attractive candidates for tumor vaccines. They can be rendered safe for human use because the deletions of essential regions of their genome can abolish their pathogenicity, without impairing their infectivity; their tropism is broad, and they can infect both dividing and nondividing cells; they can be administered through both systemic and mucosal routes; and, of crucial importance, they can induce a strong transgene product-specific antibody and CD8+ T-cell response (18). In particular, the Ad5 serotype has been extensively tested in the vaccine setting (19-23), and Ad5-based HER2 vaccine constructs were shown to induce protective and therapeutic tumor protection via both CTL and antibody responses (24-26).

We have here produced an Ad5-based HER2 construct derived from the same kinase-deficient HER2 DNA sequence (E2A) previously shown to confer protection in experimental models (27-29). This Ad-E2A construct was able to induce tumor protection in wild-type (WT) and HER2 transgenic mice, with natural killer (NK) cell-mediated antibody-dependent cellular cytotoxicity (ADCC) as a major component in the vaccine-induced tumor protection.

Materials and Methods

Recombinant adenoviruses and plasmid vectors

Both Ad-E2A and Ad-PSA recombinant viruses were prepared using the pAdEasy system as described previously (30). Briefly, electrocompetent Escherichia coli B/J5183 cells (Qbiogene) were cotransformed with the pAdEasy-1 plasmid together with Pmel-linearized pShuttle-E2A or pShuttle-PSA pDNA. The pAdEasy plasmids containing the E2A and PSA inserts were verified by restriction enzyme cleavage. The recombinant viruses Ad-E2A and Ad-PSA were generated by restriction enzyme cleavage of the pAdEasy plasmids with PacI and transfection of the DNA onto HEK-293 (31) cells using Lipofectamine (Invitrogen). Seven to 10 days after transfection, cells were harvested and viral seed stock was obtained by three to four freeze/thaw cycles. The seed stocks were expanded by infection of increasing numbers of HEK-293 cell plates. Final high-titer stocks were purified with the ViraBind Adenovirus Purification kit (Cell Biolabs, Inc.), and the titer of each virus was determined by the QuickTiter Adenovirus Titer Immunoassay kit (Cell Biolabs) following the manufacturer’s instructions.

The plasmid vector pVax was purchased from Invitrogen; pVax-E2A, encoding the full-length, kinase-deficient HER2, was constructed as described (27).

Cells

D2F2 cells were established from a spontaneous mammary tumor arisen in BALB/c mice, as described (32); D2F2/E2 cells have been obtained by stably transfecting D2F2 with a WT human HER2-encoding plasmid (33). Their mouse origin was verified by spectral karyotyping as described (34). Cell lines were maintained in Iscove’s modified Dulbecco’s medium supplemented with 10% heat-inactivated FCS (Life Technologies), 100 IU/mL penicillin, and 100 μg/mL streptomycin. Selective antibiotic G418 (Sigma-Aldrich Co.) was added at 800 μg/mL to the described medium for the D2F2/E2 cell line culture.

Animals

BALB/c mice were purchased from Microbiology and Tumor Biology Center inner breeding (Karolinska Institutet, Stockholm, Sweden) or from Charles River; Fc-γ RI/III knockout (KO) BALB/c mice were obtained from Taconic Farms. BALB/c B-cell–deficient mice (μMT-/- mice, deficient for IgM transmembrane tail exons, and whose B-cell development is thus blocked at the pro-B stage) were kindly provided by Prof. Thomas Blankenstein (Max-Delbrück Center for Molecular Medicine, Berlin, Germany). BALB/c HER2 transgenic mice have been previously described (8).

Immunizations

Adenoviral constructs. Ad-E2A or Ad-PSA, diluted in 100 μL sterile 1× PBS (Sigma-Aldrich), was administered once i.p. at the dose of 10⁶ plaque-forming units (pfu).

Prime/boost. Mice, anaesthetized with 4% isoflurane, were injected twice, 2 weeks apart, 25 μg pVax or pVax-E2A in PBS in each flank by i.d. injections using a 29-gauge insulin-grade syringe (Micro-Fine U-100, BD Consumer Healthcare). Immediately after DNA administration, a needle array electrode was placed over the area of injection, and two groups of pulses of different voltages were applied: first, two 450-V pulses, 0.05 ms long, were given at a 0.2-ms interval, and 50 ms later, eight 110-V pulses, 10 ms long, were applied at a 20-ms interval. The electrode and the Derma Vax Electroporation System were purchased from Cyto Pulse Sciences, Inc. Adenoviral constructs were administered as described above. Mice received the DNA prime at weeks 0 and 2, followed by adeno-boosts at weeks 4 and 6.

Challenge

In the preventive immunization experiments, WT BALB/c mice received, 2 weeks after immunization, 10⁷ D2F2 or D2F2/E2 cells s.c. in the right flank. Such dose was doubled when HER2 transgenic BALB/c mice were used (8).

In the therapeutic setting experiments, mice were challenged the same way with 10⁷ D2F2/E2 and then immunized when the tumors were ∼2 mm in diameter. Tumor growth was monitored by palpation and caliper measurement twice a week. When tumors reached the size of 10 mm in diameter, mice were sacrificed for ethical reasons.

T-cell and NK cell depletion

To deplete CD8+ or CD4+ T cells, immunized mice were injected i.p. with 200 μg of TIB 105 (American Type Culture Collection, clone number 53-6,2, purchased from Mabtech) or GK1.5 mAb (purchased from Mabtech; ref. 35), respectively, 1 day before tumor challenge and then every 3 days thereafter. NK cells were depleted with anti-asialo-GM1 (Wako Pure Chemical Industries Ltd.), given as 200 μL of suspension diluted in PBS according to the manufacturer’s instructions. Untreated mice were used as controls.
Anti–HER2-specific antibody detection and titration
Blood samples from immunized mice were collected 15 days after immunization, and anti-HER2 IgG was measured in a flow cytometry assay. All fluorescence-activated cell sorting (FACS)–related reagents were purchased from Becton Dickinson unless otherwise specified. Serum samples were diluted 1:50 and incubated with D2F2 or D2F2/E2 cells for 30 minutes at +4°C. Cells were washed and stained with phycoerythrin (PE)–conjugated rabbit anti-mouse IgG antibody (DAKO), also used alone as negative control.

Antibody titers were calculated as described (36).
IgG subclasses were then identified by using FITC-conjugated anti-IgG1, anti-IgG2a, and anti-IgG2b as secondary antibodies.
The mean fluorescence intensity (MFI) was measured with a FACS Calibur cytometer, and analysis was performed with CellQuest Pro software (both from Becton Dickinson).

Measurement of T-cell response by flow cytometry
Lymphocytes were isolated from immunized mice spleens squeezed through a 70-μm strainer (Becton Dickinson). RBCs were lysed using hypotonic buffer PharmLyse (Becton Dickinson). Splenocytes (10^6 per well) were seeded in U-bottomed 96-well plates and stimulated for 8 hours with each one of the following: 10 μg/mL of the human HER2 Kd–restricted peptide 63–70 (TYLPTNASL), 10 μg/mL of HIV Gag A2–restricted peptide 77–85 (SLYNTVATL), 10^5 D2F2/E2, and 10^5 D2F2. IFN-γ production was detected by intracellular cytokine staining following addition of GolgiPlug (Becton Dickinson) for the last 6 hours of stimulation, performed using the Cytofix/Cytoperm Fixation/Permeabilization Solution kit (Becton Dickinson) according to the manufacturer’s instructions. All antibodies were purchased from Becton Dickinson.

NK cell isolation and ^51^Cr ADCC assay
For ADCC assay, an effector population that consisted of isolated NK cells was used. Positive NK selection was performed with MACS DX5-coated separation beads (Miltenyi Biotec) according to the manufacturer’s instructions. The phenotype of the cells obtained at the end of each process was confirmed by FACS using anti-CD3, anti-NKp46, and anti-CD11b (Becton Dickinson) antibodies. D2F2 or D2F2/E2 targets were labeled with 100 μCi ^51^Cr (Perkin-Elmer) per million cells for 30 minutes. After being diluted as 80,000 cells/mL, they were dispensed as 100 μL per well into U-bottomed 96-well plates and allowed to adhere. Sera and effectors were then added and coincubated with the targets overnight. ^51^Cr release in the supernatants was measured by a gamma counter (Wallac Sverige AB).

Analysis of HER2 expression in established tumors
Tumors were explanted when an average size of 10 mm^3 was reached. A single-cell suspension was obtained by squeezing the samples through a strainer with 70-μm pores (Becton Dickinson). Cells were stained with anti-human HER2 PE-conjugated antibody (Becton Dickinson).

The MFI was measured with a FACS Calibur cytometer, and analysis was performed with CellQuest Pro software.

Statistical analysis
Data were analyzed using Student’s t test and Mantel-Cox test, performed with GraphPad Prism software (GraphPad Software, Inc.).

Results
Ad-E2A–preventive immunization can fully protect WT BALB/c mice from a HER2-positive tumor challenge
We first examined the efficacy of the Ad-E2A vaccine to induce tumor-preventive immunity. With a dose of 10^9 pfu of Ad-E2A, a complete protection from tumor development was obtained throughout the observation period (100 days), whereas mice administered the same dose of a control Ad-PSA vaccine all succumbed to the tumor between 30 and 42 days (Fig. 1A). The immune response was HER2 specific, as mice challenged with the D2F2 control tumor were not protected (data not shown).

Thus, we conclude that one single dose of Ad-E2A can completely and durably protect mice from challenge with a HER2-positive syngeneic mouse mammary carcinoma.

Ad-E2A therapeutic immunization can partially protect WT BALB/c mice from a HER2-positive tumor challenge and cause tumor regression
Based on these results, we focused on exploring if the same Ad-E2A recombinant virus could be used in a therapeutic setting. BALB/c mice were s.c. inoculated with D2F2/E2 and subsequently vaccinated when the tumors were ∼2 mm in diameter. The immunization resulted in the survival of ∼50% of the mice (Fig. 1B) and in a significant delay in tumor development. Interestingly, in 6 of 20 of the immunized animals, tumors that first progressed up to 6 mm in diameter subsequently regressed and were not recurring during the observation period of 100 days (data not shown).

Given that we were unable to achieve 100% of protection and only delayed the growth of tumors in the majority of the treated mice, we considered the possibility that loss of HER2 expression may be the underlying explanation. Indeed, when analyzed by FACS, tumors that were progressively growing in Ad-E2A–immunized mice only expressed very low levels of HER2, comparable with the background staining of D2F2, and significantly (**, P < 0.01) lower than in tumors from control Ad-PSA–treated mice (Fig. 1C). We therefore conclude that the limited efficacy of this therapeutic HER2 vaccination could be due to the loss of cell surface HER2 expression.

Ad-E2A immunization can partially protect HER2 transgenic BALB/c mice and delay the onset and growth of HER2-positive tumors
Next, we tested if Ad-E2A immunization could overcome the partial tolerance to HER2 immunization described in HER2 transgenic BALB/c mice (37). As a result of a single dose of Ad-E2A immunization, 50% of the immunized mice were protected from growth of the D2F2/E2 tumor, and the
onset of tumor growth was significantly delayed in all mice ($P < 0.001$). In contrast, all of the Ad-PSA–immunized mice rapidly succumbed to the tumor (Fig. 2).

**Humoral and cellular response to Ad-E2A immunization in WT and HER2 transgenic BALB/c mice**

We next investigated the ability of the Ad-E2A vaccine to induce humoral responses in WT and HER2 transgenic mice. All sera from the Ad-E2A–immunized WT mice, collected 2 weeks after the vaccination, contained significant amounts (average $= 60.6 \pm 14.4 \mu g/mL$) of specific anti-HER2 antibodies, whereas sera from control Ad-PSA–immunized mice were negative. A lower concentration of specific antibodies could be detected also in sera from Ad-E2A–vaccinated HER2 transgenic mice (average $= 9.3 \pm 0.6 \mu g/mL$). Importantly, no response toward D2F2 cells was detected (Fig. 3A). In both WT and transgenic mice, the antibodies were shown to be mainly of IgG2a isotype (Fig. 3B).

The cellular response was also monitored ex vivo, analyzing the presence of HER2-specific IFN-$\gamma$–secreting CD8$^+$ T cells in freshly isolated splenocytes from immunized mice. As shown in Fig. 3C, Ad-E2A vaccination resulted in a specific production of IFN-$\gamma$ in CD8$^+$ leukocytes from Ad-E2A–vaccinated WT BALB/c mice on 8-hour stimulation with either HER2 63–70 peptide or D2F2/E2 cells. Ad-E2A immunization failed to elicit such cellular response in HER2 transgenic mice. Representative FACS plots are shown in Supplementary Fig. S1.
Effector T cells do not contribute to tumor protection following Ad-E2A immunization

To study the importance of Ad-E2A–induced effector CD8+ and CD4+ T cells in tumor protection, each of these T-cell subsets was depleted by antibody treatment of immunized mice, initiated at the time of D2F2/E2 tumor challenge. Tumor protection was not impaired in immunized mice depleted of either CD4+ or CD8+ cells (Fig. 4A). The efficacy of the depletion is shown in Supplementary Fig. S2A and B.

Antibodies elicited by Ad-E2A immunization are crucial in protecting mice from a HER2-positive tumor challenge and are likely to act through an Fc receptor–dependent mechanism

We next examined whether the tumor-protective effect exerted by Ad-E2A vaccination was dependent on antibodies. An Ad-E2A vaccination in B-cell–deficient BALB/c mice resulted in no protection, whereas the same vaccine protected all the WT mice (Fig. 4B). Because Fc receptor–dependent ADC is a major mechanism for anti-HER2 antibody–induced protection in mice (38), also FcγRI/III KO mice were tested for Ad-E2A–induced tumor protection. No protective immunity and only a slight delay in tumor development were induced by Ad-E2A immunization in these mice (Fig. 4C). Taken together, these results suggest that an FcγRI/III–dependent mechanism mediated by IgG2a, which is the isotype most active in ADC (39), is the major one responsible for Ad-E2A–induced tumor protection.

In vivo elimination of NK cells abolishes tumor resistance in Ad-E2A–immunized mice

Because monocytes and NK cells are the major effector cells in ADC (40), we further analyzed the role of NK cells in Ad-E2A–induced tumor rejection by repeated administration of anti–asialo-GM1. This resulted in a total depletion of NK cells in the treated mice, as shown in Supplementary Fig. S2C. Of major importance, the Ad-E2A–immunized mice depleted of NK cells were no longer able to resist tumor growth, and only a slight delay in tumor development could be observed in the antibody-treated mice (Fig. 5A).

Purified NK cells are highly active in mediating ADC with sera from Ad-E2A–immunized WT and HER2 transgenic mice

The capacity of sera from Ad-E2A–immunized WT and HER2 transgenic mice to participate in NK-mediated ADC was tested in 51Cr cytotoxicity assays. Different dilutions of sera from immunized WT, transgenic, or naive mice were co-incubated with nonactivated NK cells isolated from syngeneic spleens as effectors and D2F2/E2 cells as targets. As shown in Fig. 5B, >90% of the population used for the assays consisted of NKp46+ cells (left plot). Residual CD3+ or NKp46−/CD11b+ cells represented <3% of the population (Fig. 5B, middle and right plots). NK cells from both naive and vaccinated mice were able to efficiently kill D2F2/E2 in the presence, but not in the absence, of sera from Ad-E2A–immunized WT mice. The NKp46 population that remained after the passage on DX5-coated magnetic beads was inactive in mediating ADC against D2F2/E2 cells (Fig. 5C). Of note, also sera from Ad-E2A–vaccinated transgenic mice were able to mediate lysis of D2F2/E2 cells, although significantly less efficiently than WT sera (P < 0.05 for 1:100 dilution; P < 0.01 for 1:500 and 1:1,000 dilutions; Fig. 5D).

A prime/boost regimen can improve the outcome of Ad-E2A–based immunization in HER2 transgenic mice

In an attempt to further enhance the tumor protection in the HER2 transgenic model, mice were primed with two pDNA E2A immunizations delivered i.d. plus electroporation before receiving two boosts with Ad-E2A, each immunization with 2-week interval. One week after the last immunization, mice received 2 × 10^5 D2F2/E2 cells s.c. This prime/boost regimen resulted in a more pronounced delay in tumor growth and in an increased proportion (65%) of protected mice (Fig. 6A, compared with Fig. 2).
All sera from E2A prime/boost–immunized mice contained significant amounts (average = 19.7 ± 4.7 μg/mL) of specific anti-HER2 antibodies (36), whereas sera from control mice were negative (Fig. 6B).

Discussion

Here, we have directly shown the capacity of NK cells to work in concert with a vaccine-induced humoral response, resulting in resistance to the growth of HER2-expressing tumors. Our results extend to a HER2 transgenic mouse model, where the approach of adenoviral vaccination proved efficient in inducing antibodies endowed with the ability to trigger NK-mediated ADCC activity against HER2-expressing tumors.

We show that a single dose of the Ad-E2A vaccine is sufficient to completely and durably protect WT BALB/c mice from the D2F2/E2 mouse mammary carcinoma expressing HER2, and furthermore show delayed tumor growth and induction of tumor regression in mice with established small tumors. In BALB/c HER2 transgenic mice, shown to be relatively tolerant to human HER2 (37), 50% of the vaccinated mice were protected, which could be further strengthened by priming twice with pDNA E2A electroporation. This protection is more efficient than what described by us (W-Z.W.; ref. 8) in the same strain of transgenic mice after four doses of a truncated HER2 pDNA vaccine (E2TM) electroporated in combination with pDNA granulocyte macrophage colony-stimulating factor (GM-CSF), where only ~20% of mice were protected, probably reflecting the superior efficacy of the adenoviral vector approach.

The Ad-E2A vaccine efficiently induced antibodies in WT BALB/c mice, whereas the comparatively low antibody response we found in the vaccinated HER2 transgenic mice confirmed the partial tolerance in this strain (8). Importantly, antibodies proved necessary for the HER2-specific tumor resistance, as concluded from the lack of effect seen in antibody-deficient mice. This pivotal importance of antibodies agrees with several other reports on HER2 vaccine–induced tumor protection based on adenoviral construct or pDNA (24, 25, 41, 42). Notably, however, when the same mutant full-length E2A gene as used in our Ad-E2A construct was administered as an i.m. pDNA vaccine together with a GM-CSF plasmid, tumor rejection was efficient also in B-cell–deficient mice and was critically T-cell dependent (27). Therefore, the type of vector, the mode of administration, and the specific adjuvant used seem critical for determining whether antibodies or T cells will dominate tumor protection.

Importantly, our data provide strong evidence that antibodies alone are not sufficient, and specifically point at the role of NK cells in the vaccine-induced HER2-specific tumor rejection. Mice depleted of NK cells through repeated administration of anti–asialo-GM1 antibody lost their ability to reject HER2-expressing tumor cells. Because in vivo depletion of CD4+ or CD8+ cells did not impair Ad-E2A vaccine–induced tumor rejection, and Gr-1high neutrophils or Mac-1+ macrophages/monocytes are not affected by anti–asialo-GM1 administration (43), we can exclude that the protection is mediated by cellular compartments other than NK cells.

The central role for ADCC in the tumor rejection was further supported by a lack of protection in the FcγRIII KO BALB/c mice. Furthermore, the IgG2a antibody subclass,
which dominated our vaccine-induced antibody response, is the most effective one in mediating ADCC in mice (39). This role for Fc receptors in tumor-protective activity is similar to that described for the mAb trastuzumab, as tested in mice (38) and as recently implicated in breast cancer patients receiving trastuzumab therapy (44).

In contrast, Park and colleagues (45), following vaccination with an adeno-vaccine based on a truncated rat HER2/neu receptor, recently were able to show an Fc receptor-independent rejection, which instead was dependent on a direct growth-inhibitory effect of HER2 antibodies. These contrasting findings may depend on different types of vaccines in the two studies, where the vaccine used by Park and colleagues is based on the rat neu gene, whereas we use a human HER2-based one. In addition, the D2F2/E2 tumor is insensitive to direct growth-inhibitory effects of anti-HER2 antibodies (data not shown), whereas the TUBO tumor in the study by Park and colleagues is induced and driven by the rat neu gene in BALB/neuT mice and is therefore susceptible to such effect. Because in our study there was a slight delay

Figure 4. B cells and Fc-y RI/III receptors, but not effector T cells, contribute to tumor protection following Ad-E2A immunization. A, WT BALB/c mice were immunized once i.p. with 10⁶ pfu of Ad-E2A or Ad-PSA as control. Two weeks later, the animals were challenged s.c. with 10⁵ D2F2/E2 cells. CD4- or CD8-depleting antibodies were given i.p. as described. The graphic shows the pooled results from two independent experiments. B-cell-deficient (B) or Fc-y RI/III KO (C) BALB/c mice received 10⁶ pfu of Ad-E2A or Ad-PSA once i.p. and were challenged s.c. 2 wk thereafter with 10⁵ D2F2/E2 cells. ***, P < 0.001.
Figure 5. Tumor rejection relies on NK cells through an ADCC-mediated mechanism. A, WT BALB/c mice were immunized and treated with anti-asialo-GM1 antibody as described. On s.c. inoculation of 10^5 D2F2/E2, all mice succumbed. *, P < 0.05. B, FACS analysis of the cell population obtained after positive NK cell selection. C, splenocytes from naive or vaccinated mice, isolated as described, were coincubated with D2F2/E2 cells at different E:T ratios in the presence or absence of serum (diluted 1:100) derived from Ad-E2A–immunized WT mice. D, different concentrations of sera derived from Ad-E2A–vaccinated WT and transgenic BALB/c mice were coincubated with NK cells from naive syngeneic mice, and D2F2/E2 cells were added at a 30:1 E:T ratio. *, P < 0.05; **, P < 0.01.
in tumor development induced by vaccination also in the Fc-γ RI/III KO BALB/c mice (Fig. 4C) and in anti-asialo-GM1–treated mice (Fig. 5A), this may indicate an ADCC-independent direct effect by antibodies, possibly mediated by complement.

Another convincing evidence for an NK-mediated ADCC as the major effector arm was provided by “classic” in vitro NK cytotoxicity assays. Here, highly purified, freshly isolated, and nonactivated NK cells were able to efficiently kill D2F2/E2 cells in a HER2-specific manner even at low concentrations of sera (1:1,000 dilution), underlining the power of ADCC in eliminating antibody-coated target cells (40). Although the concentration of HER2-specific antibodies in the HER2 transgenic mice was considerably lower than that in WT mice, confirming the partial HER2 tolerance (37), their sera were still efficient in inducing NK-mediated ADCC activity. This shows that NK-mediated ADCC is an efficient cell-mediated antitumor activity to consider even in tolerant or partially tolerant hosts.

Morse and colleagues (46) administered an adeno-based vaccine construct, which, similarly to our construct, expressed a kinase-deficient, full-length human HER2 molecule. These authors were able to induce polyclonal HER2-specific antibodies, which mediated receptor internalization and also induced ADCC of HER2+ target cells in vitro. As the effector cells in their assays were adherent interleukin-2–activated splenocytes, and in vivo evidence of NK cell–mediated tumor rejection was lacking, the involvement of other cells including activated monocytes known to express Fc receptors capable of mediating ADCC (40) cannot be excluded.

Although the Ad-E2A–vaccinated WT mice were found to contain low but significant numbers of IFN-γ–producing CD8+ T cells, which responded specifically to both D2F2/E2 cells and the immunodominant HER2 63–70 epitope, immunized mice depleted in vivo of CD8+ T cells were able to fully resist tumor challenge. We therefore conclude that CD8+ cells do not seem to play a major role in tumor resistance in our model. Further stressing this conclusion, the HER2 transgenic mice were unable to respond by induction of specific CD8+ cells following our vaccine approach and yet were partially resistant to D2F2/E2 challenge. Alternative modes of immunization may, however, induce CD8+ responses also in the HER2 transgenic mice, as recently shown (8). Overcoming CD8+ T-cell tolerance to HER2 may therefore pose a greater challenge compared with breaking tolerance in CD4+ T cells and inducing a humoral response, as also concluded in the BALB/neuT model transgenic for mutant rat neu (47). The propensity of HER2 to downregulate MHC class I in tumor cells further adds to the potential inefficiency of tumor vaccine approaches based solely on CD8+ T cells (28).
Collectively, our results motivate further efforts toward clinical development of treatment plans where HER2 vaccine strategies known to induce a powerful antibody response are combined with approaches optimizing NK cell–mediated ADCC. Because the insights into the molecular basis for NK cell recognition of cancer have markedly increased in recent years, efforts to combine HER2 tumor vaccines with drugs or other immune-modulatory agents affecting NK cells or their tumor ligands should be encouraged.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

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