

HER2-Targeted Polyinosine/Polycytosine Therapy Inhibits Tumor Growth and Modulates the Tumor Immune Microenvironment

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Abstract

The development of targeted therapies that affect multiple signaling pathways and stimulate antitumor immunity is greatly needed. About 20% of patients with breast cancer overexpress HER2. Small molecules and antibodies targeting HER2 convey some survival benefits; however, patients with advanced disease succumb to the disease under these treatment regimens, possibly because HER2 is not completely necessary for the survival of the targeted cancer cells. In the present study, we show that a polyinosine/polycytosine (pIC) HER2-homing chemical vector

induced the demise of HER2-overexpressing breast cancer cells, including trastuzumab-resistant cells. Targeting pIC to the tumor evoked a number of cell-killing mechanisms, as well as strong bystander effects. These bystander mechanisms included type I IFN induction, immune cell recruitment, and activation. The HER2-targeted pIC strongly inhibited the growth of HER2-overexpressing tumors in immunocompetent mice. The data presented here could open additional avenues in the treatment of HER2-positive breast cancer. *Cancer Immunol Res*; 4(8); 688–97. ©2016 AACR.

Introduction

Despite treatment advances and earlier diagnosis, breast cancer remains a major contributor to cancer morbidity and mortality. The human epidermal growth factor receptor type 2 (HER2) is amplified and/or overexpressed in 20% of breast cancers (1–3). Overexpression of HER2 is associated with increased aggressiveness and significantly shortened disease-free and overall survival (4–7). Several targeted therapies have been approved for the treatment of HER2-positive breast cancer. These include the small molecular tyrosine kinase inhibitor (TKI) lapatinib, the humanized antibodies trastuzumab and pertuzumab, and the HER2-directed antibody–drug conjugate trastuzumab emtansine (T-DM1; ref. 8).

The most commonly used targeted therapy for HER2-positive breast cancer patients is trastuzumab (Herceptin), a humanized

monoclonal antibody to HER2 that blocks the activity of HER2 and actively mediates antibody-dependent cell-mediated cytotoxicity (ADCC; ref. 9). Although trastuzumab significantly improves the disease-free survival and overall survival of breast cancer patients (10, 11), many patients develop resistance and succumb to the disease. Moreover, some patients show primary resistance to trastuzumab (12). Resistance to targeted therapy emerges due to the intrinsic genetic instability and heterogeneity of tumors (13, 14). Putative mechanisms of resistance to HER2-targeted therapies include shedding of the extracellular domain and upregulation of compensatory signal transduction pathways (15). Escape from immune cell surveillance and co-option of the immune system to promote tumor progression also have been recognized as hallmarks of cancer (16).

The need for targeted therapies that affect multiple signaling pathways and simultaneously stimulate the anticancer action of the immune system led us to develop a novel treatment modality that utilizes the overexpression of HER2, but not its activity *per se*. Our treatment modality induces several apoptotic pathways and activates the immune system against the tumor, reducing the likelihood of resistance. This strategy is based on our recent success in the eradication of EGFR-overexpressing tumors, in which a nonviral vector, polyethylenimine–polyethyleneglycol (PP) conjugated to epidermal growth factor (PPE) selectively delivers a synthetic analogue of viral double-stranded RNA (dsRNA), polyinosinic–polycytidylic acid (pIC; refs. 17, 18). dsRNA is a strong inducer of antiviral host defense, leading to innate and adaptive immune responses (19). Cellular uptake of dsRNA triggers several signaling pathways, including proapoptotic and proinflammatory pathways (20). The binding of dsRNA to Toll-like receptor 3 (TLR3) induces the synthesis of cytokines such as IFN type I, a strong immune activator (20), as well as IFN γ -induced protein 10 kDa (IP-10), and growth-regulated protein α (GRO- α), which are potent chemoattractants for T cells (21). As

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such, pIC is a potent immune activator. In clinical trials, pIC was associated with statistically significant clinical benefit, but induced severe systemic toxic effects (22, 23), likely because the pIC was administered in a nontargeted way.

In vivo, pIC targeted to EGFR-overexpressing glioblastoma tumors, as well as to disseminated tumors, eradicates tumors by activating multiple cell-killing pathways and through antitumor immune bystander effects (17, 18). We have improved the chemistry of the pIC-targeting vector and created a platform (24) that can be used to generate a vector with any desired ligand. The first vector developed with the new platform targets the HER2 receptor.

Because the HER2 receptor lacks a natural ligand that can be used for targeting, we conjugated the nonviral vector, PP (24), to a HER2 affibody (25) to generate PPHAffibody (24). Affibody molecules are small, soluble, robust affinity ligands that were developed from the B domain of staphylococcal protein A (26). HER2 affibodies possess high affinity and high selectivity (25) and have been used for tumor labeling and targeting (27). The HER2 affibody ZHER2:2891 binds to HER2 with high affinity and is internalized by HER2-expressing cells (25, 28). The chemical and physicochemical properties of the HER2-targeting vector used in this study have been described (24).

In this article we report the eradication of HER2-overexpressing cells, using targeted delivery of pIC. PPHAffibody complexed with pIC (pIC/PPHAffibody) induced target-cell apoptosis, the secretion of proinflammatory cytokines, MHC class I expression, and the recruitment of lymphocytes to the treated cells. We show that targeted pIC, delivered systemically, significantly inhibited tumor growth, especially in immunocompetent mice.

Materials and Methods

Cell lines and reagents

BT-474^{GFP/LUC+} cells are BT-474 cells transduced with lentivirus containing GFP/LUC plasmid. RENCA-HER2 cells (which overexpress human HER2/neu; ref. 29), BT-474, BT-474^{GFP/LUC+}, and MCF-7 cells were maintained in RPMI-1640 medium (supplemented with 50 µg/mL G418 for RENCA-HER2), SK-BR-3 cells in DMEM, MDA-MB-231 cells in Leibovitz L-15 medium, BT-474R cells in Dulbecco's modified Eagle medium/Ham F12 1:1 (DMEM/F12; Life Technologies) supplemented with 1 µmol/L trastuzumab, and 2 mmol/L L-glutamine and MCF-7-HER2 cells (which overexpress human HER2/neu; ref. 30) in DMEM/F12 medium (Gibco) supplemented with 250 µg/mL G418. All media were supplemented with 10% fetal bovine serum (FBS), 10⁴ U/L penicillin, and 10 mg/L streptomycin. Cells were cultured for ≤5 passages, were in logarithmic growth phase for all experiments, and were tested periodically for *Mycoplasma*. All cell lines were obtained from the ATCC (1999–2002), except RENCA-HER2 (kindly provided in 2002 by Prof. W. Wels, Chemotherapeutisches Forschungsinstitut, Georg-Speyer-Haus, Frankfurt am Main, Germany), BT-474R (trastuzumab-resistant) cells (ref. 31; kindly provided in 2013 by Dr. Maurizio Scaltriti and Dr. José Baselga, Memorial Sloan Kettering Cancer Center, New York, NY), and MCF-7-HER2 (kindly provided in 2012 by Dr. M. C. Hung, University of Texas MD Anderson Cancer Center, Houston, TX). Cells were maintained at 37°C and 5% CO₂, except MDA-MB-231 (37°C without 5% CO₂). Except where noted otherwise, cell culture reagents were from Biological Industries, Bet Ha'emek, Israel. MCF-7-HER2 cells were authenticated using STR DNA

fingerprinting in 2011. BT-474R cells were validated by ATCC and confirmed by SNP array in 2008. RENCA-HER2 cells were validated for HER2 expression using immunohistochemistry following cell inoculation in BALB/c mice. All other cell lines were not authenticated; however, cell morphology in culture was monitored and was consistent with ATCC description and relative HER2 amounts were confirmed by flow cytometry.

Peripheral blood mononuclear cells (PBMC) were isolated and maintained as described previously (17).

Low-molecular-weight pIC was purchased from InvivoGen (France). PPHAffibody and PPCys were generated as described (24). Throughout the text, the concentration of "pIC/PPHAffibody" or "pIC/PPCys" refers to the concentration of pIC in the complex. The N:P ratio (amine:phosphate) was 6 for *in vitro* studies and 6.6 for *in vivo* studies. pIC/PPHAffibody complexes were 206 nm as measured using a Nano-ZS Zetasizer (24).

Chemotaxis

A total of 1 × 10⁵ BT474 cells/well were plated in 24-well plates. The following day, the medium was replaced by medium containing 0.15% FCS, and pIC/PPHAffibody was added at the indicated concentrations. After 48 hours, medium from the treated cells was placed in the bottom chamber of a Transwell plate (5.0-µm diameter pores; Corning), and 1 × 10⁶ PBMCs (in 0.15% FCS) were placed in the upper chamber. After 4 hours, medium from the bottom chamber was collected and lymphocytes were gated and counted by flow cytometry (BD FACS ARIAI; BD Biosciences).

Cell survival

Cells (0.5 × 10⁴–1 × 10⁴)/well were plated in triplicates in a 96-well plate and treated 24 hours later with pIC/PPHAffibody for 72 hours. Cell survival was analyzed using CellTiter-Glo (Promega) and a Luminoskan Ascent Microplate Luminometer (Thermo Fisher Scientific Inc., France).

Flow cytometry

Cells were treated with pIC/PPHAffibody (1 µg/mL), labeled with FITC-conjugated mouse anti-human class I, HLA-ABC (clone G46-2.6; BD Pharmingen), and subjected to flow cytometry (BD FACS ARIAI, BD Biosciences) and analyzed using FlowJo software. Debris, dead cells, and doublets were gated out.

Confocal microscopy

Cells (1 × 10⁴ cells/well) were plated in an 8-well µ-slide (iBidi). The next day, the cells were treated with pIC/PPHAffibody for 2 or 4 hours. Cells were fixed with 4% paraformaldehyde and permeabilized with 1% Triton X-100, incubated with antibody against affibody (Abcam, 1:200) followed by a secondary Alexa 488-conjugated antibody (Jackson ImmunoResearch 1:400). Confocal microscope (FV-1000 Olympus) pictures were taken at ×10 magnification.

mRNA extraction and analysis by qRT-PCR

Cells were treated with pIC/PPHAffibody or pIC/PPCys (1 µg/mL) for 2 or 4 hours. mRNA was extracted using the EZ-10 DNAaway RNA Miniprep Kit (Bio Basic Canada INC) and cDNA was prepared using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Life Technologies). mRNA expression was determined by quantitative real-time PCR (qRT-PCR) as described in the Supplementary Materials and Methods.

Western blot analysis

Cells were treated with pIC/PPHAffibody (2 µg/mL) for 4 or 8 hours. Proteins were extracted using NP-40 lysis buffer and analyzed by Western blot using primary antibodies against cleaved caspase-3, caspase-3, tubulin, and PARP (1:1000; Cell Signaling Technology). Experiments were repeated at least three times.

Apoptosis

BT-474 or MDA-MB-231 cells (1.5×10^5 /well) were plated in a 24-well plate in duplicates. Cells were treated with pIC/PPHAffibody (2 µg/mL) for 4 and 8 hours, stained with Annexin V/propidium iodide (PI; MLB MEBCYTO apoptosis kit) and analyzed using flow cytometry. Each experiment was repeated at least three times.

Quantification of RANTES, IP-10, and GRO- α by ELISA

BT-474 or SK-BR-3 cells (1×10^4 cells/well) were treated with pIC/PPHAffibody. Forty-eight hours later, 100 to 200 µL of medium was collected and subjected to RANTES, IP-10, or GRO- α ELISA assays (PeproTech) and read using a Bio-Tek Synergy H1 plate reader.

PBMC coculture

BT-474^{GFP/LUC+} cells (1×10^4 cells/well) were plated in 96-well plates, in triplicate. After 24 hours, the cells were treated with pIC/PPHAffibody for 48 hours. PBMCs (5×10^5 /well) were added to the culture for an additional 48 hours. Luciferase activity, which corresponds to the number of live BT-474^{GFP/LUC+} cells, was measured using the Luciferase Assay system (Promega) and a Luminoskan Ascent Microplate Luminometer (Thermo Scientific).

PBMC medium transfer

BT-474 cells (1.5×10^5 cells/well) were plated in 24-well plates. After 24 hours, the cells were treated with pIC/PPHAffibody or pIC/PPCys for another 24 hours. Medium from duplicate wells was combined and transferred to a 6-well plate, and PBMCs (1.3×10^7 cells/well) were added for 12 additional hours. RNA was isolated from PBMCs.

Animals, tumor growth, and treatment

MCF-7-HER2 model. 17 β -estradiol pellets (Innovative Research of America, Inc.) were inserted s.c. into 6- to 8-week-old female athymic Nude-Foxn1nu mice (Harlan Laboratories) 1 day before cell injection. MCF-7-HER2 cells (5×10^6) were injected into the mammary fat pad of each mouse ($n = 6$ –9/group). Mice were randomized and divided into treatment groups when the tumors reached an average size of 100 mm³. pIC/PPHAffibody (1 mg/Kg) was injected intravenously (i.v.) every 24 hours. Trastuzumab (10 mg/kg) was injected intraperitoneally (i.p.) once a week.

RENCA-HER2 model. RENCA-HER2 cells (5×10^6) were injected subcutaneously (s.c.) into the right flanks of immunocompetent BALB/c female mice (6–8-week-old; Harlan Laboratories). Mice were randomized into treatment groups ($n = 6$ –8/group) when tumors reached 200 mm³ and injected i.v. with pIC/PPHAffibody (0.25 mg/kg) every 24 hours for 12 days.

For the immune-cell depletion experiment, mice were randomized into treatment groups when tumors reached 100 mm³. NK cells were depleted by antibody to asialo-GM1 (50 µL/mouse, i.p.,

Wako Chemicals), starting 2 days before pIC/PPHAffibody treatment, again on the day of the treatment (d 0), and every 4 days thereafter. CD8 cells were depleted using anti-m-Lyt-2.2 (CD8a) (Bio X Cell) i.p. at 0.1 mg/mouse, starting 2 days before pIC/PPHAffibody treatment and every 2 days thereafter. Depletion of both NK and T cells was confirmed using flow cytometry (data not shown). pIC/PPHAffibody (0.25 mg/kg) was administered i.v. every 24 hours.

MMTV-huHER2 model. Tumor cells were isolated from spontaneous mammary tumors arising in MMTV-huHER2 transgenic mice (32). Single-cell suspensions derived from 3 transgenic mice with MMTV-huHER2 tumors were mixed with matrigel 1:1 (vol/vol; BD Bioscience) and injected into the mammary fat pads of immunocompetent FVB, 6- to 8-week-old female mice (Harlan Laboratories; $n = 6$ /group). When tumors reached 100 mm³, the mice were randomized and treated for 12 days. Mice were injected i.v. with pIC/PPHAffibody (0.25 mg/kg) every 24 hours.

All animal protocols were approved by the Hebrew University of Jerusalem Institutional Animal Care and Use Committee.

Syngeneic mouse model of MMTV-HER2 Founder #5 (Fo#5). The mammary tumors were propagated into the thoracic mammary fat pads of female FVB mice (Charles River Laboratories). When tumors reached a volume of 100 to 300 mm³, animals were randomized ($n = 9$) and received a daily i.v. dose of PPHAffibody (0.215 mg/kg), or PPHAffibody (0.215 mg/kg) plus pIC (0.25 mg/kg) for up to 15 consecutive days. The fit curve of the tumor volumes for each group was generated using nonlinear mixed modeling and was plotted over time. The median time to tumor progression of 1,000 mm³ was estimated with Kaplan–Meier analysis. Studies were performed in compliance with NIH guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at Genentech, Inc.

Tumor xenografts were measured with calipers and tumor volumes were determined using the formula: length \times width²/2, and plotted as means \pm SEM.

Immune cell infiltration

BALB/c female mice were inoculated with RENCA-HER2 cells (5×10^6). Mice ($n = 4$ –5) and treated for 4 days with pIC/PPHAffibody (i.v.). On day 5, tumors were excised and digested using collagenase type IV and DNase (Roche). Cells were washed, counted, and stained with antibodies against NKp46, CD45.2, CD3, and CD8 (Miltenyi Biotech). Flow cytometry was performed as above.

Statistical analysis

Statistical significance of differences between groups was determined by applying an unpaired Student *t* test (Prism software). Kaplan–Meier analysis (nonparametric log-rank test, JMP software) was used to determine time to tumor progression. *P* values < 0.05 were considered statistically significant.

Results

Targeted delivery of pIC/PPHAffibody induced HER2-dependent tumor cell death

To determine the effect of PPHAffibody on pIC delivery and the induction of targeted tumor cell death, several breast cancer cell

lines with differential HER2 expression were utilized. Expression of HER2 on MDA-MB-231, MCF-7, SK-BR-3, and BT-474 cells had been determined (33, 34) to be approximately 8×10^3 , 1.5×10^4 , 1.9×10^6 , and 1×10^6 receptors per cell, respectively. pIC/PPHAffibody efficiently induced the death of HER2-overexpressing breast cancer cells as compared with low HER2-expressing cells, in a concentration-dependent manner (Fig. 1A). Less than 20% of BT-474 and SK-BR-3 cells survived treatment with 2 $\mu\text{g}/\text{mL}$ pIC/PPHAffibody, whereas no effect was observed for MDA-MB-231 (***, $P < 0.001$). The survival of MDA-MB-231 cells was not due to lack of TLR3 expression (Supplementary Fig. S1) but rather due to lack of HER2 overexpression. Delivery of pIC with a nontargeted vector, polyethyleneimine–polyethyleneglycol–cysteine (PPCys), did not induce cell death in any of the cell lines.

Specific binding and time-dependent internalization of the pIC/PPHAffibody into BT-474 were observed by confocal microscopy (Fig. 1B). After 2 hours of treatment with pIC/PPHAffibody the complex was bound to the cell membrane of BT-474 cells, and

was internalized after 4 hours. However, the complex did not bind or internalize into MDA-MB-231 cells (Fig. 1B) even after longer incubation (data not shown). Thus, pIC was selectively delivered into HER2-overexpressing cells, and cell death was induced in a HER2-dependent manner.

Many HER2-positive cancer patients are either resistant to trastuzumab before treatment or develop resistance afterward. Comparing the efficacy of pIC/PPHAffibody treatment with that of trastuzumab, we found that pIC/PPHAffibody was more potent than trastuzumab in inducing cell death in BT-474 and SK-BR-3 cells (Fig. 1C). The survival of trastuzumab-resistant BT-474-derived cells (BT-474R cells), which maintain HER2 overexpression (31), was significantly decreased by pIC/PPHAffibody treatment (Fig. 1D).

pIC/PPHAffibody induced apoptosis in HER2-overexpressing cells

To elucidate whether pIC/PPHAffibody treatment induces apoptotic cell death, cleavage of caspase-3 and poly (ADP-ribose)

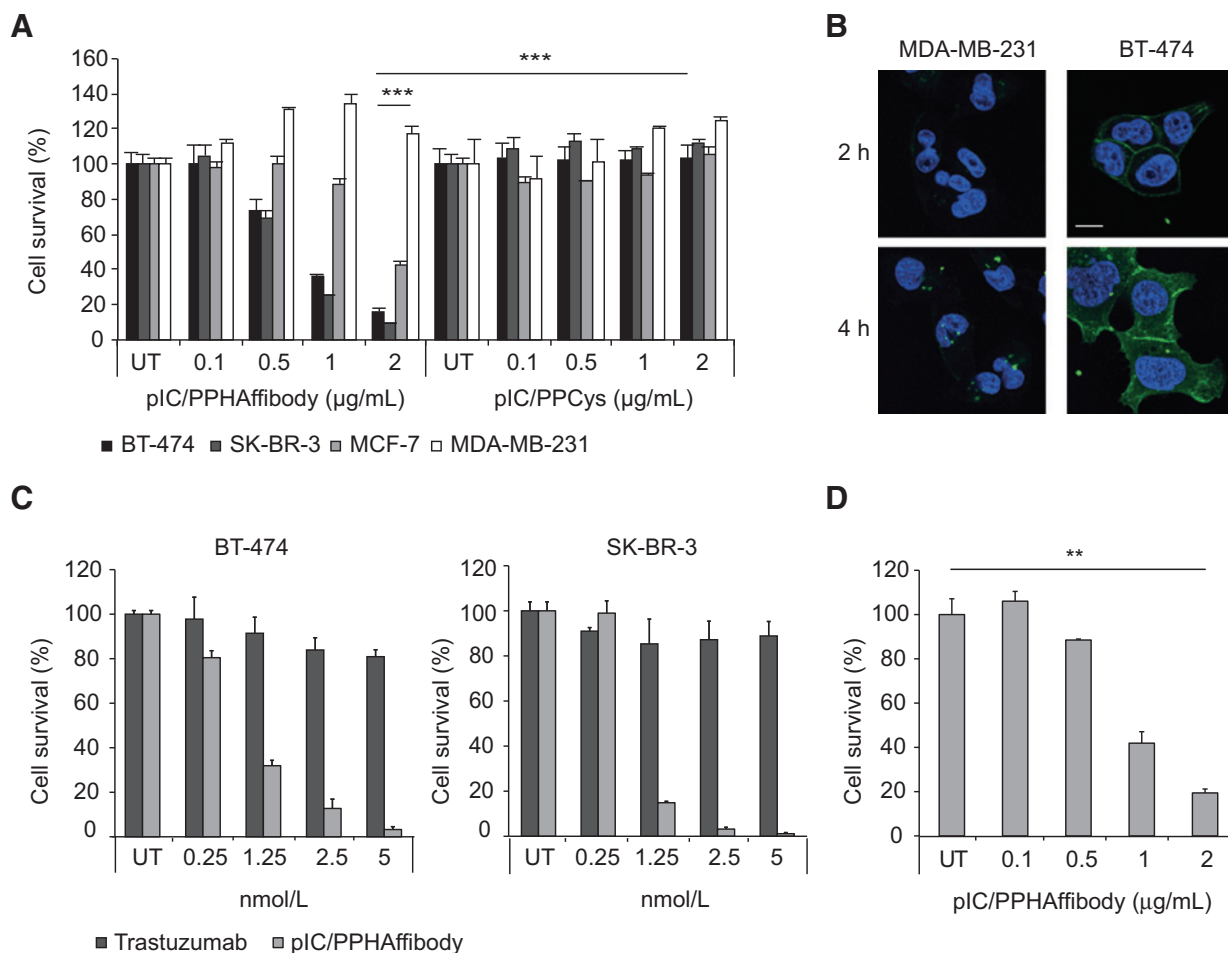


Figure 1.

Selective delivery of pIC/PPHAffibody decreases the survival of HER2-overexpressing cells. **A**, survival of BT-474, SK-BR-3, MCF-7, and MDA-MB-231 cells treated with pIC/PPHAffibody or pIC/PPCys at the indicated concentrations for 72 hours (***, $P < 0.001$ BT-474 vs. MDA-MB-231 or for pIC/PPHAffibody vs. pIC/PPCys). **B**, internalization of pIC/PPHAffibody into BT-474 and MDA-MB-231 after 2 and 4 hours of treatment (described in Materials and Methods) as shown by confocal microscopy. Pictures were taken at $\times 10$ magnification; scale bar, 4 μm . **C**, survival of BT-474 and SK-BR-3 cells following treatment with pIC/PPHAffibody or trastuzumab. **D**, survival of BT-474 trastuzumab-resistant cells following treatment with pIC/PPHAffibody (**, $P < 0.01$ 2 $\mu\text{g}/\text{mL}$ vs. untreated cells). In **A**, **C**, and **D**, cell survival is presented as the percentage of untreated (UT) cells of the same cell line. Error bars, SD. Representative figures of at least three independent experiments.

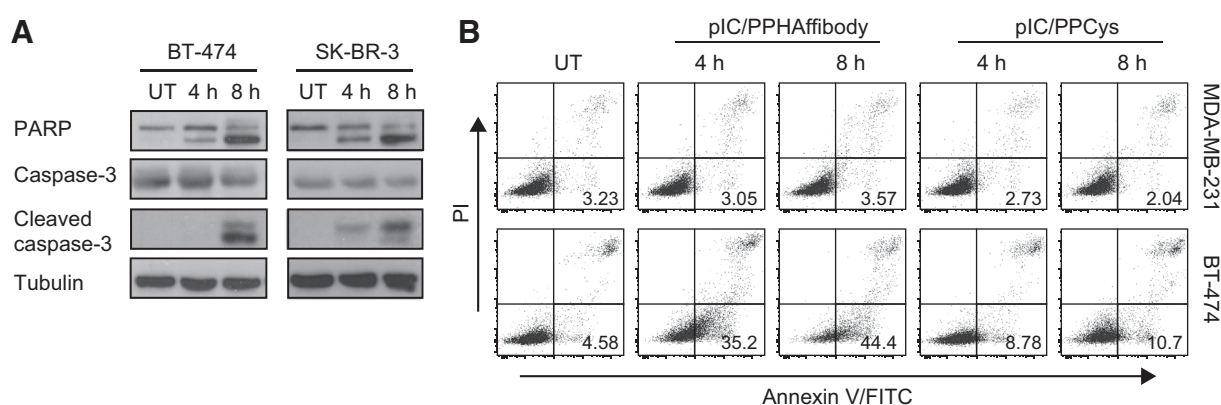


Figure 2.

pIC/PPHAffibody induces cellular apoptosis. **A**, Western blot analysis of PARP and caspase-3 cleavage in BT-474 and SK-BR-3 cells treated with 2 μ g/mL pIC/PPHAffibody for 4 and 8 hours, Tubulin served as a loading control. UT, untreated. **B**, BT-474 and MDA-MB-231 cells were treated with pIC/PPHAffibody or pIC/PPCys (2 μ g/mL) for 4 and 8 hours. Annexin V-FITC and PI-PE binding were measured by flow cytometry. Numbers represent the percentage of cells in the respective quadrants. Representative figures of at least three independent experiments.

polymerase (PARP) was analyzed by Western blot (Fig. 2A). A slight apoptotic effect was detected at 4 hours of treatment with pIC/PPHAffibody (2 μ g/mL), with more extensive cleavage of caspase-3 and PARP after 8 hours, in both BT-474 and SK-BR-3 cells (Fig. 2A). Treatment of BT-474 cells with pIC/PPHAffibody for 4 or 8 hours led to a robust increase in Annexin V-positive cells, as compared with untreated or MDA-MB-231 cells (Fig. 2B). In contrast, cells treated with pIC/PPCys showed little Annexin V staining (Fig. 2B). These results indicated that pIC/PPHAffibody induces apoptotic cell death, and that the decreased survival of cells with high amounts of HER2 receptor was due to apoptosis.

Treatment with pIC/PPHAffibody promotes a proinflammatory effect

Escape of immune surveillance is a hallmark of cancer (16). Accordingly, to generate an effective therapy it is essential to reactivate immune surveillance against the tumor. pIC induces secretion of type I IFN and other cytokines, all of which are associated with immune cell recruitment and activation (35). Therefore, we examined whether targeted delivery of pIC via PPHAffibody into HER2-overexpressing cells could induce anti-tumor cytokines. Profound increases in IFN α and IFN β mRNA expression were seen in BT-474 and SK-BR-3 cells after treatment with pIC/PPHAffibody (Fig. 3A and B). In addition, treatment of BT-474 with pIC/PPCys did not induce an increase in IFN β secretion (Supplementary Fig. S2A).

Several proinflammatory chemokines that are induced by pIC are known to promote lymphocyte recruitment, including IP-10, RANTES (CCL5), and GRO- α (35–37). Additionally, IP-10 not only promotes T-cell recruitment, but also induces the generation of effector T cells (38). To determine whether targeted delivery of pIC induces the expression and secretion of these cytokines, qRT-PCR and ELISA assays were performed. mRNAs for IP-10, RANTES, and GRO- α were strongly induced in BT-474 cells treated with pIC/PPHAffibody, whereas almost no stimulation was induced by pIC/PPCys (Supplementary Fig. S2B). ELISA assays showed that treatment with pIC/PPHAffibody increased IP-10, RANTES, and GRO- α secretion in both BT-474 cells and SK-BR-3 cells. At 2 μ g/mL of pIC, lower concentrations of cyto-

kines were observed, due to the rapidity of cell death at this concentration, which precludes cytokine accumulation (Fig. 3C and D, respectively). In MCF-7 cells, pIC/PPHAffibody induced IP-10 secretion but to a lesser extent than that in BT-474 and SK-BR-3 cells, whereas in MDA-MB-231 cells IP-10 did not change (Supplementary Fig. S2C). RANTES and GRO- α were undetectable in both MCF-7 and MDA-MB-231 (data not shown).

Induction of PBMC-mediated killing after pIC/PPHAffibody treatment

Having demonstrated that pIC/PPHAffibody treatment induces the secretion of activators of the innate and adaptive immune responses, we next tested whether pIC/PPHAffibody treatment could induce lymphocyte recruitment. Medium was collected from BT-474 or SK-BR-3 cells treated with pIC/PPHAffibody for 48 hours and placed in the bottom of a Boyden chamber. PBMCs were placed in the upper chamber. After 4 hours, lymphocyte chemotaxis was increased by ≥ 3 -fold (Fig. 4A). Thus, treatment with pIC/PPHAffibody induced cytokine secretion, which in turn promoted lymphocyte chemotaxis.

To test whether pIC/PPHAffibody treatment increased PBMC cytotoxicity, BT-474^{GFP/LUC+} cells were first treated with pIC/PPHAffibody for 48 hours and then cocultured with PBMCs for 48 additional hours. The addition of PBMCs led to a strong decrease in BT-474^{GFP/LUC+} cell survival (Fig. 4B). Increased IFN γ mRNA expression was seen in PBMCs cultured with medium from pIC/PPHAffibody-treated BT-474 cells, but not in PBMCs that received medium from cells treated with pIC/PPCys (Fig. 4C). Our results suggest that the treatment of HER2-overexpressing cells with pIC/PPHAffibody activated PBMCs to further attack any remaining tumor cells, in accordance with our previous findings with EGFR-overexpressing tumors (17).

Upregulation of MHC-I by pIC/PPHAffibody treatment

Downregulation of MHC-I is one of the mechanisms associated with tumor immune escape and decreased lysis by cytotoxic T cells. pIC promotes MHC I expression and antigen presentation (39–42). To establish whether pIC/PPHAffibody treatment could induce upregulation of MHC-I expression in HER2-overexpressing cells, BT-474 and SK-BR-3 cells were treated

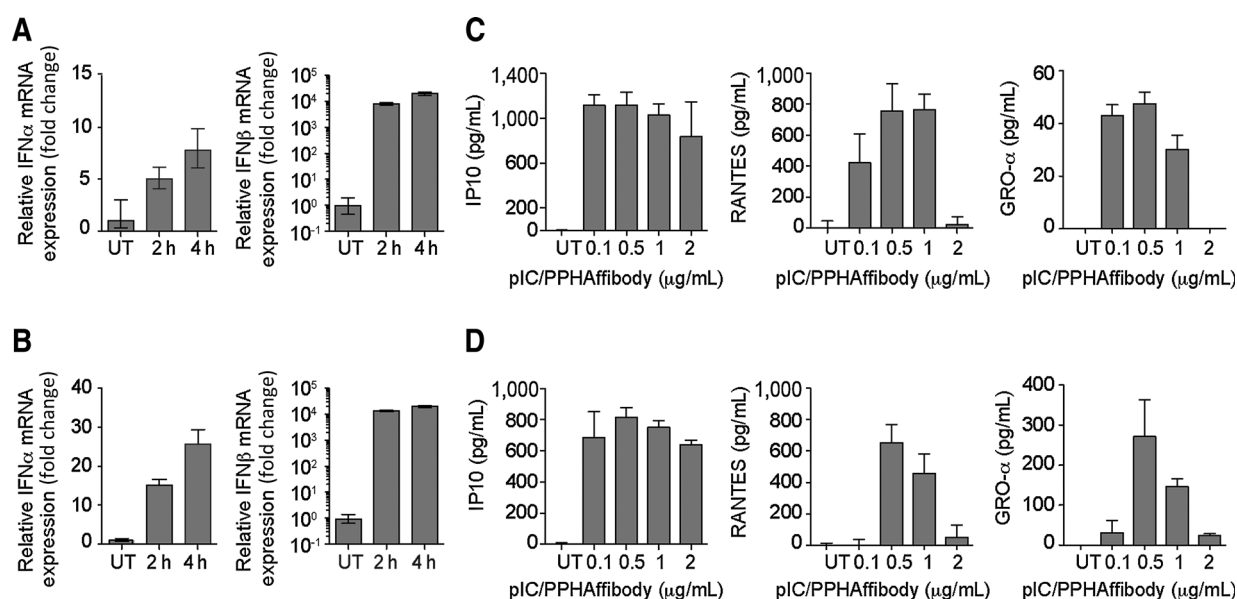


Figure 3.

pIC/PPHAffibody leads to the induction of proinflammatory cytokine expression. qRT-PCR analysis of type I IFN expression in BT-474 (A) and SK-BR-3 cells (B) treated with pIC/PPHAffibody (1 μ g/mL) for 2 or 4 hours. Data are expressed as fold change [relative quantification (RQ)] in IFN α or IFN β expression in pIC/PPHAffibody-treated samples, normalized to GAPDH and compared with untreated (UT) cells. Error bars, \pm RQ_{max}/RQ_{min}. For IFN β , log₁₀ scale is used for the y-axis. ELISA assays for IP-10, RANTES, and GRO- α were performed on supernatants of BT-474 (C) and SK-BR-3 cells (D) treated for 48 hours with pIC/PPHAffibody at the indicated pIC concentrations. Each assay was conducted in triplicate and data are presented as mean concentration + SD.

with pIC/PPHAffibody for 24 hours and subjected to flow cytometry. pIC/PPHAffibody treatment greatly increased HLA class I surface expression on both BT-474 and SK-BR-3 cells as compared with untreated cells (Fig. 5). Thus, treatment with pIC/PPHAffibody can potentiate the detection and activation of tumor-infiltrating T cells against HER2-overexpressing tumors.

pIC/PPHAffibody treatment inhibits tumor growth *in vivo*

To investigate whether targeted delivery of pIC can abrogate *in vivo* tumor growth, HER2-overexpressing MCF-7 cells (MCF-7-HER2) were inoculated into nude mice. The rate of tumor growth

was significantly slower in mice treated with pIC/PPHAffibody than in trastuzumab treated or control mice (*, $P < 0.05$; Fig. 6A).

Because our *in vitro* studies showed that pIC/PPHAffibody induced the recruitment and activation of immune cells against treated tumor cells, we hypothesized that pIC/PPHAffibody treatment would be more effective in immunocompetent mice. Immunocompetent BALB/c mice were inoculated s.c. with HER2-overexpressing RENCA cells (RENCA-HER2). Although pIC/PPHAffibody treatment did not have a strong effect on RENCA-HER2 cell survival *in vitro* (Supplementary Fig. S3A), the growth of RENCA-HER2 tumors in BALB/c mice was suppressed (***, $P < 0.001$;

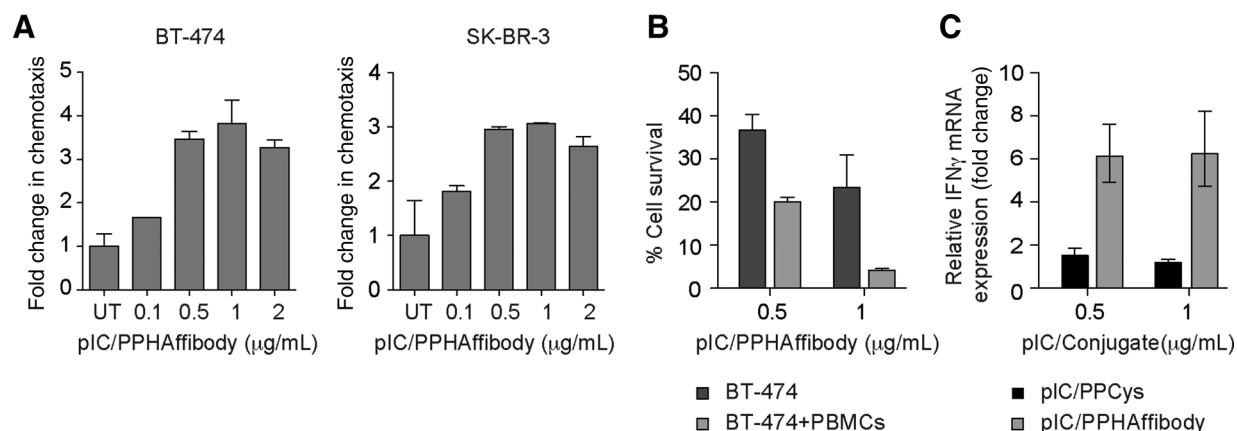


Figure 4.

A, PBMC chemotaxis toward medium from BT-474 and SK-BR-3 cells treated with pIC/PPHAffibody at the indicated concentrations for 48 hours. B, luciferase assay showing survival of BT-474^{GFP/LUC+} cells treated with pIC/PPHAffibody incubated with and without PBMCs (survival is the percentage of untreated cells). C, qRT-PCR analysis of IFN γ mRNA expression in PBMCs cultured in medium from BT474 cells treated with pIC/PPHAffibody. Representative data from at least three independent experiments.

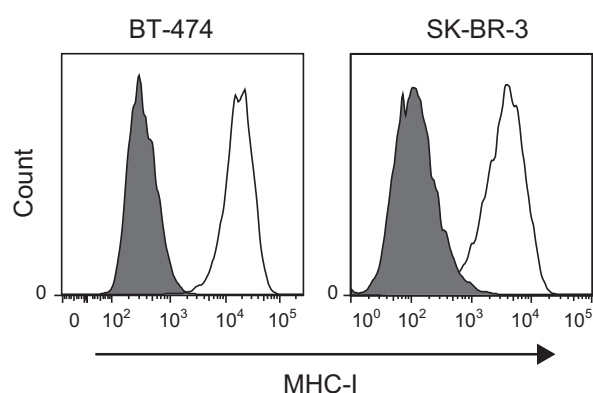


Figure 5. pIC/PPHAffibody treatment induces upregulation of HLA class I expression. Flow cytometry analysis of MHC-I surface expression in BT-474 and SK-BR-3 cells treated with pIC/PPHAffibody compared with untreated cells. Representative data from at least three independent experiments.

Fig. 6B). Moreover, immunohistochemistry for cleaved caspase-3 showed strong induction of apoptosis (Supplementary Fig. S3B). To confirm that the profound inhibition of tumor growth was not tumor type specific, we tested the efficacy of pIC/PPHAffibody

treatment in another immunocompetent model, MMTV-huHER2 mammary tumor transplants. Tumor cells were isolated from spontaneous mammary tumors arising in MMTV-huHER2 transgenic mice (32) and were inoculated into the mammary fat pad of FVB mice. As in the BALB/c model, tumor growth was inhibited by pIC/PPHAffibody (**, $P < 0.01$; Fig. 6C). In both tumor models, a statistically significant survival benefit was achieved in mice treated with pIC/PPHAffibody.

We also assessed the effects of pIC/PPHAffibody on the infiltration of immune cells into the RENCA-HER2 tumors by flow cytometry (Fig. 6D). Treatment with pIC/PPHAffibody significantly increased NK cell (21.5% control vs. 32.6% treated) and CD8⁺T-cell (13.8% control vs. 23.9% treated) infiltration into the tumors (**, $P < 0.01$). When NK or CD8⁺T cells were depleted from the RENCA-HER2 mice treated with pIC/PPHAffibody, tumor growth increased in both of the depleted groups as compared with mice treated with pIC/PPHAffibody alone (pIC/PPHAffibody alone vs. pIC/PPHAffibody-NK or pIC/PPHAffibody-CD8⁺, *, $P < 0.05$; Fig. 6E). These results strongly support our hypothesis that pIC/PPHAffibody not only directly causes tumor cell apoptosis, but also engages the innate and adaptive immune responses to repress tumor growth.

To determine whether pIC/PPHAffibody treatment could be used to treat trastuzumab-resistant tumors in which the extracellular domain of HER2 was shed (43), we used the trastuzumab-

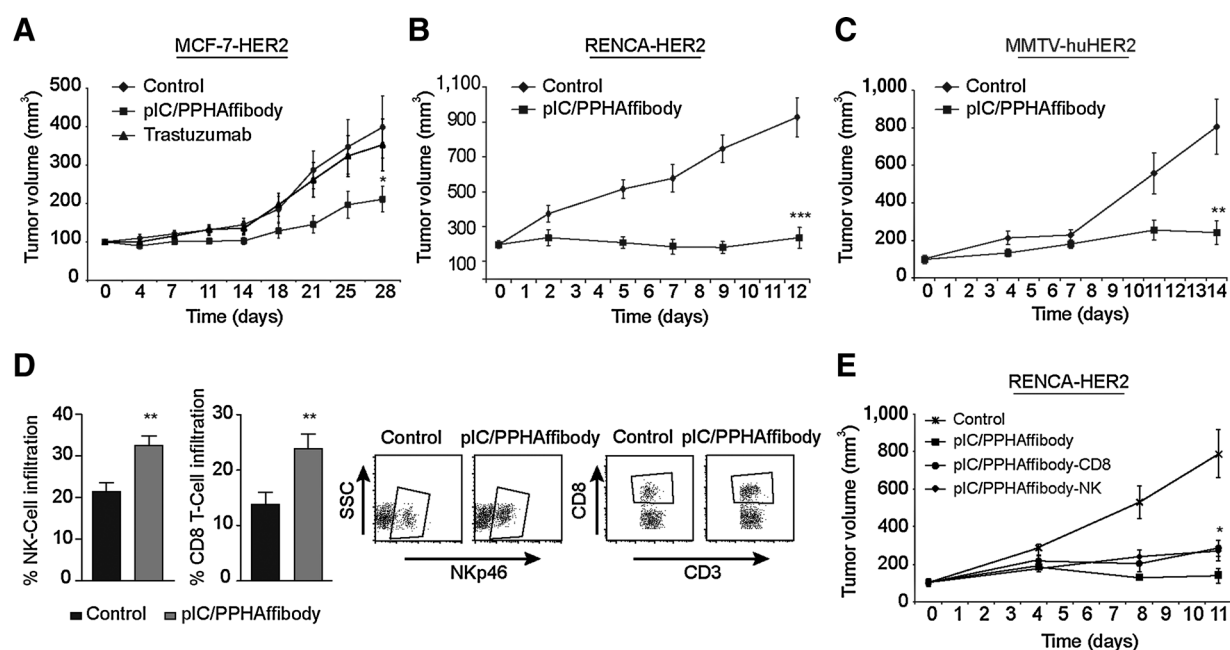
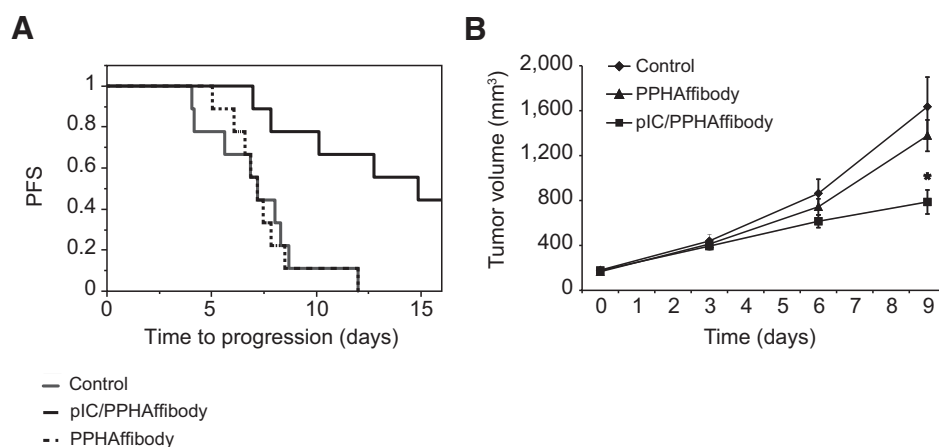


Figure 6. Antitumor activity of pIC/PPHAffibody on HER2-overexpressing tumors in immunodeficient and immunocompetent mouse models. **A**, MCF-7-HER2 cells were inoculated into the mammary fat pads of nude mice. Mice bearing ~100-mm³ tumors were divided into 3 different groups: control (untreated), trastuzumab, and pIC/PPHAffibody. (*, $P < 0.05$, pIC/PPHAffibody vs. control). **B**, effect of pIC/PPHAffibody on RENCA-HER2 tumors (s.c.) in BALB/c mice (***, $P < 0.001$; treatment vs. untreated control). **C**, tumor cells derived from established MMTV-huHER2 tumors were inoculated into the mammary fat pads of FVB mice. Treatment with pIC/PPHAffibody began when tumors reached ~200 mm³ (**, $P < 0.01$; treatment vs. untreated control). **D**, RENCA-HER2 cells were inoculated s.c. into BALB/c mice ($n = 5-7$). When the tumors reached ~150 mm³, mice were treated for 4 days with pIC/PPHAffibody. Tumors were analyzed by flow cytometry. NK cells (NKp46/CD45.2⁺) and CD8 T-cells (CD8⁺/CD3⁺) were gated out of total live cells. Representative dot plots are shown. (**, $P < 0.01$, treatment vs. control). **E**, RENCA-HER2 cells were inoculated s.c. into BALB/c mice. When the tumors reached 100 mm³, the mice were divided into 4 treatment groups ($n = 6-7$): control (untreated); pIC/PPHAffibody; CD8 depleting antibody (i.p. every 2 days) + pIC/PPHAffibody; NK depleting antibody (i.p. every 4 days) + pIC/PPHAffibody. pIC/PPHAffibody was administered i.v. every 24 hours. (*, $P < 0.05$, pIC/PPHAffibody vs. NK or CD8 depleted mice). Data, mean \pm SEM.

Figure 7.

Efficacy of pIC/PPHAffibody on a trastuzumab-resistant mouse model. Fo5 mammary tumors were propagated into the thoracic mammary fat pad of female FVB mice. When tumors reached a volume of 100 to 300 mm³, mice were treated with pIC/PPHAffibody. **A**, progression-free survival (PFS) analysis (*, $P = 0.0165$; pIC/PPHAffibody vs. control). **B**, tumor volume (*, $P < 0.05$; control vs. pIC/PPHAffibody-treated mice). Data, mean \pm SEM.



resistant syngeneic Fo5 mouse model, derived by serial tumor transplantation from MMTV-HER2 Founder #5 (32, 43). These tumors express p95HER2, and the mice have high amounts of circulating HER2 extracellular domain (43). Treatment with pIC/PPHAffibody significantly increased the time to tumor progression (Fig. 7A). The median time to progression for treated mice was 14.9 days, compared with 7.2 days in controls (log-rank $P = 0.0165$). Moreover, a statistically significant reduction in tumor volume was also observed (*, $P < 0.01$; Fig. 7B). These results indicate that pIC/PPHAffibody has potential as a treatment even for trastuzumab-resistant patients.

Discussion

Early diagnosis and targeted treatments have improved the progression-free survival of breast cancer patients with HER2 overexpression. Yet, many patients experience a relapse and have limited treatment options. This situation motivated us to seek new therapeutic modalities that would effectively target HER2-overexpressing tumors. Here, we introduce a targeted therapy that selectively delivers synthetic dsRNA, pIC, into HER2-overexpressing breast tumor cells to induce targeted cell killing and promote an immune response against the tumor. Although it is a potent activator of the adaptive and innate immune responses (44), pIC caused severe toxicity when used systemically as naked pIC in clinical trials (45, 46). The targeted delivery of pIC into HER2-overexpressing tumors is expected to abrogate the toxicity observed in previous trials, while stimulating an overall therapeutic effect.

The internalization of pIC into HER2-overexpressing tumor cells stimulated the activation of proapoptotic and proinflammatory processes, specifically in HER2-overexpressing cells. In contrast with trastuzumab and T-DM1 (43), the kinetics of cell death caused by pIC/PPHAffibody were very rapid; caspase-3 and PARP cleavage were detected after 4 to 8 hours, and the cells were almost completely eradicated after 72 hours of treatment. Treatment with pIC/PPHAffibody led to the expression of antiproliferative (e.g., IFN) and immunostimulatory cytokines (e.g., IP-10, GRO- α , and RANTES), triggering immune cell recruitment and activation and leading to extensive cancer cell killing *in vitro*.

There are several mechanisms whereby HER2-targeted pIC could restore antitumor immune surveillance. Tumor-infiltrating lymphocytes may be incompletely activated, anergic, or function-

ally defective (47). pIC/PPHAffibody led to the induction of IFN α/β and additional cytokines that can activate various cells of the innate system, such as NK, macrophages, and antigen presenting cells, which in turn activate the adaptive immune system (48). Type I IFN can also directly induce the adaptive immune system by regulating T-cell survival, proliferation, and activation (49, 50). An additional mechanism is the enhanced MHC class I expression evinced by pIC/PPHAffibody, which may facilitate the activation of T cells against the tumor, thus leading to enhanced tumor killing.

Our *in vivo* data underscore the important roles of the innate and adaptive immune systems in the elimination of tumors by pIC/PPHAffibody. Despite their low sensitivity to pIC/PPHAffibody *in vitro*, RENCA-HER2 tumors grown in immunocompetent mice were strongly inhibited by pIC/PPHAffibody treatment. Tumor-infiltrating lymphocytes are predictive of improved prognosis in breast cancer (51). HER2-targeted pIC generated an influx of tumor-infiltrating NK and CD8⁺ T cells the depletion of which, in combination with pIC/PPHAffibody treatment, resulted in an increase in tumor growth. pIC/PPHAffibody was also effective against two models derived from MMTV-huHER2 tumors, including a trastuzumab-resistant tumor model, Fo#5. Although the Fo#5-derived, trastuzumab-resistant tumors have been shown to express the p95HER2 receptor, they also express the full-length receptor. pIC/PPHAffibody treatment efficiently increased the progression-free survival of these mice, indicating that pIC/PPHAffibody can be used to target trastuzumab-resistant tumors. In contrast with the immunocompetent models, MCF-7-HER2 tumors were significantly less sensitive *in vivo*, where they were propagated in nude mice with an impaired T-cell response. Our results in immunocompetent mice elucidate the immune stimulatory effect of HER2-targeted pIC.

The effects of HER2-targeted pIC—namely the attraction of immune cells to the tumor, the activation of immune cells, and the stronger presentation of tumor-specific antigens by the induced expression of MHC-I—should strongly enhance the efficacy of most cancer immune therapies.

In conclusion, we present a novel vector for targeting the delivery of pIC to HER2-positive tumors, including trastuzumab-resistant tumors. We show that HER2-targeted pIC treatment caused tumor cell apoptosis and mobilized immune surveillance against the tumor. Moving this therapy to the clinic is of high

importance as this modality is expected to improve patients' outcomes, reducing the morbidity and mortality associated with HER2-overexpression in breast and other tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Zigler, A. Shir, S. Klein, S.-F. Yu, G. Mizraji, M.X. Sliwkowski

Writing, review, and/or revision of the manuscript: M. Zigler, S. Klein, G. Mizraji, M.X. Sliwkowski, A. Levitzki

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