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Original Article

Trastuzumab in combination with PEGylated interferon-α1b exerts synergistic antitumor activity through enhanced inhibition of HER2 downstream signaling and antibody-dependent cellular cytotoxicity

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Abstract: The anti-HER2 monoclonal antibody trastuzumab is the mainstay of treatment for HER2-positive breast and gastric cancer, and its combination with multiple chemotherapeutic agents has represented an effective and rational strategy in the clinic. In this study, we report that trastuzumab in combination with PEGylated interferon-α1b (IFN-α1b), a polyethylene glycol (PEG)-conjugated form of a subtype of interferon alpha (IFN-α), synergistically inhibited the proliferation of HER2-positive cells, including BT-474 and SK-BR-3 breast cancer cells and NCI-N87 gastric cancer cells, and also induced their apoptosis, but had no effect on HER2-negative MDA-MB-231 breast cancer cells. Trastuzumab inhibited phosphorylation of HER2, AKT and ERK, an effect that was enhanced by PEGylated IFN-α1b, likely owing to PEGylated IFN-α1b-mediated downregulation of HER2 through the lysosomal degradation pathway. Moreover, PEGylated IFN-α1b significantly enhanced trastuzumab-mediated antibody-dependent cellular cytotoxicity (ADCC) in HER2-positive cells. Importantly, trastuzumab combined with PEGylated IFN-α1b exhibited significant synergistic antitumor activity in HER2-positive BT-474 xenografts, an effect that was associated with enhanced inhibition of HER2 expression and AKT and ERK phosphorylation. Strikingly, depletion of natural killer cells with anti-Asialo GM1 antibody abrogated the synergistic antitumor activity, indicating that augmented ADCC is essential for this synergy. Taken together, our findings indicate that both enhanced inhibition of HER2 downstream signaling and augmented ADCC contribute to the synergistic antitumor activity of trastuzumab with PEGylated IFN-α1b, and imply that combining trastuzumab with PEGylated IFN-α1b could be a promising strategy for HER2-positive cancers.

Keywords: Trastuzumab, PEGylated IFN-α1b, ADCC, HER2-positive cancer, synergistic effect

Introduction

HER2 is a member of the human epidermal growth factor receptor family, which is involved in regulating cell growth, differentiation, and survival through a variety of signal transduction cascades, including Ras/Raf/MEK/ERK and PI3K/AKT pathways [1, 2]. Approximately 15-20% of breast cancers with HER2 overexpression are associated with increased risk of local growth and distant metastasis [2-4]. A number of studies have demonstrated that HER2 is also present in other cancers, particularly gastric cancer. An estimated 15-20% of advanced gastric and gastroesophageal junction cancers, which are especially prevalent in East Asia, exhibit overexpression or amplification of HER2 [5]. Thus, HER2-targeted therapy is a clinically established strategy for treating HER2-positive cancers.

Trastuzumab, a humanized anti-HER2 monoclonal antibody (mAb), has profoundly improved the course of disease and survival of patients with HER2-overexpressing breast [4] and gastric cancer [5, 6]. Its activity *in vitro* is credited largely to inhibition of HER2 signaling. Studies in both experimental mouse models and patients have shown that the immune system contributes substantially to the therapeutic

effects of trastuzumab *in vivo* [7]. A growing body of preclinical and clinical evidence points to the importance of innate immunity mediated by antibody-dependent cellular cytotoxicity (ADCC) as essential for the clinical effect of trastuzumab, with Fc receptor-expressing natural killer (NK) cells and monocytes playing major roles in the resulting antitumor response [8, 9]. In addition to these components of innate immunity, reports have shown that effective anti-HER2 antibody treatment also requires an adaptive immune response [8]. The above studies provide a rationale for combining immunomodulating agents and trastuzumab to improve therapeutic effects and clinical efficacy. Indeed, combining trastuzumab with immune modulators, such as anti-CD137 and anti-PD-1 mAb, activates trastuzumab, resulting in an augmented response [10, 11].

Interferon alpha (IFN-α), a member of the type I interferon family of cytokines, consists of several subtypes, all of which bind to the IFN-α/β-receptor (IFNAR) and activate the JAK/STAT signaling pathway, leading to the transcription and translation of genes whose protein products mediate antiviral, antiproliferative, antitumor, and immune-modulating effects [12-14]. Recombinant IFN-α has been approved to treat hematological tumors as well as solid tumors. In clinical practice, IFN-α is used in the treatment of multiple myeloma (MM) [15], chronic myeloid leukemia (CML) [16], non-Hodgkin's lymphoma (NHL) [17], renal cell carcinoma (RCC) [18], head and neck tumors (H&NT) [19], melanoma, and medullary thyroid carcinoma (MTC) [12]. In addition to monotherapy against tumor cells, IFN-α administration can potentially synergize with chemotherapy and targeted therapy through different mechanisms of action that affect apoptosis, cell dormancy, and activation of antitumor immune responses [20]. Polyethylene glycol (PEG) modification (PEGylation) of interferon reduces interferon clearance and thereby extends the duration of its therapeutic effects, resulting in a more convenient less-frequent schedule of injections and less immunogenicity [21]. PEGylated IFN-α2a and PEGylated IFN-α2b have been widely used to treat patients with chronic hepatitis B or C virus infection [22].

Interestingly, type I interferons appear to be involved in the mechanism of action of anti-HER2 mAb therapy [10, 23, 24], as evidenced by the fact that neutralization of IFNAR1 abrogates the therapeutic efficacy of anti-HER2 antibodies [10]. These findings prompted us to investigate whether HER2-targeted therapy in combination with type I IFN-α might produce enhanced antitumor efficacy.

In the present study, we investigated the efficacy of trastuzumab in combination with PEGylated IFN-α1b against HER2-positive cancers both *in vitro* and *in vivo*. We found that trastuzumab combined with PEGylated IFN-α1b exerted synergistic antitumor effects against HER2-positive cancer cells both *in vitro* and *in vivo*, actions that reflected enhanced inhibition of HER2-mediated downstream signaling and enhanced trastuzumab-mediated ADCC. Thus, our data provide a basis for clinical application of combined trastuzumab and PEGylated IFN-α1b therapy for HER2-positive cancers.

Materials and methods

Materials

Trastuzumab (Herceptin) was purchased from Roche (Basel, Switzerland). PEGylated IFN-α1b was provided by the Shanghai Institute of Biological Products Co., Ltd (Shanghai, China). Sulforhodamine B (SRB) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Antibodies against HER2, phospho-HER2 (Tyr-1221/1222), Stat1, phospho-Stat1 (Tyr701), AKT, phospho-AKT (Ser473), phospho-ERK1/2 (Thr202/Tyr204), β-tubulin and GAPDH were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against ERK1/2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-Asialo GM1 antibody was purchased from Thermo-Fisher Scientific (Sunnyvale, CA, USA).

Cell culture

BT-474, SK-BR-3, NCI-N87, and MDA-MB-231 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The NCI-H292 cell line was obtained from the Cell Bank of the Chinese Academy of Sciences. All cell lines were cultured according to instructions provided by the ATCC.

Cell proliferation assay

Tumor cells were seeded in 96-wells plates overnight, after which they were treated with

increasing concentrations of compounds (in triplicate) for the indicated time. Antiproliferative activity was accessed using SRB assays, as described previously [25]. The combination index (CI) value, which defines the interaction between two drugs as synergistic (CI<1), additive (CI=1) or antagonistic (CI>1), was determined on the basis of the median effect principle using CalcuSyn software.

Western blotting

Cells were collected and washed with phosphate-buffered saline (PBS; pH 7.4) after treating with drugs for the indicated time, and then were lysed in sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris-HCl pH 6.8, 2% SDS, 20% glycerol, 1 mM dithiothreitol). Equal amounts of protein were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then were transferred to polyvinylidene difluoride (PVDF) membranes. Blots were probed with primary antibodies and then incubated with the appropriate secondary antibodies (Millipore, Bedford, MA, USA). Proteins were detected by immunoblotting using a Western blot imaging System (Clinx Science Instruments, Shanghai, China) with Chemi Capture software.

Quantitative real-time polymerase chain reaction (qRT-PCR)

After drug treatment, RNA was isolated using the TRIzol reagent system and reverse-transcribed using the PrimeScript Reverse Transcription reagent Kit (Takara, Dalian, China). qPCR was performed on a StepOnePlus Real-Time PCR System (ThermoFisher Scientific) according to the instructions for the SYBR Premix Ex Taq II kit (Takara, Dalian, China). mRNA was quantified using the $2^{-\Delta\Delta CT}$ method, and target mRNA levels were normalized to those of the housekeeping gene, *GAPDH*. The following primer pairs were used for qRT-PCR analysis: HER2, 5'-CTG CAC CCA CTC CTG TGT GGA CCT G-3' (forward) and 5'-CTG CCG TCG CTT GAT GAG GAT C-3' (reverse); GAPDH, 5'-GGG GAA GGT GAA GGT CGG AGT C-3' (forward) and 5'-CAA GCT TCC CGT TCT CAG CCT T-3' (reverse).

ADCC

A nonradioactive, fluorometric cytotoxicity assay employing calcein-acetoxymethyl (Calcein-AM) (Dojindo, Kyushu, Japan) was used to measure BT-474 cell lysis and trastuzumab-mediated ADCC [26]. Peripheral blood mononuclear cells (PBMCs) (Changhai Hospital, Shanghai, China) were incubated with PEGylated IFN-α1b (30 μg/mL) or PBS (control) overnight and wash out the agent before coincubation with target cells. BT-474 cells were treated with 1 μM Calcein-AM for 20 minutes and then washed two times. BT-474 cells (target [T] cells) were then treated with different concentrations of trastuzumab for 1 h at 37°C, followed by culturing with PBMCs (effector [E] cells) at an E:T ratio of 50:1 for an additional 4 h. Cells were centrifuged and the fluorescence of supernatants was analyzed using a Synergy H4 Hybrid microplate reader, with excitation at 490 nm and emission at 515 nm. Maximum fluorescence was determined by lysis in 0.1% Triton X-100 without centrifugation.

In vivo study

Female nude mice (Balb/cA-nude, 5-6 weeks old) were purchased from Jiangsu GemPharmatech Co., Ltd (Nanjing, China). BT-474 xenografts were established by inoculating nude mice subcutaneously with BT-474 cells. When tumors reached a volume of ~100 mm^3, mice were randomly assigned to control (n=10~12) and treatment (n=6) groups. Control groups were given vehicle alone, and treatment groups received trastuzumab (intravenous injection, i.v.), PEGylated IFN-α1b (subcutaneous injection, s.c.), or both, twice a week for a total of 3 weeks. Tumor volume was calculated as (length × $width^2$)/2, and body weight was monitored as an indicator of general health. Tumor tissues were collected 24 h after the last dosing. Tumor samples were analyzed by Western blotting. NK cell depletion was performed by intraperitoneal injection (i.p.) of 50 μg anti-Asialo GM1 antibody (Thermo-Fisher Scientific) on days 0, 7 and 14. NK cell depletion in peripheral blood was confirmed by flow cytometry analysis using an NKp46-FITC antibody (Thermo-Fisher Scientific). Animal experiments were conducted in accordance with the Institutional Animal Care and Use Committee guidelines of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

Data analysis

Data were analyzed with GraphPad Prism Version 7 software (GraphPad Software, Inc.,

San Diego, USA). Dose-response curves were generated based on non-linear regression analyses. CalcuSyn software was used to calculate CI values. Data are presented as means ± standard deviation (SD) for *in vitro* experiments and means ± standard error of the mean (SEM) for *in vivo* studies. A two-tailed Student's t-test was used to test for significance where indicated. Differences were considered significant at a *P*-value <0.05.

Results

Trastuzumab combined with PEGylated IFN-α1b exerts synergistic antiproliferative effects in HER2-positive cancer cells

Because trastuzumab is the first-line drug for HER2-positive breast cancer [27], we first focused on the combined effects of trastuzumab and PEGylated IFN-α1b against HER2-positive breast cancer cells. Trastuzumab combined with PEGylated IFN-α1b exerted synergistic cytotoxicity in BT-474 (CI=0.31) and SK-BR-3 (CI=0.28) cells, two breast cancer cell lines that overexpress HER2. Similar synergistic effects were observed in HER2-positive NCI-N87 gastric cancer cells (CI=0.33) (**Figure 1A**, **1B**). In contrast, no synergy was observed in HER2-negative MDA-MB-231 breast cancer cells (CI=1.08) or EGFR-positive NCI-H292 lung cancer cells (CI=1.18) (**Figure 1A**, **1B**). Furthermore, cotreatment with trastuzumab and PEGylated IFN-α1b induced an increase in cleavage of PARP and caspase-3 and -8, resulting in enhanced apoptosis (**Figure 1C**). Thus, these results demonstrate that the combination of trastuzumab and PEGylated IFN-α1b produces enhanced antiproliferative and pro-apoptotic effects in HER2-positive cancer cells.

Trastuzumab combined with PEGylated IFN-α1b produces enhanced inhibition of HER2-mediated downstream signaling

To explore the mechanism underlying the synergistic anti-proliferative effect, we next examined the combined effects on HER2-mediated and IFNAR-mediated downstream signaling. Cotreatment with trastuzumab and PEGylated IFN-α1b produced a greater suppression of HER2 expression and HER2, AKT and ERK phosphorylation in HER2-positive BT-474, SK-BR-3, and NCI-N87 cells compared with that produced by each drug alone; these effects were not observed in HER2-negative MDA-MB-231 cells (**Figure 2A**). Notably, combined treatment did not alter phosphorylation of Stat1, a key downstream signaling factor of IFNAR, in any of the four cell lines, irrespective of HER2 expression (**Figure 2A**). The enhancement in trastuzumab-induced downregulation of HER2, P-HER2, P-AKT, P-ERK by co-treatment with PEGylated IFN-α1b was abolished by a specific IFNAR-blocking antibody, indicating that PEGylated IFN-α1b acts on IFNAR (**Figure 2B**). Taken together, these results suggest that trastuzumab combined with PEGylated IFN-α1b synergistically inhibits cell proliferation through enhanced HER2 downregulation and inhibition of HER2, AKT and ERK phosphorylation in HER2-positive cancer cells.

PEGylated IFN-α1b downregulates HER2 through lysosomal degradation in HER2-positive cancer cells

Given that trastuzumab-mediated HER2 endocytosis induces a limited decrease in HER2 levels [28], the above results prompted us to explore the effect of PEGylated IFN-α1b on HER2. As shown in **Figure 3A**, PEGylated IFN-α1b downregulated HER2 as well as phosphorylation of HER2, AKT and ERK in HER2-positive BT-474 and SK-BR-3 breast cancer cells and NCI-N87 gastric cancer cells. We further found that PEGylated IFN-α1b downregulated HER2 expression and HER2, AKT and ERK phosphorylation in a concentration-dependent and time-dependent manner in BT-474 cells (**Figure 3B**, **3C**). To further understand the possible mechanism of HER2 downregulation, we cotreated cells with PEGylated IFN-α1b and the proteasome inhibitor bortezomib or the lysosomal inhibitor, chloroquine diphosphate (CQ). CQ reversed the degradation of HER2 induced by PEGylated IFN-α1b in BT-474 cells, whereas bortezomib had no such effect (**Figure 3D**). A further quantitative real-time PCR analysis showed that PEGylated IFN-α1b did not modulate *HER2* gene transcription (**Figure 3E**). Collectively, these analyses show that PEGylated IFN-α1b induces HER2 degradation through the lysosomal pathway, which contributes to the enhanced inhibition of HER2 by trastuzumab in combination with PEGylated IFN-α1b in HER2-positive cancer cells.

Trastuzumab combined with PEGylated IFN-α1b exerts enhanced antitumor efficacy against BT-474 xenografts

Given the synergistic effects observed *in vitro*, we next evaluated the antitumor efficacy of

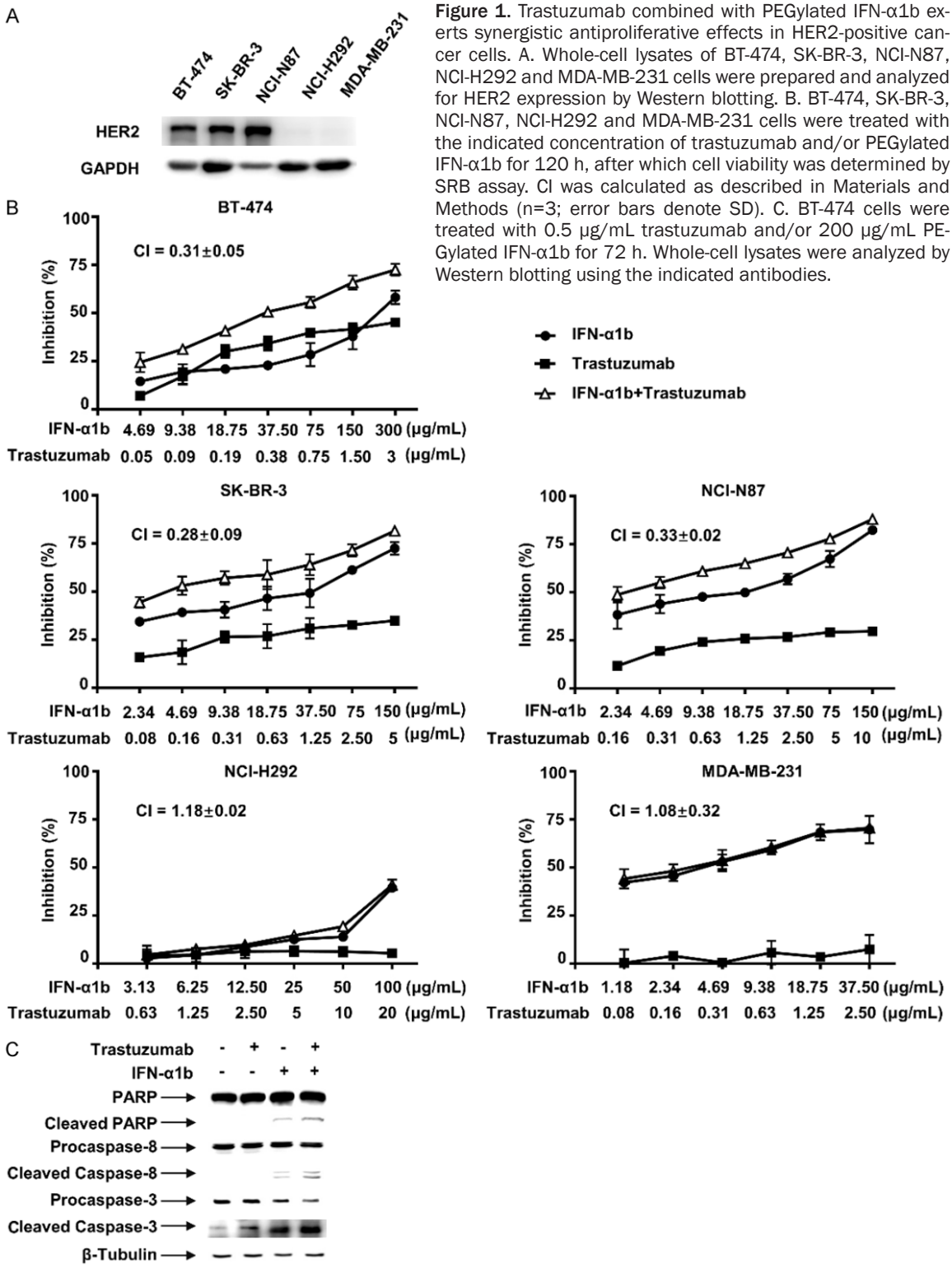

Figure 1. Trastuzumab combined with PEGylated IFN-α1b exerts synergistic antiproliferative effects in HER2-positive cancer cells. A. Whole-cell lysates of BT-474, SK-BR-3, NCI-N87, NCI-H292 and MDA-MB-231 cells were prepared and analyzed for HER2 expression by Western blotting. B. BT-474, SK-BR-3, NCI-N87, NCI-H292 and MDA-MB-231 cells were treated with the indicated concentration of trastuzumab and/or PEGylated IFN-α1b for 120 h, after which cell viability was determined by SRB assay. CI was calculated as described in Materials and Methods (n=3; error bars denote SD). C. BT-474 cells were treated with 0.5 μg/mL trastuzumab and/or 200 μg/mL PEGylated IFN-α1b for 72 h. Whole-cell lysates were analyzed by Western blotting using the indicated antibodies.

combined treatment with trastuzumab and PEGylated IFN-α1b in HER2-positive BT-474 breast cancer xenografts. PEGylated IFN-α1b alone caused dose-dependent inhibition of tumor growth (**Figure 4A**, **4B**). Trastuzumab combined with PEGylated IFN-α1b produced an antitumor efficacy that was significantly ($P<0.05$) superior to that of single agents on day 20 (**Figure 4A**). More importantly, tumors in the group treated with PEGylated IFN-α1b

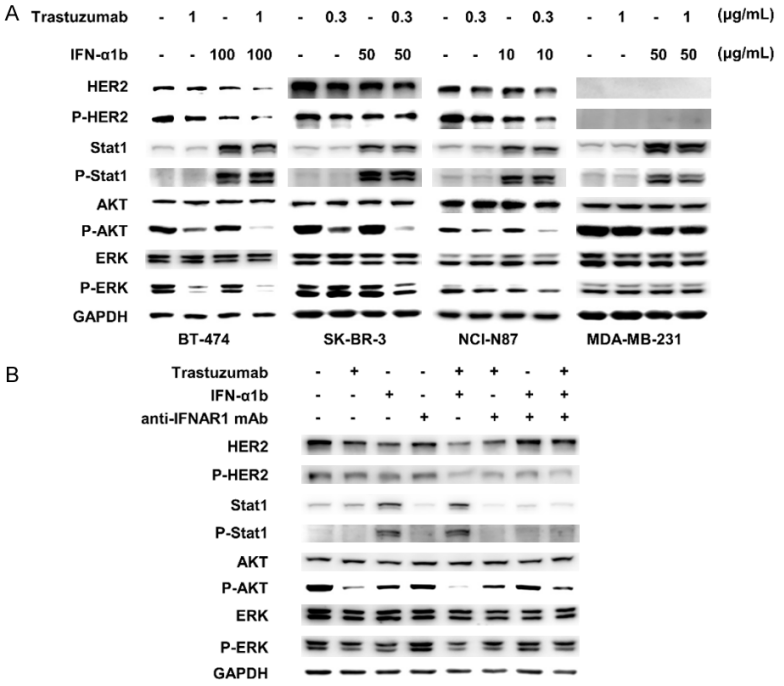

Figure 2. Trastuzumab combined with PEGylated IFN-α1b enhances HER2 downregulation and inhibition of HER2, AKT and ERK phosphorylation. A. BT-474, SK-BR-3, NCI-N87 and MDA-MB-231 cells were treated with the indicated concentration of trastuzumab and/or PEGylated IFN-α1b for 24 h. Whole-cell lysates were analyzed by Western blotting using the indicated antibodies. B. BT-474 cells were pretreated with 100 μg/mL anti-IFNAR1 mAb for 2 h, and then treated with 1 μg/mL trastuzumab and/or 100 μg/mL PEGylated IFN-α1b for 24 h. Whole-cell lysates were analyzed by Western blotting using the indicated antibodies.

alone began to regrow for an additional 40 d, whereas tumors in the cotreatment group remained markedly suppressed over this period (**Figure 4A**). Notably, complete tumor regression was observed in three of six tumors in the cotreatment group, whereas no regression of tumors was observed following treatment with either agent alone (**Figure 4B**). These observations further confirm the enhanced antitumor efficacy of trastuzumab combined with PEGylated IFN-α1b against BT-474 xenografts. Moreover, the combination of trastuzumab with PEGylated IFN-α1b was generally well tolerated, with no significant body weight loss observed during the course of the experiment in any group (**Figure 4A**). Consistent with *in vitro* results, cotreatment with trastuzumab and PEGylated IFN-α1b potentiated the reduction in HER2 expression and AKT and ERK phosphorylation in tumor tissues (**Figure 4C**). Collectively, these findings indicate that cotreatment with trastuzumab and PEGylated IFN-α1b produces enhanced antitumor effects in HER2-positive BT474 breast cancer xenografts that are associated with enhanced downregulation of HER2 and inhibition of AKT and ERK phosphorylation.

PEGylated IFN-α1b significantly augments trastuzumab-mediated ADCC

Notably, in addition to inhibition of the HER2 signaling pathway, trastuzumab-mediated ADCC is an essential component of the antitumor activity of trastuzumab *in vivo* [29]. Thus, we next investigated whether PEGylated IFN-α1b influenced trastuzumab-mediated ADCC. ADCC-mediated cell lysis was measured using a Calcein-AM release assay in BT-474 cells in the presence of PBMCs. Trastuzumab effectively activated ADCC against BT-474 cells, an effect that was significantly enhanced by PEGylated IFN-α1b (**Figure 5A**, **5B**). To define the role of ADCC *in vivo*, we depleted NK cells in a HER2-positive BT-474 breast cancer xenograft animal model using an anti-Asialo GM1 antibody. The proportion of NKp46-positive NK cells in the peripheral blood of mice treated with anti-Asialo GM1 mAb was significantly lower than that in the untreated group, indicating the successful depletion of systemic NK cells (**Figure 5C**). In the absence of functional NK cells, the antitumor activity of trastuzumab combined with PEGylated IFN-α1b (**Figure 5D**, **5E**) was significantly suppressed, demonstrating that NK-mediated ADCC plays an essential role in the enhanced antitumor effect of trastuzumab combined with PEGylated IFN-α1b against BT-474 xenografts.

Taken together, these results suggest that cotreatment with trastuzumab and PEGylated IFN-α1b augments trastuzumab-mediated ADCC activity and enhances inhibition of HER2-mediated downstream signaling, thereby improving antitumor efficacy *in vivo*.

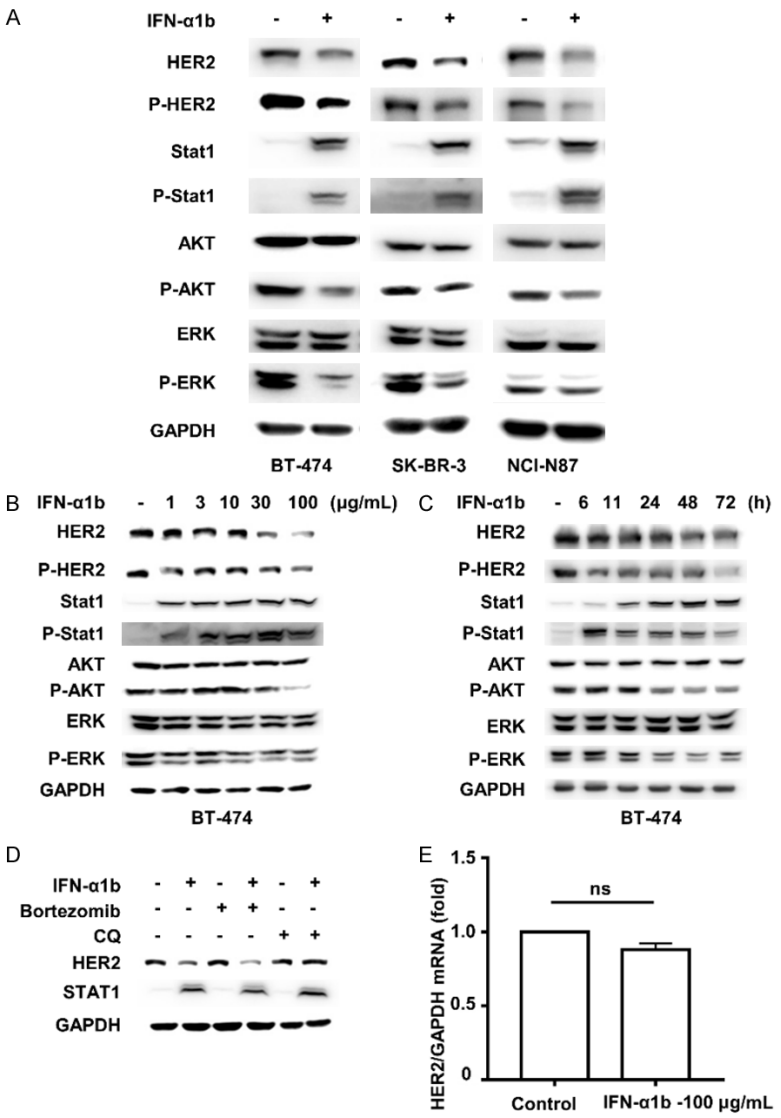

Figure 3. PEGylated IFN-α1b downregulates HER2 through lysosomal degradation. (A) BT-474, SK-BR-3 and NCI-N87 cells were treated with 50 μg/mL PEGylated IFN-α1b for 72 h. Whole-cell lysates were analyzed by Western blotting using the indicated antibodies. (B and C) BT-474 cells were treated with increasing concentrations of PEGylated IFN-α1b for 72 h (B) or treated with 50 μg/mL PEGylated IFN-α1b for increasing time (C). Whole-cell lysates were analyzed by Western blotting using the indicated antibodies. (D) BT-474 cells were pre-treated with 50 μg/mL PEGylated IFN-α1b for 64 h and then treated with 100 nM bortezomib or 1 μM chloroquine diphosphate (CQ) for 8 h. Whole-cell lysates were analyzed by Western blotting using the indicated antibodies. (E) BT-474 cells were treated with 100 μg/mL PEGylated IFN-α1b for 72 h. Total RNA was extracted and HER2 mRNA levels were determined by qPCR using GAPDH as an internal control (n=3; error bars denote SD; ns, no significance).

Discussion

Although the clinical benefit of trastuzumab has been well established, more potent targeted therapies for HER2-positive cancers are necessary to overcome limitations in its efficacy [30]. In the present study, we found that cotreatment with trastuzumab and PEGylated IFN-α1b exerted synergistic antitumor activity in HER2-positive cancer cells both *in vitro* and *in vivo*. Enhanced inhibition of HER2-mediated downstream signaling and augmented ADCC were observed only with concurrent trastuzumab and PEGylated IFN-α1b treatment and were the major mechanisms of synergistic antitumor activity. As the first investigation of trastuzumab in combination with PEGylated IFN-α1b as a treatment for HER2-positive cancers, our study helps set the stage for future clinical applications of combined treatment with trastuzumab and PEGylated IFN-α1b in HER2-positive cancers.

IFN-α, a cytokine with multiple functions, exerts a chemo-sensitizing effect against several tumors [20, 31]. IFN-α in combination with cell-targeted drugs including monoclonal antibodies, tyrosine kinase inhibitors shows synergistic antitumor activity against different cancers [32-34]. Additionally, the combination of trastuzumab with immunomodulating agents, including anti-PD-1 and anti-CD137 antibodies, enhances the antitumor efficacy of trastuzumab [10, 11, 35]. It has been reported that the *in vivo* antitumor activity of HER2-targeted antibodies requires the release of type I interferons [10]. However, few studies have examined the effect of a combination treatment comprising IFN-α and HER2-targeted mAbs. Here, we focused on whether cotreatment with trastuzumab and pegylated

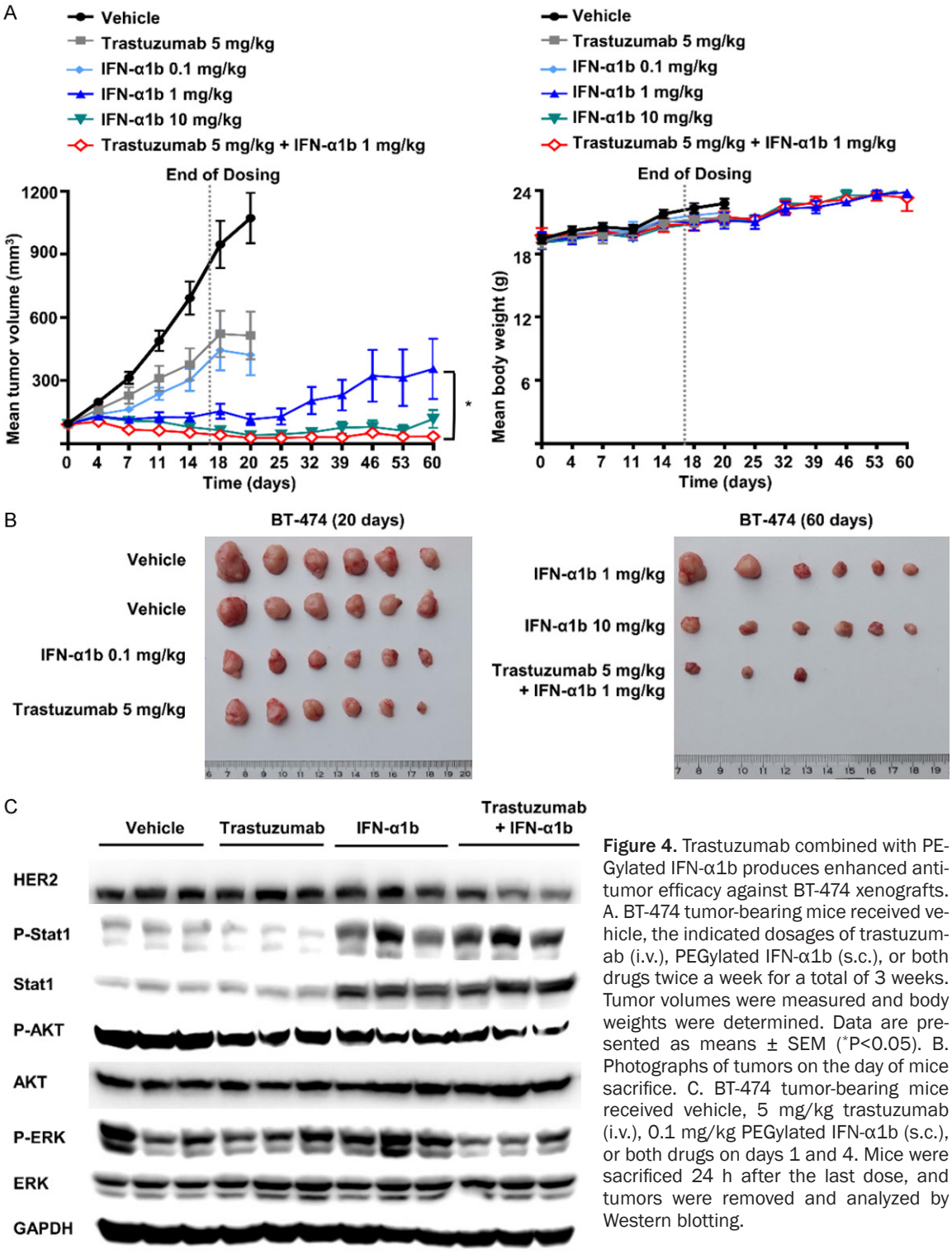

Figure 4. Trastuzumab combined with PEGylated IFN-α1b produces enhanced antitumor efficacy against BT-474 xenografts. A. BT-474 tumor-bearing mice received vehicle, the indicated dosages of trastuzumab (i.v.), PEGylated IFN-α1b (s.c.), or both drugs twice a week for a total of 3 weeks. Tumor volumes were measured and body weights were determined. Data are presented as means ± SEM (*P<0.05). B. Photographs of tumors on the day of mice sacrifice. C. BT-474 tumor-bearing mice received vehicle, 5 mg/kg trastuzumab (i.v.), 0.1 mg/kg PEGylated IFN-α1b (s.c.), or both drugs on days 1 and 4. Mice were sacrificed 24 h after the last dose, and tumors were removed and analyzed by Western blotting.

IFN-α1b produces enhanced antitumor efficacy, demonstrating that combination of the two exerts synergistic antitumor activity against HER2-positive cancers both *in vitro* and *in vivo*.

Previous reports have demonstrated downregulation of HER2 by interferon, showing that IFN-γ activates Stat1-mediated transcriptional downregulation of HER2 in cancer cells [36]. In

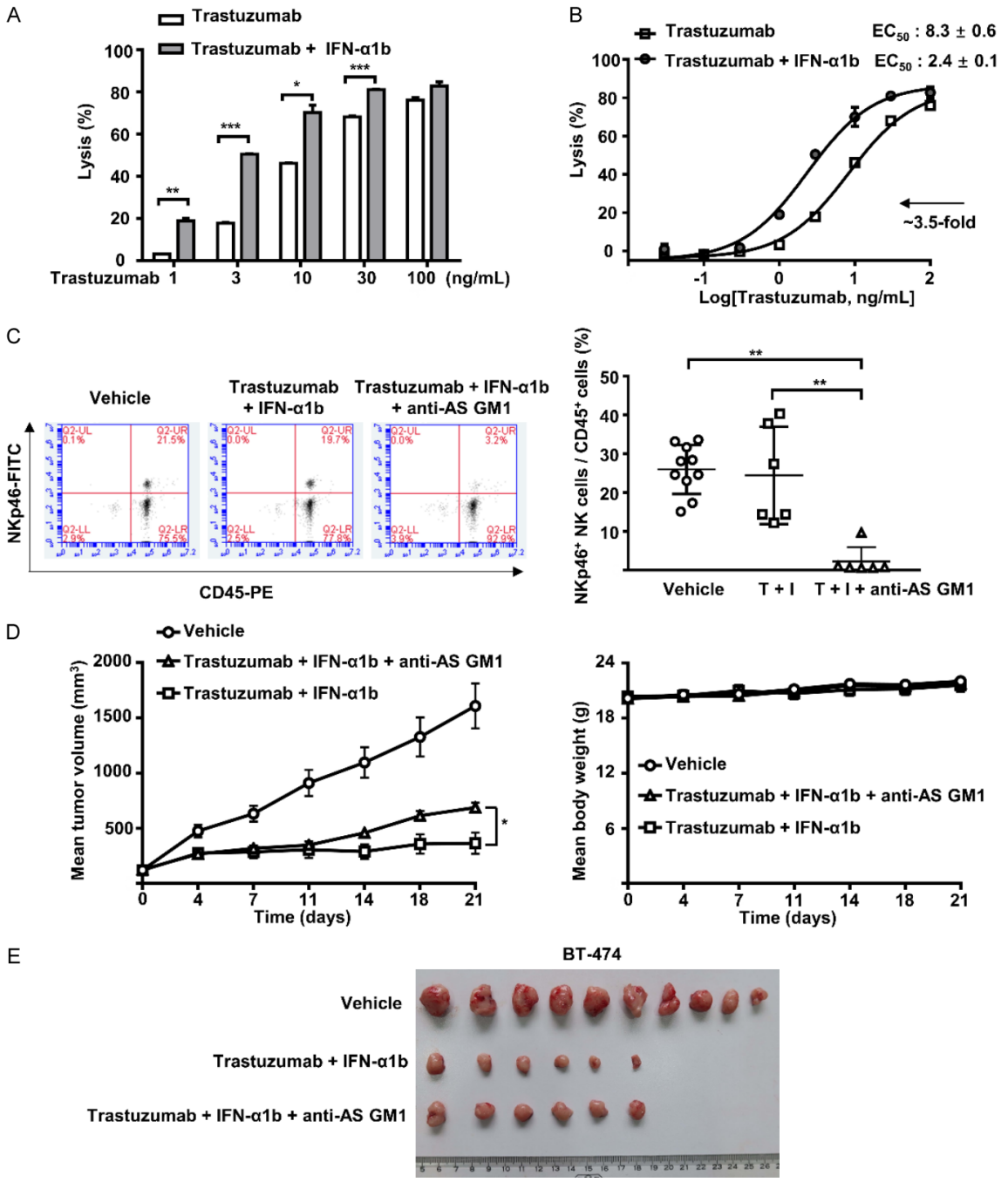

Figure 5. PEGylated IFN-α1b significantly augments trastuzumab-mediated ADCC both *in vitro* and *in vivo*. A and B. BT-474 cells were treated for 4 h with different concentrations of trastuzumab in the presence of 30 μg/mL PE-Gylated IFN-α1b-stimulated or -unstimulated PBMCs at an effector-to-target cell ratio of 50:1, and ADCC lysis was determined by measuring Calcein-AM release. Data are presented as means ± SD (*P<0.05; **P<0.01, ***P<0.005). C. The percentage of NK cells (NKp46+) in lymphocytes (CD45+) of mouse peripheral blood was analyzed by flow cytometry after treating with anti-AS GM1 antibody for 2 weeks. One representative dot plot from each group and the statistical graph of all samples were shown. (**P<0.01, T, trastuzumab, I, IFN-α1b). D. BT-474 tumor-bearing mice received vehicle or the combination of 5 mg/kg trastuzumab (i.v.) and 1 mg/kg PEGylated IFN-α1b (s.c.) twice a week for 21 d. A subgroup of mice treated with combination therapy simultaneously received an injection (i.p.) of 50 μg anti-AS GM1 antibody per mouse once a week to deplete NK cells. Tumor volumes were measured and body weights were determined. Data are presented as means ± SEM (*P<0.05, anti-AS GM1, anti-Asialo GM1). E. Photographs of tumors on the day of mice sacrifice.

the present study, we found that PEGylated IFN-α1b induced HER2 downregulation through lysosomal degradation rather than modulation of transcription, and thereby inhibited its downstream signaling. The underlying mechanism by which PEGylated IFN-α1b regulates HER2 remains unclear and warrants further investigation. The antitumor effect of trastuzumab requires the engagement of mechanisms that inhibit HER2-mediated signaling pathways. Combining with agents that enhance the inhibition of HER2-mediated downstream signaling has proven to be an effective approach for improving the antitumor activity of trastuzumab [37]. In the present study, PEGylated IFN-α1b enhanced trastuzumab-induced downregulation of HER2 and its downstream signaling both *in vitro* and *in vivo*, thereby contributing to the synergistic antiproliferative effects of the two in HER2-positive cancers.

On the other hand, the antitumor effect of trastuzumab *in vivo* mainly depends on ADCC. Thus, enhancing ADCC has also proven to be a promising strategy for improving the antitumor efficacy of trastuzumab [38, 39]. The effector cells involved in trastuzumab-mediated ADCC include NK cells and monocytes [40]. It has been reported that cytokines, including IL-2 [41] and IL-12 [35], enhance NK cell activity and thereby augment trastuzumab-mediated ADCC [42]. Furthermore, IFN-α is known to activate NK cell function [43]. Addition of IFN-α to mAb therapy augments antitumor ADCC *in vitro*, which could be attributable to effects on NK cells as well as macrophages [39]. In our study, overnight treatment of PBMCs with PEGylated IFN-α1b greatly enhanced trastuzumab-mediated ADCC activity. Eliminating NK cells using an anti-Asialo GM1 antibody significantly suppressed the antitumor activity of trastuzumab combined with PEGylated IFN-α1b *in vivo*, indicating that NK cells are the primary effector cell type in trastuzumab-mediated ADCC. Whether other effector cells also play a role in enhancing trastuzumab-mediated ADCC in addition to NK cells needs further investigation. Unlike other reports, our results, taken together, suggest that cotreatment with trastuzumab and PEGylated IFN-α1b simultaneously enhances both trastuzumab-mediated ADCC and suppression of HER2 expression and downstream signaling, which together likely constitute the main mechanism of the observed *in vivo* antitumor activity.

A growing body of evidence implicates HER2 in immune regulation [23, 44]. Innate and adaptive immune mechanisms have emerged as key players in modulating the effects of trastuzumab [7, 8]. IFN-α, a member of the cytokine family, also has important immune regulatory functions. In the current study, our investigation of the mechanism underlying the efficacy of combination therapy mainly focused on the enhancing effects of PEGylated IFN-α1b on the action of trastuzumab. A question for future research is whether the synergistic antitumor activity of the two also depends on their joint promotion of tumor immunity. Combining targeted therapies with immunotherapies including cytokines is becoming a new type of antitumor strategy as a result of the development of immunomodulatory drugs with remarkable activity against many solid tumors. Our findings further support this strategy.

In summary, our study demonstrates that trastuzumab combined with PEGylated IFN-α1b exerts synergistic antitumor activity by enhancing inhibition of HER2-mediated downstream signaling and potentiating ADCC against HER2-positive breast cancer both *in vitro* and *in vivo*. This report provides a strong rationale for investigating the therapeutic efficacy of trastuzumab in combination with PEGylated IFN-α1b as a treatment for HER2-positive breast cancers in future clinical trials.

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Disclosure of conflict of interest

None.

Abbreviations

mAb, monoclonal antibody; IFN-α, interferon alpha; ADCC, antibody-dependent cellular cytotoxicity; NK cell, natural killer cell; PBMC, peripheral blood mononuclear cell.

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