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ORIGINAL RESEARCH

A targeted immunotherapy approach for HER2/neu transformed tumors by coupling an engineered effector domain with interferon-γ

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ABSTRACT

Despite substantial clinical progress with targeted therapies, current antibody-based approaches have limited efficacy at controlling HER2/neu-positive breast cancers, especially in the absence of chemotherapies. Previously, we showed that the combination of IFNγ and anti-HER2/neu antibody synergistically reduces tumor growth in an *in vivo* implanted mammary tumor model. Here, we report a recombinant approach to produce an anti-HER2/neu scFv and IFNγ fusion protein using an engineered effector domain (EED) scaffold. The new molecule induces *in vitro* apoptosis in an IFNγ receptor-dependent manner. At a very low dose in the *in vivo* xenografted tumor models, the new EED-IFNγ fusion protein demonstrates superior activity over the anti-HER2/neu antibody and is even active on tumors that are resistant to anti-HER2/neu antibody therapy. Examination of tumor infiltrated macrophages and lymphocytes reveals that the fusion protein can induce changes in tumor microenvironment to support immune reactivity against tumors. Our studies have defined a targeted immunotherapy approach for the treatment of cancers.

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Introduction

HER2/neu-targeted therapies originated in our laboratory after we described that the p185^{neu} protein residing on the cell surface[1] could serve as a downregulatable target for monoclonal anti-HER2/neu antibodies.[2-4] Those studies demonstrated that the antibodies, upon disabling the Her2/neu kinase complex, induced a reversion of the malignant phenotype.[3,5] It was also demonstrated that two antibodies binding to distinct ectodomain epitopes led to more complete phenotypic reversion and inhibition of tumor growth.

Similar antibodies have since been developed for human use. Two anti-HER2/neu antibodies (trastuzumab and pertuzumab) and an antibody-drug conjugate (trastuzumab emtansine) are used to treat HER2/neu positive breast cancer and stomach cancer.[6-9] In addition, kinase inhibitors that limit HER2/neu tyrosine kinase activity have also been developed.[10] HER2/neu-targeted therapies have greatly helped HER2/neu positive breast cancers in clinical treatments, improving overall survival even in patients with metastatic breast cancers.[11]

Despite substantial progress, current targeted therapies remain insufficient at controlling HER2/neu positive breast cancers. Trastuzumab as a single agent only demonstrates efficacy in about 30% of HER2/neu positive tumors,[12] and currently is administrated in combination with chemotherapy. Most importantly, about 30% of patients develop resistance to trastuzumab and disease will recur, and almost all patients with advanced disease develop resistance to HER2 targeted therapies over time and succumb to the disease.[13] Efforts have been made to understand the effect of immune modulation to aid targeted therapies.

It is reported that the clinical efficacy of anti-HER2/neu antibody is associated with the activation of both the adaptive and innate immune system.[14,15] Stagg et al. suggested that mAb therapy requires type 1 and 2 IFNs, and found IFNγ induced CD8$^+$ T cells were determinants for effective tumor inhibition.[16] More recently, we have showed that a combination of IFNγ and anti-HER2/neu antibody synergistically reduces tumor growth in an *in vivo* implanted mammary tumor model.[17]

We have been focusing on improving the effectiveness of antibody effector functions, and report here a recombinant approach to produce an anti-HER2/neu scFv and IFNγ fusion protein. This fusion protein is an extension of previous structurally based studies using the Grababody scaffold,[18] which is an scFv protein containing an engineered effector domain (EED). At a very low dose, the new fusion protein demonstrates superior activity over the anti-HER2/neu antibody. Furthermore, it is active even on tumors that are resistant to anti-HER2/neu antibody therapy. The IFNγ scFv–EED represents an approach to take advantage of the synergistic activity of IFNγ and the anti-HER2/neu antibody, while targeting the IFNγ activity precisely to the HER2-expression tumor cells.

Results

IFNγ scFv–EED retains the target-binding activity of the anti-HER2/neu antibody as well as the IFNγ activity

Previously, we reported that the anti-HER2/neu scFv–EED, namely 4D5scFvZZ, was able to bind the HER2/neu receptor proteins that were either immobilized on Biacore chips or

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expressed on the cell surface.[18] One structural component in the scFv–EED is the EED feature, which was originally derived from the immunoglobulin Fc binding unit of the staphylococcal protein A (SPA), and is designed to capture circulating immunoglobulins to promote antibody effector functions.[18]

We recombinantly fused the human IFNγ to the C-terminus of the 4D5scFvZZ. The recombinant protein 4D5scFvZZ-IFNγ was expressed in bacteria and purified to confirm its binding activity for HER2/neu. A control construct without EED was also generated (4D5scFv-IFNγ). We performed FACS analysis on T6–17 cells, which are mouse fibroblasts overexpressing human HER2/neu receptor.[18] As shown in Fig. 1A, both 4D5scFvZZ-IFNγ and 4D5scFv-IFNγ were able to bind T6–17 cells, indicating that both constructs were properly folded to obtain an active 4D5scFv binding unit.

IFNγ is known to induce class I MHC antigen expression in breast and ovarian cancer cells.[19] To verify that the IFNγ subunit in the fusion protein is active, we examined its activity on MHC expression in SKBR3, a human breast cancer cell line overexpressing HER2/neu. As shown in Fig. 1B, 4D5scFvZZ-IFNγ and free IFNγ were both able to induce the expression of class I MHC. Neither IFNγ nor 4D5scFvZZ-IFNγ had any effect on class II MHC antigen expression. Therefore, the engineered Fc domain fusion protein was confirmed to mediate defined IFNγ-related activities.

The EED contributes to the anti-proliferative activity of 4D5scFvZZ-IFNγ

Human HER2/neu expressing T6–17 cells were used to study the anti-proliferative activity of the IFNγ scFv–EED fusion protein. To confirm that the activity of the fusion protein is through IFNγ signaling in the transformed cells, we transfected shRNA to knock down the IFNγ receptor and established the T6–17(IFNγR KD) cell line. A control cell line, T6–17(vector) was generated with the empty shRNA vector. As shown in Fig. 1C, 4D5scFvZZ-IFNγ demonstrated dose-dependent activity to limit the proliferation of both cell lines, but it was more active in T6–17 (vector) with the intact receptor. The calculated IC_{50} for 4D5scFvZZ-IFNγ to inhibit T6–17(vector) and T–17(IFNγR KD) was 2.46 μg/mL and 5.04 μg/mL, respectively. Since the IFNγ receptor expression in T6–17(IFNγR KD) cells is only diminished, but not totally eliminated,[17] the two-fold change in IC_{50} suggests that the anti-proliferative activity of 4D5scFvZZ-IFNγ is influenced by the IFNγ receptor level on tumor cells.

We observed that 4D5scFvZZ-IFNγ caused ~75% inhibition of cell proliferation at 10 μg/mL in both cell lines, an activity that was superior to that of either 4D5scFvZZ or 4D5scFv-IFNγ. In the proliferation assay, although the scFv–IFNγ fusion (4D5scFv-IFNγ) had better activity than the original 4D5scFvZZ species,[18] the combination of the EED and IFNγ in one construct exhibited the highest inhibition activity.

To determine if the fusion protein caused apoptosis/necrosis in tumor cells, we performed 7-aminoactinomycin D (7-AAD) and PE-conjugated Annexin V staining, and analyzed the stained cells by FACS (Fig. 1D). We noticed a substantial reduction in the frequency of live cells (bottom left quadrant) after cells were treated for 2 d with 4D5scFvZZ-IFNγ (60.6% vs. 95.7% in control). Treatment by other agents, including the anti-HER2/neu antibody m4D5, failed to demonstrate a significant reduction in live cell population. Cell death induced by 4D5scFvZZ-IFNγ was determined to be primarily necrotic, as the staining of the entire cell population was shifted to upper right quadrant (Fig. 1D).

In vivo *activity of 4D5scFvZZ-IFNγ*

We next examined if 4D5scFvZZ-IFNγ could have better *in vivo* activity than the original 4D5scFvZZ species, which had been studied in the T6–17 tumor model.[18] As shown in Fig 2A, 4D5scFvZZ-IFNγ had better activity than 4D5scFvZZ in limiting the growth of T6–17 tumors. In addition, 4D5scFvZZ-IFNγ was able to dose-dependently reduce the growth of implanted tumors (Fig. 2B). The humanized anti-HER2/neu bivalent antibody 4D5 (trastuzumab) was used in these experiments as a positive control. In both experiments, the activity of 4D5scFvZZ-IFNγ was superior to that seen with 4D5.

The in vivo *activity of 4D5scFvZZ-IFNγ is greatly improved by replacing human IFNγ with the mouse homolog*

In the previous study, we have shown that the synergistic action of anti-HRE2 antibody and IFNγ is dependent on the IFNγ receptor on tumor cells (Fig. 1C). It has been reported that IFNγ is species specific, and human IFNγ activity is minimal in mouse cell lines.[20] The first construct 4D5scFvZZ-IFNγ is designed for use in human and it contains the human IFNγ sequence. Both human IFN-Rα and IFN-Rβ would be required to render mouse cells to be fully responsive to human IFNγ.[20]

To better assess the *in vivo* activity in the mouse model, we constructed 4D5scFvZZ-mIFNγ that carries the mouse IFNγ sequence. Compared with the construct containing human IFNγ, 4D5scFvZZ-mIFNγ had far more potent activity in the T6–17 tumor model and we could use the protein at a much lower dosage. We found that the fusion protein was superior to the co-treatment of 4D5scFvZZ and free IFNγ(Fig. 2C). Furthermore, we compared side-by-side the activity of 4D5scFvZZ-mIFNγ and the 4D5 antibody, both at a low dose of 0.125 mg/kg and five times per week via i.p. injection. As shown in Fig. 2D, 4D5scFvZZ-mIFNγ had much better activity than 4D5 in limiting the growth of T6–17 tumors. In our experience with the T6–17 model, the activity we observed for the fusion protein at such a low dose is comparable to that of 4D5 at the much higher dose (5–10 mg/kg).

4D5scFvZZ-mIFNγ is active on 4D5-resistant tumors

Previously, CT-26-HER2 was established by engineering the BALB/c syngeneic tumor line CT-26 to express human HER2/neu.[21] CT-26-HER2 has been confirmed to be a resistance model for HER2/neu antibody therapies as it carries the oncogenic

K-Ras^{G12D} mutation.[22,23] To investigate if 4D5scFvZZ-mIFNγ mediates activity against this resistant tumor line, we compared it with 4D5 in BALB/c mice carrying implanted CT-26-HER2 tumors. As shown in Fig. 2E, while 4D5 treatment had no effect on the *in vivo* CT-26-HER2 tumors, 4D5scFvZZ-mIFNγ was able to significantly reduce the tumor growth.

The in vivo *activity of 4D5scFvZZ-mIFNγ is less affected by the expression level of the IFNγ receptor in tumor cells*

In the *in vitro* proliferation assay (Fig. 1C), the activity of the 4D5scFvZZ-mIFNγ was reduced when the IFNγ receptor levels in the transformed cell were diminished by shRNA. However, the *in vivo* activity of the fusion protein is less dependent on the IFNγ receptor status in the tumor cells. As shown in Fig. 3A and B, even in T6–17(IFNγR KD) tumors with diminished expression of the IFNγ receptor, 4D5scFvZZ-mIFNγ clearly showed good activity comparable to that in the control T6–17(vector) tumors. This study suggests that the slightly reduced anti-proliferation activity of 4D5scFvZZ-mIFNγ toward tumor cells with reduced IFNγ receptor levels is compensated *in vivo* by the host immune response that IFNγ can induce. The IFNγ receptor-independent *in vivo* activity of scFv–EED–IFN supports this new engineering strategy for patients with tumors with low IFNγ receptor expression.

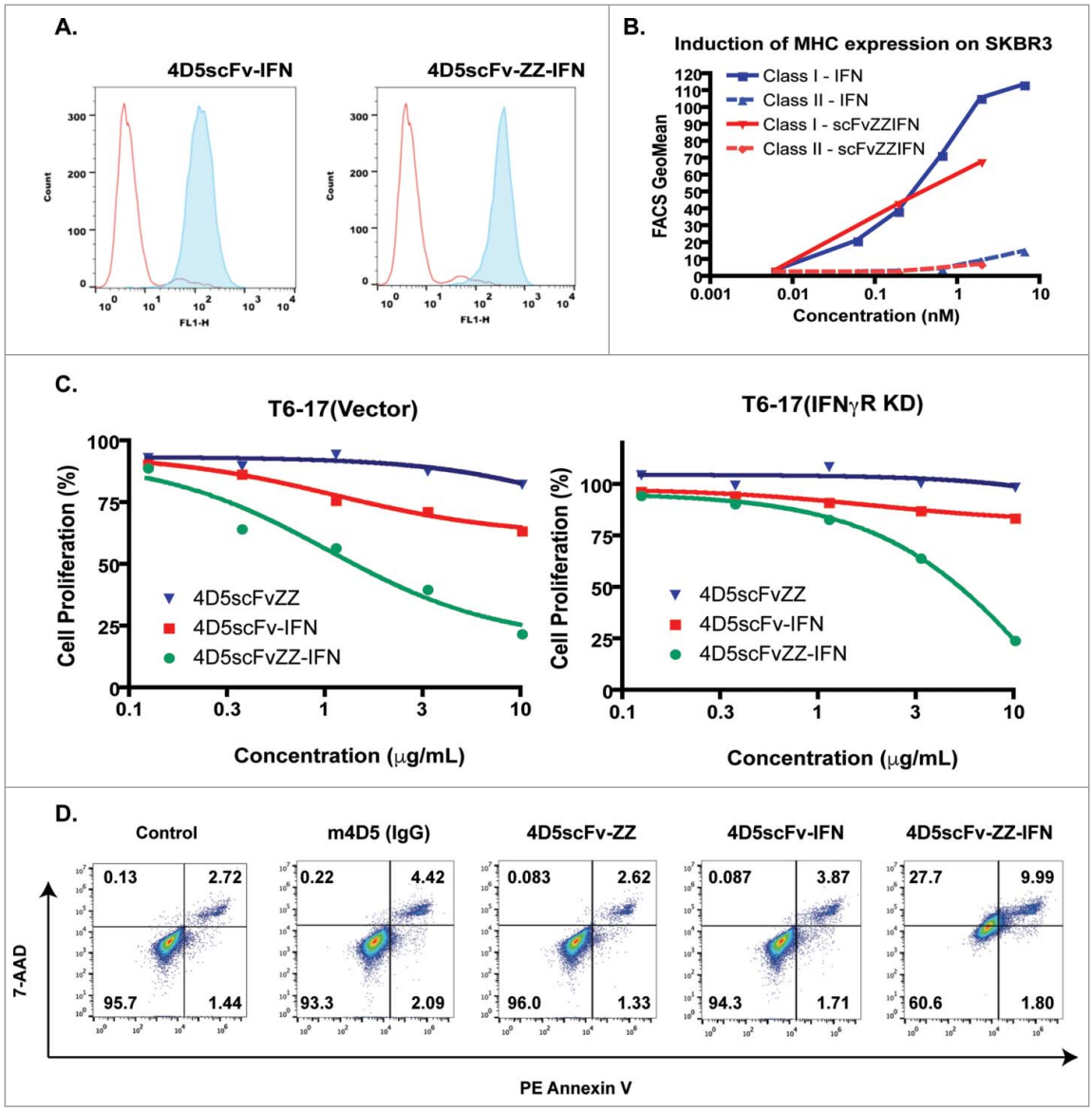

Figure 1. *In vitro* activity of 4D5scFvZZ-IFNγ. (A) Binding to the target. Both 4D5scFvZZ-IFNγ and 4D5scFv-IFNγ bind to cell surface p185$^{her2/neu}$. T6–17 cells with the expression of p185$^{her2/neu}$ were prepared for Fluorescence-activated cell sorting (FACS). Histograms represent staining with 0.5 μg of 4D5scFv-IFNγ or 4D5scFvZZ-IFNγ, as indicated in the figure, followed by His-Probe antibody and Alexa488-conjugated goat anti-rabbit antibodies (filled peak). The control staining (unfilled peak) was obtained with only the His-Probe antibody and the secondary antibody. (B) Effect of 4D5scFvZZ-IFNγ on MHC expression. SKBR3 cells were incubated with IFNγ or 4D5scFvZZ-IFNγ for 24 h at different doses. The expression levels of both class I and class II MHC antigens was analyzed by FACS using monoclonal antibodies W6/32 and L243, respectively. (C) Proliferation by MTT assay. 2000 T6–17(Vector) or IFNγR knocked-down T6–17(IFNγ R KD) cells were plated in 96-well plates and incubated with different concentrations of proteins for 72 h. Cell viability was determined by MTT assay as described in materials and methods. (D) 4D5scFvZZ-IFNγ induced apoptosis/necrosis. HER2/neu expressing T6–17 cells were treated with control, the antibody 4D5, 4D5scFvZZ, 4D5scFv-IFNγ, and 4D5scFvZZ-IFNγ (10 ug/mL each), for 2 d, then stained with Annexin V/7-AAD staining kit for FACS analysis. The lower and upper right quadrants represent early and late apoptotic cells, respectively. Only the 4D5scFvZZ-IFNγ treatment induced apoptosis/necrosis significantly.

Previously, we had reported that, in the treatment of HER2 tumors in the syngeneic MMTVneu transgenic mice, the combination of IFNγ and anti-HER2/neu antibody induced the shift of tumor associated macrophages (TAMs) from M2 to M1 and reduced MDSC infiltration into tumors.[17] Here, we want to find out whether the treatment with the new fusion protein also induced similar changes. The T6–17(vector) tumors at the end of treatments were isolated and analyzed for TAMs and tumor infiltrating lymphocytes (TIL) (Fig. 3). Treatment of 4D5scFvZZ-mIFNγ slightly increased M1 types of TAM and clearly reduced M2 type in a dose-dependent manner. As a result, the M1:M2 ratio was increased after the treatment. In addition, the treatment significantly increased CD45$^+$ TILs in the high dose group. We also observed modestly increased PD-L1 levels in tumors treated with 4D5scFvZZ-mIFNγ, which is in consistent with the known mIFNγ effect on the expression of this checkpoint molecule (Fig. 3).

Discussion

There is evidence that ADCC plays a role in the clinical activity of HER2-targeted antibody therapy. In a study of 18 HER2$^+$ operable breast cancer cases, 15 patients (83%) showed a trastuzumab-induced ADCC activity.[24] One patient with strong

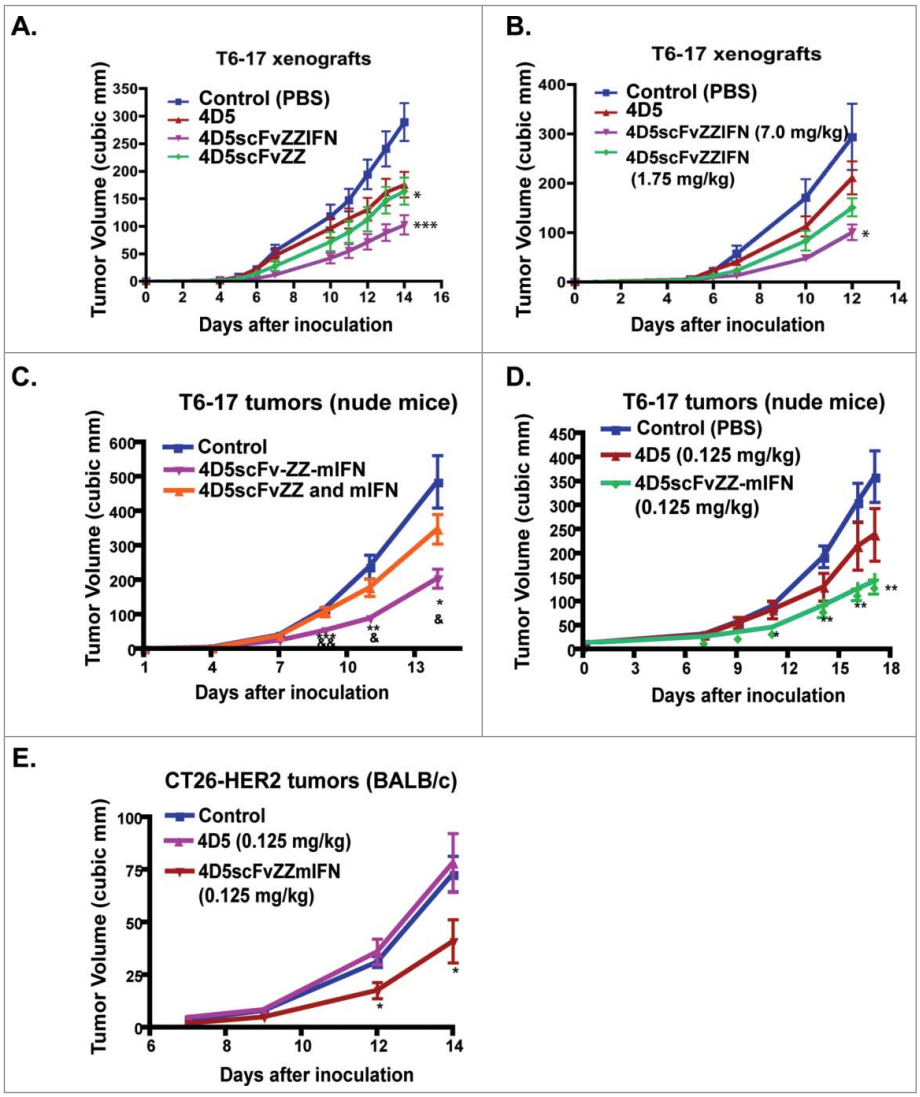

Figure 2. *In vivo* activity of scFv–EED–IFNγ on the growth of xenografted tumors. (A & B) *In vivo* activity of 4D5scFvZZ-IFNγ. (A) 4D5scFvZZ-IFNγ has better activity than 4D5scFvZZ. T6–17 tumor cells (5×10^5) were injected subcutaneously into both sides of the back of 6∼10-week old female athymic nude mice. Tumors were palpable 5 d after the inoculation of transformed T6–17 cells. Mice were treated with control (PBS), 4D5 mAb (1 mg/kg, twice; then 7 mg/kg twice, for a total of four treatments in 2 weeks), 4D5scFvZZI-IFNγ (7 mg/kg, five times per week), or 4D5scFvZZ (7 mg/kg, five times per week). Tumor growth in the 4D5scFvZZ-IFNγ group was highly suppressed compared with other groups. (B.) Dose-dependent activity of 4D5scFvZZ-IFNγ. Mice were treated with control (PBS), 4D5 mAb (1 mg/kg, twice per week), or 4D5scFvZZ-IFNγ (7 mg/kg or 1.75 mg/kg; five times per week). Tumor growth was dose-dependently suppressed by 4D5scFvZZ-IFNγ. (C) 4D5scFvZZ-mIFNγ has better activity than the combination of 4D5scFvZZ and free mouse IFNγ. Doses for each construct: 4D5scFvZZ-mIFNγ: 0.05 mg/kg; 4D5scFvZZ: 0.05 mg/kg; mIFNγ: 0.015 mg/kg. The dose of mIFNγ was adjusted to contain the equal molar amount of IFNγ as the 4D5scFvZZ-mIFNγ. (D) 4D5scFvZZ-mIFNγ has better activity than the HER2/neu antibody 4D5. For both C & D, T6–17 tumor cells (5×10^5) were injected subcutaneously into both flanks of the back of 6–10 weeks nude mice. Treatment started the next day after tumor implantation. (E) *In vivo* activity of 4D5scFvZZ-mIFNγ on 4D5 resistant tumors. CT26-HER2 tumor cells (1×10^6) were injected subcutaneously into both sides of the back of 6–10 weeks BALB/c female mice. Treatment started the next day after tumor implantation. The dosages of each treatment are indicated in the chart. Treatments were administrated five times per week via i.p.. Data represent mean + SEM *t*-test (two-sided) was performed to compare the difference in the tumor size for different treatment groups. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with control. All experimental groups in A–D that were compared for statistical difference had unequal variance, except the "4D5scFvZZ-mIFN" and "4D5scFvZZ and mIFN" comparison, which and Fig. 2E had equal variance.

ADCC activity had complete pathologic response to the treatment, while four patients with intermediate induced ADCC activity had partial response. All three patients with no induced ADCC had significant tumor regression. In addition, objective response rate (ORR) and progression-free survival (PFS) after trastuzumab-based therapies are reported to be correlated with the 158 V/V genotype of the Fc receptor FcγRIIIa, which demonstrates a significantly higher trastuzumab-mediated ADCC than other genotypes.[25-27]

However, studies also show that ADCC is weakened over time in adjuvant and metastatic breast cancer patients as compared with healthy controls.[28] This is in contrast to the trastuzumab-induced ADCC observed in the neoadjuvant setting, which only lasts about 5 week.[24] It is speculated that long time trastuzumab treatment leads to an immune suppressive mechanism that inhibits ADCC. Therefore, an immunotherapy that limits the ADCC inhibition may enhance the clinical activity of trastuzumab.

For solid tumors, one immunosuppressive mechanism is the hypoxia-driven adenosine accumulation in the tumor microenvironment. Through the A2A adenosine receptor, adenosine can prevent T cells and NK cells from killing tumor cells. A2AR antagonists have been tested to prevent the inhibition of antitumor T cells and NK cells.[29] Another approach is to change immune profiles of tumor microenvironment with immune-stimulatory cytokines. In a clinical trial with trastuzumab plus IL-12 in metastatic breast cancer patients, sustained production of IFNγ and other cytokines was associated with response to the treatment.[30]

Ever since the prototype of the anti-HER2/neu antibody was developed 30 y ago,[3] we and others have tried to produce engineered peptides or proteins that might provide even better therapeutic activity.[18,31,32] The IFNγ scFv–EED now represents the first engineered antibody-like protein that demonstrates superior *in vivo* activity. Endowed with multiple capabilities including disabling the receptor target, promoting the infiltration of T cells, and shifting TAMs to M1 type, this new species of mAb-like protein is active on trastuzumab- resistant tumors. The fusion protein approach is an improvement from our recent observation that IFNγ and anti-HER2/neu antibody synergistically inhibit *in vivo* tumor growth.[17] By linking IFNγ recombinantly to scFv–EED, this new therapeutic protein is

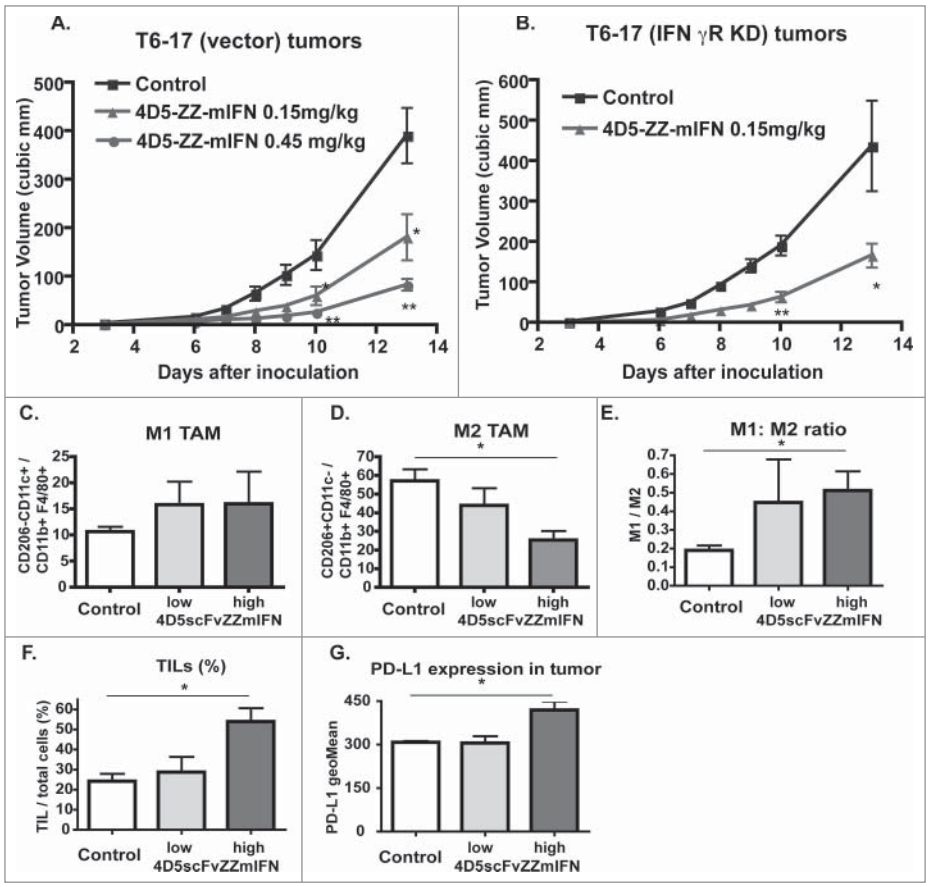

Figure 3. Effect of 4D5scFvZZ-mIFNγ on host immune cells. (A & B) *In vivo* activity of 4D5scFvZZ-mIFNγ is independent on the IFNγ receptor level. T6–17(Vector) or T6–17(IFNγR KD) tumor cells (5×10^5) were injected subcutaneously into the back of 6 ~10 week old female nude mice. The next day, mice were treated with control or 4D5scFvZZ-mIFNγ, five times per week. *t*-test (two-sided) was performed to compare the difference in the tumor size of different groups. (A) At the end of the experiment, tumors in the low dose group were significantly smaller than those in the control group ($^*p < 0.05$, equal variance). Tumors in the high dose group were very significantly smaller than those in the control group ($^{**}p < 0.01$, unequal variance). (B) Tumors in the treatment group were significantly smaller than those in the control group ($^*p < 0.05$, unequal variance). (C–G) At the end of the treatments, tumors from mice in Fig. 3A were collected and examined for macrophages (C D & E) and TILs (F). (G) Expression levels of PD-L1 in the tumors after treatments. Compared with the control group, tumors in the mice treated with high dose 4D5scFvZZ-mIFN had less M2 TAM, higher M1:M2 ratio, and more TILs (two tail *t*-test, equal variance, $p < 0.05$). In addition, there was a significant difference in the PD-L1 expression levels between treatment and control groups (one-way ANOVA, equal variance, $p < 0.05$). Data represent mean + SEM.

able to target the IFNγ effect toward the tumor and reduce the unwanted systemic activity of this cytokine.

Currently, immunocytokines in clinical development are mostly in the format of fusion protein with either antibodies or antibody fragments.[33] While antibody-cytokine fusion proteins generally have longer *in vivo* half-lives due to binding to neonatal Fc receptors, their tissue penetration into solid tumor is less than optimal.[34] In contrast, fusion proteins with smaller antibody fragments, such as scFv, tend to have better tumor penetration but shorter half-lives.[35] In this study, we directly fused IFNγ to anti-HER2/neu scFv–EED. Since IgG binding domain was shown before to extend the plasma half-life of scFv fusion protein,[36] this recombinant fusion protein is thought able to penetrate better into solid tumor with a prolonged *in vivo* half-life.

Bacterially expressed scFv–EED–IFNγ was used at a very low *in vivo* dose of 0.1–0.45 mg/kg. That is about 10–20-fold less than the usual dose for a recombinant protein in this type of experiment. The high potency of the molecule provides an exciting opportunity to dramatically reduce the medical cost for targeted cancer therapies. This is a very critical feature for this approach considering the problem and burden of the high cost of cancer drugs to the health care system.

In summary, the scFv–EED–IFNγ fusion protein represents a novel targeted immunotherapy. Its effects on TMAs repolarization and TILs infiltration indicate that this new type of molecules can fundamentally change the tumor microenvironment to support immune reactivity against tumors. We have started to generate similar species using scFvs targeting other oncogene encoded cancer-specific receptors. The 4D5scFvZZ-IFNγ fusion protein, once properly humanized, has the potential to be developed clinically as an immune-competent treatment of HER2/neu positive cancers.

Materials and methods

Cells and antibodies

T6–17, a gift from Dr JH Pierce, was derived from NIH3T3 by overexpressing the p185$^{her2/neu}$ receptor.[37] CT26-HER2 was kindly provided by Drs Cristina Jaime-Ramirez and William Carson in OSUMC. The cell line was originally from Dr Sherie L. Morrison of UCLA. SKBR3 was obtained from the American Type Culture Collection. Authenticity of these cells was determined by confirming their known expression profiles for receptors using fluorescence-activated cell sorting (FACS) periodically. CT26-HER2 was cultured in RPMI 1640 media, plus L-glutamine with 10% FBS, 1% Antibiotic–Antimycotic (100X, ThermoFisher, Cat.# 15240062) and prophylactic Plasmocin (InvivoGen, Cat.# ant-mpp). All other cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat inactivated fetal calf serum, L-glutamine (2mM), penicillin (100 U/mL) and streptomycin (100 mg/mL) at 37°C in a humidified 5% CO_2 atmosphere. All cell lines are routinely checked to confirm the absence of mycoplasma. Both T6–17 and CT26HER2 were confirmed virus free by the IMPACT II test (IDEXX Bioresearch). Anti-class I and class II MHC antigens antibodies, FITC anti-human HLA-A,B,C antibody (clone W6/32, Cat.# 311403) and FITC anti-human HLA-DR antibody (clone L243, Cat.# 307603), respectively, were purchased from Biolegend (San Diego, CA).

Cell proliferation assay

To measure cell proliferation, we used the modified MTT assay as described previously.[18,32] For T6–17 cells, exponentially growing cells were seeded at a density of 5,000 cells/well in 96-well plates. For each data point, five wells of cells were prepared for the same treatment to obtain the average value. The MTT assay was usually repeated at least once to confirm.

Flow cytometry assays (Fluorescence-activated cell sorting, FACS)

For the determination of cell surface expression of MHC receptors in response to IFNγ, cells were incubated with appropriate FITC labeled primary antibodies for 1 h on ice. After wash and collection, the cell pellets were resuspended in FACS buffer. 10,000 cells were run for each sample on a FACSCalibur^{TM} flow cytometer and analyzed using the CellQuest software.

For apoptosis assay, T6–17 cells were treated for 2 d with 10 ug/mL of control antibody 9BJ5, 4D5, 4D5scFvZZ, 4D5scFv-IFNγ and 4D5scFvZZ-IFNγ, respectively. Cells were harvested and washed with phosphate-buffered saline (PBS), and then stained with PE-Annexin V and 7-AAD according to the manufacturer's instructions (eBioscience, San Diego, CA). Stained cells were analyzed with Accuri C6 flow cytometer (BD Biosciences) and the acquired FACS data were analyzed with FlowJo software (Tree Star, Ashland, OR).

For TILs, tumors were collected for single cell suspensions at 1 d after final treatment. Tumor tissues were cut and digested with Collagenase D (Roche, 1 mg/mL) and DNAse (Sigma, 1 mg/mL) in 2% FBS RPMI for 30 min. Then cells were filtered through a cell strainer (Falcon). Cell surface antigens were stained with anti-CD45, F4/80, CD11b, CD11c, CD206 and PD-L1 antibodies (Biolegend). Cells were analyzed with FACS LSR (BD Biosciences) and FACS data were analyzed with FlowJo software (Tree Star, Ashland, OR).

IFNγR knock down by shRNA

TRC mouse lentiviral shRNA clones targeting IFNγR1 and the control pLKO.1 were obtained from The Open Biosystems Expression Arrest^{TM}TRC Library (Thermo Scientific). Lentiviruses were produced by VairaSafe^{TM} Lentivirus expression system (Cell Biolabs inc., San Diego, CA) as manufacturer's instructions. T6–17 cells were transfected with those lentiviruses and selected with 1 μg/mL puromycin and analyzed by FACS with anti-IFNγR antibody (Biolegend).

In vivo tumor studies

All mouse procedures were performed according to the guidelines and protocols approved by the IACUC of University of Pennsylvania. In general, we used four mice for each treatment group, and we routinely implanted two tumors on each mouse. Mice were randomly grouped and treated with various reagents as described in the text. Control mice were treated with PBS.

Tumor size was measured with a Vernier caliper, and tumor volume was calculated by the formula: 3.14* length * width * height/6.

Statistical analysis

For our routine tumor study, the minimal number of tumors in each group is 6. By having at least six tumors in each group, the probability is 87% that the study will detect a treatment difference at a two-sided 0.05 significance level, if the true difference between treatments is two times the standard deviation. In our experience, this will allow us to detect a 40% reduction in the average tumor size of the treatment group as compared with the control group. All data expressed as mean ± s.e.m. Student's *t*-test and one-way analysis of variance (ANOVA) were used to compare two groups (GraphPad Prism 6, La Jolla, CA, USA). p-value < 0.05 was considered statistically significant. *F*-test was performed to check for variance.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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