

Targeting IFN- α to B Cell Lymphoma by a Tumor-Specific Antibody Elicits Potent Antitumor Activities¹

Tzu-Hsuan Huang,^{2*} Koteswara R. Chintalacheruvu,[†] and Sherie L. Morrison[†]

IFN- α , a cytokine crucial for the innate immune response, also demonstrates antitumor activity. However, use of IFN- α as an anticancer drug is hampered by its short half-life and toxicity. One approach to improving IFN- α 's therapeutic index is to increase its half-life and tumor localization by fusing it to a tumor-specific Ab. In the present study, we constructed a fusion protein consisting of anti-HER2/*neu*-IgG3 and IFN- α (anti-HER2/*neu*-IgG3-IFN- α) and investigated its effect on a murine B cell lymphoma, 38C13, expressing human HER2/*neu*. Anti-HER2/*neu*-IgG3-IFN- α exhibited potent inhibition of 38C13/HER2 tumor growth in vivo. Administration of three daily 1- μ g doses of anti-HER2/*neu*-IgG3-IFN- α beginning 1 day after tumor challenge resulted in 88% of the mice remaining tumor free. Remarkably, anti-HER2/*neu*-IgG3-IFN- α demonstrated potent activity against established 38C13/HER2 tumors, with complete tumor remission observed in 38% of the mice treated with three daily doses of 5 μ g of the fusion protein ($p = 0.0001$). Ab-mediated targeting of IFN- α induced growth arrest and apoptosis of lymphoma cells contributing to the antitumor effect. The fusion protein also had a longer in vivo half-life than rIFN- α . These results suggest that IFN- α Ab fusion proteins may be effective in the treatment of B cell lymphoma. *The Journal of Immunology*, 2007, 179: 6881–6888.

Although spontaneous immune responses against tumor-associated Ags (TAAs)³ can be detected (1), malignant cells causing disease fail to elicit an immune response that leads to rejection. Many studies have demonstrated that it is possible to enhance the immunogenicity of tumor cells by introducing immunostimulatory molecules such as cytokines and costimulatory molecules into them (2–4); however, effective gene transfer still remains a challenge. In addition, eradication of residual cancer cells may require the targeting of widely scattered micrometastatic tumor deposits that are not accessible to direct gene transfer. An alternative approach would be to use Abs specific for TAAs to direct the immunostimulatory molecules to the tumor. Tumor-specific Abs fused to immunostimulatory molecules have been demonstrated to be effective in murine tumor models (5–7) and are currently being evaluated in a clinical trial (7).

Both the innate and the adaptive immune responses are essential for providing protection against infectious pathogens and tumors. The cross-talk between innate and adaptive immunity is regulated by interactions between cells and cytokines. Cytokines produced by cells of the innate immune system can, directly or indirectly,

activate the cells of the adaptive immune response and play an important role in eliciting protective antitumor immunity (8). Central to the activation of the innate immune system is the detection of bacterial products or “danger” signals that lead to the release of proinflammatory cytokines, such as IFN- α , TNF- α , and IL-1.

IFN- α is a proinflammatory cytokine with potent antiviral and immunomodulatory activities and is a stimulator of differentiation and activity of dendritic cells (DCs) (9). Type I IFNs (IFN- α and IFN- β) have multiple effects on the immune response (10). IFN- α plays a role in the differentiation of Th1 cells (11) and the long-term survival of CD8⁺ T cells in response to specific Ags (12). Multiple studies have shown that IFNs are also capable of exerting antitumor effects in both animal models (13) and cancer patients (14). In addition to enhancing the adaptive antitumor immune response, IFN- α can increase expression of the tumor suppressor gene *P53* (15), inhibit angiogenesis (16), and prime apoptosis (17) in tumor cells. Although these properties suggest that IFN- α should be an effective therapeutic for the treatment of cancer, its short half-life and systemic toxicity have limited its usage. The exploitation of a tumor-specific Ab to both increase the half-life of IFN- α and to deliver higher doses to the tumor site is one promising approach for improving the therapeutic index of IFN- α .

The HER2/*neu* (*c-erbB-2*) protooncogene encodes a transmembrane protein tyrosine kinase growth factor receptor, p185^{HER2} (18), with extensive homology to the human epidermal growth factor receptors. Abundant evidence has supported the role of this protooncogene in tumorigenesis with overexpression correlated with poor prognosis in cancer patients. The elevated levels of the HER2/*neu* protein in malignancies and the extracellular accessibility of this molecule make it an excellent TAA for tumor-specific therapeutic agents. In fact, treatment of patients with advanced breast cancer using the anti-HER2/*neu* Ab trastuzumab (herceptin; Genentech), directed at the extracellular domain of HER2/*neu*, can lead to an objective response in some patients with tumors overexpressing the HER2/*neu* oncoprotein (19).

In the present study, we constructed a fusion protein consisting of anti-HER2/*neu*-IgG3 with the variable region of C6MH3-B1 (20) and IFN- α , and investigated its effect on a murine B cell

*Department of Microbiology and Immunology, University of California, San Francisco, CA 94143; and [†]Department of Microbiology, Immunology and Molecular Genetics and the Molecular Biology Institute, University of California, Los Angeles, CA 90095

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² Address correspondence and reprint requests to Dr. Tzu-Hsuan Huang, Department of Microbiology and Immunology, University of California, San Francisco, 513 Parnassus Avenue, HSW 1002A, San Francisco, CA 94143-0534. E-mail address: lhuang@diabetes.ucsf.edu

³ Abbreviations used in this paper: TAA, tumor-associated Ag; DC, dendritic cell; GPS, glutamine/penicillin/streptomycin; FPLC, fast protein liquid chromatography; VSV, vesicular stomatitis virus; MTS, mouse thymic stroma; PI, propidium iodide.

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lymphoma, 38C13, expressing human HER2/*neu* (38C13/HER2). We chose to evaluate IFN- α targeting to tumor in this model given the responsiveness of this B cell lymphoma to IFN- α (21). Fusion of IFN- α to an Ab significantly increased its *in vivo* half-life. Anti-HER2/*neu*-IgG3-IFN- α was found to be efficient in inhibiting the growth *in vivo* of both small and established 38C13/HER2 tumors with no signs of systemic toxicity observed at effective doses. Anti-HER2/*neu*-IgG3-IFN- α inhibited the growth of and induced apoptosis in 38C13/HER2 cells. These results suggest that fusion of IFN- α to a tumor-specific Ab will result in an agent effective for the treatment of B cell lymphoma.

Materials and Methods

Cell lines and culture conditions

38C13 is a highly malignant murine B cell lymphoma derived from C3H/HeN mice. The construction and characterization of 38C13 expressing human HER2/*neu* (38C13/HER2) has been previously described (6). Both 38C13 and 38C13/HER2 were cultured in IMDM (Irvine Scientific) supplemented with 2 mM L-glutamine, 10 U/ml penicillin, 10 μ g/ml streptomycin (GPS; Sigma-Aldrich) and 10% calf serum (Atlanta Biologicals). Murine myeloma P3X63Ag8.653 (American Type Culture Collection) and its derivatives expressing anti-HER2 IgG3-IFN- α or IgG3-IFN- α were grown in IMDM supplemented with 10% calf serum and GPS. L929 fibroblasts (American Type Culture Collection) were cultured in IMDM with 5% calf serum and GPS. The construction and characterization of CT26/HER2, a murine colon adenocarcinoma cell line overexpressing human HER2/*neu*, has been previously described (6). CT26/HER2 was cultured in IMDM with 5% calf serum and GPS.

Plasmid construction

The H and L chain variable regions of C6MH3-B1, an anti-human HER2/*neu* ScFv provided by Dr. J. D. Marks (University of California, San Francisco, CA) were inserted into the human γ 3 H chain (pAH4802) and κ L chain (pAG4622) expression vectors, respectively (22), and used to produce chimeric IgG3 of this specificity. To construct the anti-human HER2/*neu*-IgG3(C6MH3-B1)-IFN- α fusion protein, PCR was first used to introduce a *Bam*H1 restriction enzyme site upstream and *Xba*I restriction enzyme site downstream of the mature murine IFN- α 1 gene amplified by PCR from genomic DNA of BALB/c mice with the forward primer 5'-CGCGGATCCTGTGACCTGCCTCAGACTC-3' and the reverse primer 5'-GCTCTAGATCATTTCTTCTCTCAGTCTTC-3'. The final PCR product was ligated into a TA vector. The resulting vector, after sequencing, was digested with *Bam*H1 and *Xba*I to release the DNA fragment which was inserted into the vector pAH9612 containing the IgG3 constant region with the C6MH3-B1 H chain variable region and a GGGGS GGGSGGGGS peptide linker at the end of C_H3. The final PCR product, pAH9616, contained anti-HER2/*neu*-IgG3 followed by a GGGGS GGGSGGGGS peptide linker and murine IFN- α .

Production and purification of recombinant proteins

Plasmid encoding the IgG3 H chain with the C6MH3-B1 variable region fused to IFN- α was transfected into P3X63Ag8.653 cells expressing either L chain with the C6MH3-B1 variable region (23) to produce anti-HER2/*neu*-IgG3-IFN- α or nonspecific L chain (4D5; Genentech) (6) to produce IgG3-IFN- α by electroporation with a pulse of 960 μ Fd capacitance and 0.2 V. Transfectants producing anti-HER2/*neu*(C6MH3-B1)-IgG3, anti-HER2/*neu*(C6MH3-B1)-IgG3-IFN- α , or IgG3-IFN- α were selected and characterized as previously described (6). Anti-HER2/*neu*(C6MH3-B1)-IgG3 was purified from culture supernatants using protein G immobilized on Sepharose 4B fast flow (Sigma-Aldrich), and anti-HER2/*neu*(C6MH3-B1)-IgG3-IFN- α and IgG3-IFN- α were purified from culture supernatants using protein A immobilized on Sepharose 4B fast flow (Sigma-Aldrich). Purity and integrity were assessed by Coomassie blue staining of proteins separated by SDS-PAGE. The international reference standard for mouse IFN- α provided by the National Institutes of Health was used to determine IFN activity of the fusion proteins. rIFN- α was obtained from PBL Biomedical Laboratories.

FPLC analysis of IgG3-IFN- α fusion protein

To determine whether the fusion protein exists as monomer and/or polymers in solution, 100 μ g of IgG3-IFN- α mixed with 400 μ g of OVA to provide an internal control was analyzed by gel filtration on a 30 \times 1.5-cm Superose 6 column attached in a fast protein liquid chromatography

(FPLC) using PBS and 0.5 ml/min flow rate. Gel filtration on the same column of IgA2m that exists predominantly as dimer Ab with a molecular mass of 350 kDa and a mixture of Miles IgG of molecular mass 150 kDa and OVA of molecular mass 45 kDa were used to provide molecular mass standards.

Flow cytometry analysis of HER2/*neu*-binding activity

To detect the reactivity of various anti-HER2/*neu* fusion proteins with CT26/HER2 cells, 1×10^6 cells were incubated at 4°C for 1 h with 10 pM of the fusion protein. For some experiments, the fusion proteins were preincubated with 900 U of heparin at 4°C for 17 h before incubation with CT26/HER2 cells. Cells were then reacted with biotinylated rat anti-human IgG (BD Biosciences) diluted 1/100. The bound biotinylated Abs were detected with PE-labeled streptavidin (BD Biosciences) diluted 1/1500 and cells were analyzed by flow cytometry using a FACScan (BD Biosciences).

IFN- α antiviral activity

The L-929 fibroblast cell line sensitive to the vesicular stomatitis virus (VSV) infection was used to quantify the biological activity of IFN- α . L-929 cells were plated in a 96-well tissue culture plate (Falcon; BD Biosciences) at a density of 4×10^4 cells/well and incubated overnight at 37°C in a 5% CO₂ atmosphere. Afterward, serial dilutions of different IFN- α fusion proteins or standard IFN- α (international reference standard for mouse IFN- α ; National Institutes of Health, Bethesda, MD) were added and the plate was incubated at 37°C for 24 h. Four thousand PFU of VSV was then added to each well and incubated at 37°C for another 48 h. Surviving adherent cells were stained with 50 μ l of crystal violet (0.05% in 20% ethanol) for 10 min. The plates were washed with water and the remaining dye was solubilized by the addition of 100 μ l of 100% methanol. Plates were read using an ELISA reader at 595 nm.

Assay for the antiproliferative effect of anti-HER2/*neu*-IgG3-IFN- α

In brief, 38C13 or 38C13/HER2 cells were plated in a 96-well tissue culture plate at a density of 1.25×10^4 cells/well and serial dilutions of different fusion proteins were added. The plates were then incubated for 48 h at 37°C in a 5% CO₂ atmosphere. Plates were developed by addition of 20 μ l of MTS solution (Promega) and analyzed at 490 nm using an ELISA reader. Inhibition of proliferation (percent) was calculated as: $100 - [(OD_{exp} - OD_{blank}) / (OD_{medium} - OD_{blank})] \times 100$.

Assay for apoptosis

In brief, 1×10^6 cells were treated with different fusion proteins for 72 h. The cells were then washed with ice-cold PBS. The annexin V/propidium iodide (PI) assay was conducted following procedures suggested by the manufacturer using the Vybrant Apoptosis Assay Kit 2 (Molecular Probes).

Proliferation of CFSE-labeled 38C13/HER2 tumor cells

In brief, 1×10^6 cells were incubated with 2.5 μ M CFSE (Molecular Probes) for 10 min at 37°C. Cells were then treated with 1 nM of different fusion proteins for 48 h and analyzed by flow cytometry following procedures suggested by the manufacturer using the CellTrace CFSE Cell Proliferation Kit (Molecular Probes).

Mice

Female C3H/HeN mice 6–8 wk of age obtained from Taconic Farms were used. Animals were housed in a facility using autoclaved polycarbonate cages containing wood-shaving bedding. The animals received food and water *ad libitum*. Artificial light was provided under a 12/12-h light/dark cycle. The temperature of the facility was 20°C with 10–15 air exchanges per hour.

Half-life

Murine rIFN- α (PBL Biomedical Laboratories), IgG3-IFN- α , and anti-HER2/*neu*-IgG3-IFN- α were iodinated to 10 μ Ci/ μ g with ¹²⁵I using Iodo-Beads (Pierce) according to the manufacturer's protocol. Mice were injected i.p. with 66 μ Ci of ¹²⁵I-labeled proteins. At various intervals after injection of ¹²⁵I-labeled rIFN- α , IgG3-IFN- α , or anti-HER2/*neu*-IgG3-IFN- α , residual radioactivity was measured using a mouse whole body counter (Wm. B. Johnson and Associates).

Tumor challenge and Ab therapy

C3H/HeN mice received 1000 38C13/HER2 tumor cells s.c. Treatment was given by i.p. injection either 1, 3, and 5 days or 12, 13, and 14 days

after tumor challenge. Tumors were measured every other day, and the tumor volume (in cubic millimeters) was approximated using the following formula: $[\text{length (mm)} \times \text{width (mm)} \times \text{width (mm)}] / 2$ (24). Animals were observed until the length of the s.c. tumor reached 15 mm or until any mouse was observed to be suffering or appeared to be moribund. Animals under these conditions were euthanized humanely according to institutional policy.

Western blot analysis and Ab

In brief, 38C13/HER2 cells were treated with different fusion proteins for the indicated times, washed with ice-cold PBS, and lysed on ice for 10 min in lysis buffer (0.125% Nonidet P-40, 0.875% Brij 97, 10 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.15 M NaCl, 0.4 mM Na_3VO_4 , 0.4 mM NaF, 1 mM PMSF, 2.5 μM leupeptin, and 2.5 μM aprotinin). Cell lysates were clarified at $10,000 \times g$ for 10 min at 4°C . Protein samples were then boiled in sample buffer before separation on 8% SDS-PAGE gels and transferred onto polyvinylidene fluoride microporous membranes (Millipore). After blocking with 3% BSA in 150 mM NaCl, 50 mM Tris-HCl (pH 7.6; TBS) for 1 h at room temperature, blots were probed with the indicated primary Abs overnight at 4°C . The blots were then washed three times at room temperature with 0.05% Tween 20 in TBS, incubated with the appropriate secondary Abs conjugated with HRP, and detected by a peroxidase-catalyzed ECL detection system (ECL; Pierce). Polyclonal rabbit anti-phosphoSTAT1 was obtained from Cell Signaling Technology. Polyclonal HRP-conjugated donkey anti-rabbit IgG was obtained from Amersham Biosciences. Polyclonal rabbit anti-GAPDH was obtained from Abcam.

Statistical analysis

Statistical analyses were performed using a two-tailed Student's *t* test for in vitro studies and log-rank (Mantel-Cox) analysis for animal survival curves.

Results

Production and characterization of anti-HER2/neu-IgG3-IFN- α

The construction and expression of anti-HER2/neu-IgG3 with the C6MH3-B1 (20) variable region has been described previously (23). The amino-terminal end of mature murine IFN- α was fused to the carboxyl-terminal end of anti-HER2/neu-IgG3 separated by a flexible $[(\text{Gly}_4)_3 \text{Ser}]_3$ linker (Fig. 1A). An identical fusion protein, IgG3-IFN- α , lacking HER2/neu specificity was constructed by replacing the C6MH3-B1 L chain with the 4D5 (rhuMab HER2, herceptin; Genentech) L chain. The proteins purified from culture supernatants using protein G were analyzed by SDS-PAGE under nonreducing and reducing conditions (Fig. 1B). In the absence of reducing agents, anti-HER2/neu-IgG3 (Fig. 1B, lane 1) migrates with a molecular mass of 170 kDa, whereas anti-HER2/neu-IgG3-IFN- α (Fig. 1B, lane 2) and IgG3-IFN- α (Fig. 1B, lane 3) are 210 kDa, the size expected for a complete IgG3 with two molecules of murine IFN- α attached (Fig. 1A). After treatment with the reducing agent, L chains migrating with a molecular mass of 25 kDa are seen for these proteins (Fig. 1B, lanes 4–6). However, the anti-HER2/neu-IgG3 has an H chain with a molecular mass of 60 kDa (Fig. 1B, lane 4), whereas IgG3-IFN- α (Fig. 1B, lane 5) and anti-HER2/neu-IgG3-IFN- α (Fig. 1B, lane 6) have an H chain with a molecular mass of 80 kDa as expected. The lower band in lane 1 (Fig. 1B) is bovine IgG which also bound to the protein G column; and the bovine H and L chains are also seen in lane 4 (Fig. 1B) and to a lesser degree in lanes 5 and 6 (Fig. 1B). FPLC analysis showed that the IgG3-IFN- α fusion protein existed as a monomer in solution (data not shown).

Ag binding and antiviral activity of anti-HER2/neu-IgG3-IFN- α

Both anti-HER2/neu-IgG3 and anti-HER2/neu-IgG3-IFN- α bound CT26/HER2 cells, which express high levels of human HER2/neu, while IgG3-IFN- α bound CT26/HER2 weakly (Fig. 1C). Many cytokines including IL-1, IL-2, IL-6 (25) and IFN- γ (26) have been shown to interact with heparin. To determine whether the weak interaction between IgG3-IFN- α and CT26/HER2 is due to the heparin binding, proteins were incubated with heparin before

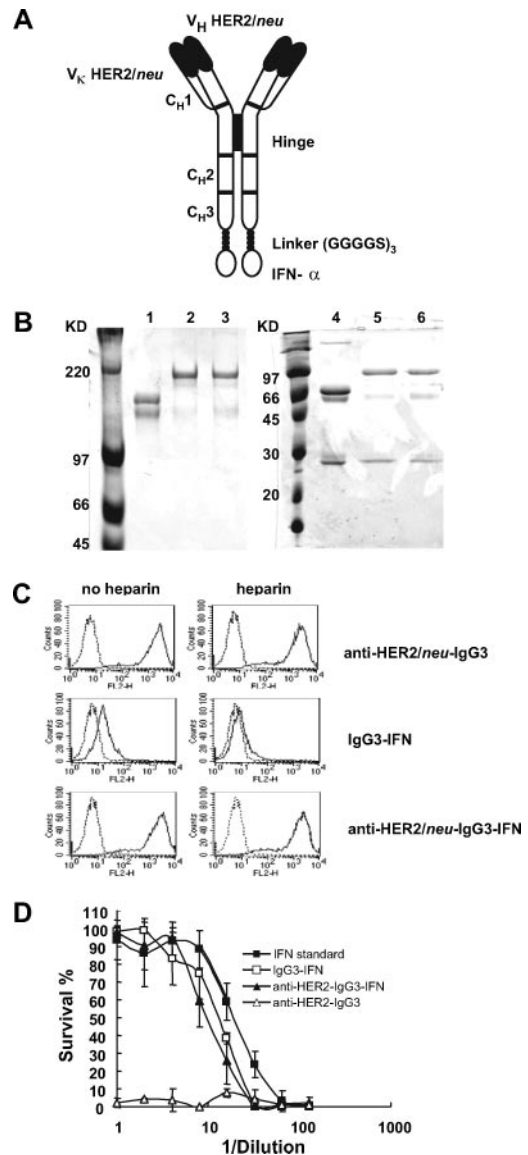


FIGURE 1. Construction and characterization of anti-HER2/neu IgG3-IFN- α . *A*, Schematic diagram of anti-HER2/neu-IgG3-IFN- α . Solid areas represent anti-HER2/neu variable regions. Open areas represent human IgG3 and κ constant regions. White circles represent murine IFN- α . *B*, SDS-PAGE of purified anti-HER2/neu-IgG3 (lanes 1 and 4), IgG3-IFN- α (lanes 2 and 5), and anti-HER2/neu-IgG3-IFN- α (lanes 3 and 6) under nonreducing (lanes 1–3) or reducing (lanes 4–6) conditions. The molecular mass marker proteins are shown at the left of each gel. *C*, Anti-HER2/neu-IgG3 and anti-HER2/neu-IgG3-IFN- α bind HER2/neu. CT26/HER2, a murine colonic cell line expressing high levels of human HER2/neu, was reacted with anti-HER2/neu-IgG3, IgG3-IFN- α , or anti-HER2/neu-IgG3-IFN- α with or without heparin followed by PE-labeled rabbit anti-human IgG. Dashed lines represent signal from cells without addition of recombinant protein. *D*, The protective activity of the IFN- α standard and different IFN- α fusion proteins against VSV. Dilutions of 1 U of IFN- α standard, 0.21 ng (10 pM) of anti-HER2/neu-IgG3-IFN- α , 0.21 ng (10 pM) of IgG3-IFN- α , or 0.17 ng (10 pM) of anti-HER2/neu-IgG3 in 100 μl were prepared and added to L-929 cells. After a 24-h incubation, 4000 PFU of VSV were added. Forty-eight hours later, viable cells were stained with crystal violet dye, dissolved by methanol, and solubilized dye was detected using an ELISA reader at 570 nm.

the addition to CT26/HER2. Heparin inhibited the binding of IgG3-IFN- α to CT26/HER2 cells but did not inhibit the binding of anti-HER2/neu-IgG3 and anti-HER2/neu-IgG3-IFN- α (Fig. 1C).

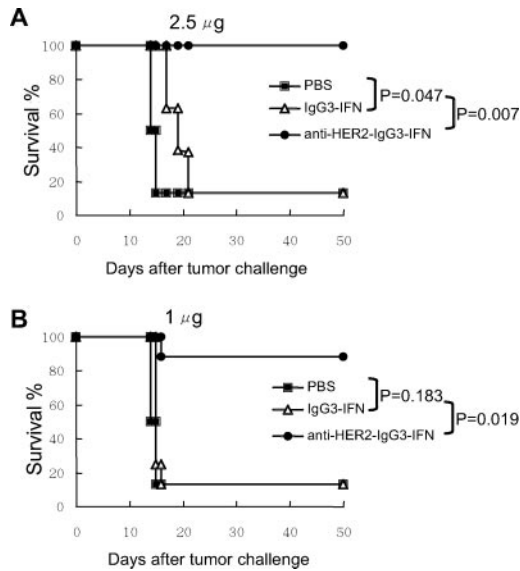


FIGURE 2. In vivo antitumor activity of different IFN- α fusion proteins and rIFN- α . C3H/HeN mice were s.c. challenged with 1×10^3 38C13/HER2 cells and i.p. treated with either 2.5 μ g (A) or 1 μ g (B) of the indicated proteins at days 1, 3, and 5 after tumor challenge. The tumor volume of each mouse is measured. Animals were observed until the diameter of the s.c. tumor reached 15 mm.

These results demonstrated that anti-HER2/*neu*-IgG3-IFN- α retained its ability to bind Ag and IgG3-IFN- α does not recognize HER2/*neu*. The L-929 fibroblast cell line sensitive to VSV infection was used to quantify the IFN- α biological activity of the fusion proteins in comparison to an IFN- α standard. Both anti-HER2/*neu*-IgG3-IFN- α and IgG3-IFN- α exhibited ~ 2400 U of IFN- α activity/ μ g activity against VSV-induced cytotoxicity in L-929 cells, while anti-HER2/*neu*-IgG3 exhibited no anti-viral activity (Fig. 1D).

In vivo antitumor activity of fusion proteins

To determine the in vivo antitumor activity of anti-HER2/*neu*-IgG3-IFN- α , syngeneic mice were inoculated s.c. with 1×10^3 38C13/HER2 tumor cells and treated on days 1, 3, and 5 after tumor challenge by i.p. administration of different doses of protein (Fig. 2). Mice treated with 2.5 μ g of IgG3-IFN- α showed some regression of tumor growth, with one (13%) of eight mice alive after 50 days (Fig. 2A). However, in vivo targeting of IFN- α to tumors using a tumor-specific Ab dramatically improved its antitumor effect. All mice treated with 2.5 μ g (Fig. 2A) of anti-HER2/*neu*-IgG3-IFN- α remained tumor free 50 days after tumor challenge ($p = 0.0048$ compared with PBS control), and none of the treated mice showed evidence of toxicity. Thus, targeting of IFN- α to the tumor cell surface resulted in significant antitumor activity compared with IFN- α linked to a nonspecific Ab ($p = 0.007$). Targeted anti-HER2/*neu*-IgG3-IFN- α continued to show potent antitumor activity when a lower dose was used. Seven (88%) of eight mice treated with 1 μ g (Fig. 2B) of anti-HER2/*neu*-IgG3-IFN- α remained tumor free after 50 days. In marked contrast, at this lower dose mice treated with IgG3-IFN- α showed tumor growth similar to mice treated with PBS ($p = 0.183$) and only one (13%) of eight mice survived. When the treatment was increased to three doses of 5 μ g, both anti-HER2/*neu*-IgG3-IFN- α and IgG3-IFN- α were effective in preventing tumor growth (data not shown) undoubtedly reflecting the fact that 38C13 cells are sensitive to IFN- α treatment (21, 27, 28). Tumor growth in mice treated with 5 μ g of anti-HER2/*neu*-IgG3 Ab was the same as the PBS control,

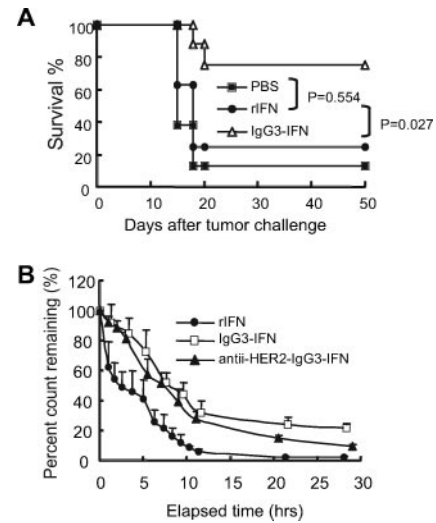


FIGURE 3. Fusion of IgG3 to IFN- α improved its antitumor activity and increased its in vivo half-life. A, Mice were treated with 9600 U of rIFN- α or 9600 U (4 μ g) of IgG3-IFN- α at days 1 and 3 after tumor challenge. Animals were followed for survival and sacrificed when the diameter of the s.c. tumor reached 15 mm. B, Groups of three C3H/HeN mice were injected i.p. with 66 μ Ci of 125 I-labeled rIFN- α , IgG3-IFN- α or, anti-HER2/*neu*-IgG3-IFN- α . At various intervals after injection of the 125 I-labeled proteins, residual radioactivity was measured using a mouse whole body counter. The results represent the mean of three mice. Bars, SD.

suggesting that Ab alone has no antitumor effect in vivo (data not shown). These results indicated that targeting of IFN- α to the tumor cells by a tumor-specific Ab can dramatically potentiate its effectiveness which was most clearly seen when low doses were administered. Importantly, this antitumor activity can be achieved without any evident toxicity.

IFN- α fused to an Ab results in improved antitumor activity compared with free IFN- α

As described above, we found that IFN- α fused to a nontumor-specific Ab exhibited antitumor activity. To compare its antitumor activity with that of soluble rIFN- α , mice were inoculated s.c. with 1×10^3 38C13/HER2 tumor cells and treated 1 and 3 days after tumor challenge by i.p. administration of 9600 U (4 μ g) of IgG3-IFN- α or 9600 U of rIFN- α (Fig. 3A). All mice treated with 9600 U of IgG3-IFN- α showed delayed tumor growth and 75% of the mice remained tumor free 50 days after tumor challenge ($p = 0.027$). In contrast, mice treated with the same number of units of rIFN- α were not statistically different from PBS controls in their tumor growth pattern.

IFN- α has a very short in vivo half-life (29). In previous study, fusion of Abs to cytokines has been shown to increase their half-life (6). The clearance of 125 I-labeled rIFN- α , IgG3-IFN- α , or anti-HER2/*neu*-IgG3-IFN- α was examined in C3H/HeN mice. Mice were injected i.p. with 66 μ Ci of 125 I-labeled proteins and the residual radioactivity was measured using a mouse whole body counter. rIFN- α was cleared rapidly with 50% eliminated by ~ 2.5 h (Fig. 3B). In contrast, both anti-HER2/*neu*-IgG3-IFN- α and IgG3-IFN- α exhibited significantly increased in vivo half-life with ~ 8 h required for elimination of 50% of the injected radioactivity. This increased half-life may contribute to the antitumor efficacy of the IFN- α fusion proteins. Thus, fusion of an IgG3 Ab to IFN- α can significantly improve its in vivo antitumor activity. However, this antitumor activity can be further improved by targeting the IFN- α to the tumor, making it effective at lower doses.

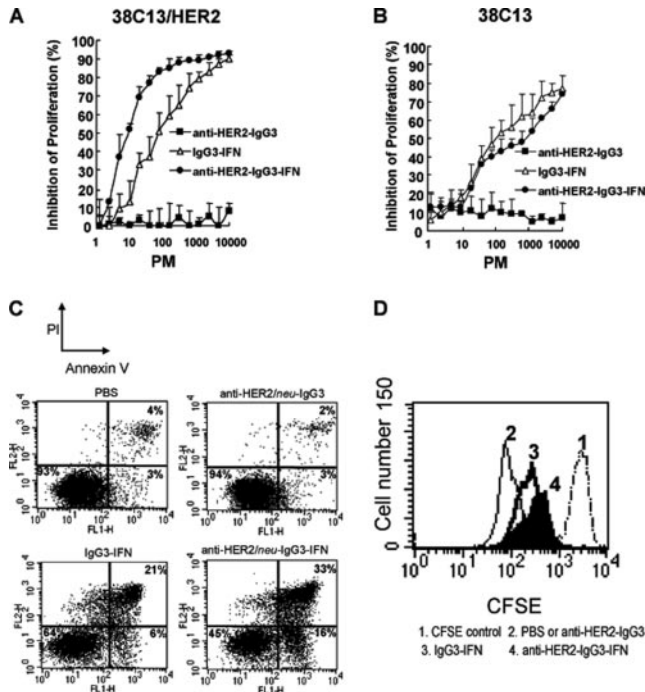


FIGURE 4. IFN- α fusion proteins inhibited cell proliferation and induced apoptosis in 38C13/HER2 cells in vitro. IFN- α fusion proteins inhibited tumor cell proliferation. After incubation for 48 h with different doses of the different fusion proteins, viable 38C13/HER2 (A) or 38C13 (B) cells were measured using the MTS assay. These experiments were performed three times in triplicate; error bars, SD of the measurements. C, IFN- α fusion proteins induce apoptosis in 38C13/HER2 cells. In brief, 1×10^6 38C13/HER2 cells were incubated with 1 nM of the indicated proteins for 72 h. The cells were then washed, stained with Alexa Fluor 488, annexin V, and PI and were analyzed by flow cytometry. The percentage of cells located in each quadrant is indicated at the corner. D, IFN- α fusion proteins inhibited proliferation of surviving 38C13/HER2 cells. In brief, 1×10^6 38C13/HER2 cells were labeled with 2.5 μ M CFSE and immediately fixed (dash line), or treated with PBS (thin black line), or 1 nM of either anti-HER2/neu IgG3 (thin black line, overlaps with PBS control), IgG3-IFN- α (thick black line), or anti-HER2/neu-IgG3-IFN- α (black area) for 48 h. The cells were then washed and analyzed by flow cytometry. The histogram was obtained by gating on the population of live cells.

Anti-HER2/neu-IgG3-IFN- α inhibited proliferation of tumor cells in vitro

IFN- α has multiple activities including activation of the immune response and direct cytotoxicity against tumors. To investigate potential mechanisms of the antitumor effects seen using either anti-HER2/neu-IgG3-IFN- α or IgG3-IFN- α , the eight mice remaining tumor free (see Fig. 2A) were challenged with 1×10^3 38C13/HER2 tumor cells. Surprisingly, all mice resembled untreated mice and quickly developed bulky tumors (data not shown). These results imply that under these experimental conditions of low tumor burden the IFN- α fusion proteins did not initiate a protective adaptive immune response, but instead the potent antitumor activity of the IFN- α fusion proteins is mediated either by the innate immune system or by a direct cytotoxic effect on tumor cells.

To determine whether IFN- α fusion proteins are directly cytotoxic to tumor cells, the 38C13/HER2 or parental 38C13 tumor cells were incubated with different proteins for 48 h and cell proliferation measured using the MTS assay. Treatment with anti-HER2/neu-IgG3 did not significantly inhibit the proliferation of either 38C13/HER2 or parental 38C13 tumor cells (Fig. 4, A, and B). Although both anti-HER2/neu-IgG3-IFN- α and IgG3-IFN- α

inhibited the proliferation of 38C13/HER2 tumor cells, anti-HER2/neu-IgG3-IFN- α was more effective than IgG3-IFN- α with IP_{50} values of 10 and 100 pM for anti-HER2/neu-IgG3-IFN- α and IgG3-IFN- α , respectively (Fig. 4A). In contrast, anti-HER2/neu-IgG3-IFN- α and IgG3-IFN- α exhibited similar antiproliferative activity against parental 38C13 tumor cells. These results provided evidence that IFN- α fusion proteins can directly inhibit the proliferation of the B cell lymphoma 38C13, and targeting IFN- α to tumor cells potentiated this effect.

Anti-HER2/neu-IgG3-IFN- α induced apoptosis in tumor cells in vitro

IFN- α signaling can induce apoptosis in some tumor cell lines. To determine whether apoptosis contributed to the antiproliferative effect we observed, 38C13/HER2 cells treated with different proteins were assayed for the translocation of phosphatidylserine using the annexin V-affinity assay (30). Dead cells were stained by PI, which enters cells with a disrupted plasma membrane and binds to DNA. Compared with the PBS control, there was no increase in the number of dead cells (annexin V/PI bright, 2%) or early apoptotic cells (annexin V bright, 3%) following treatment with anti-HER2/neu-IgG3 (Fig. 4C). In contrast, when cells were treated with IgG3-IFN- α , there was a significant increase in the number of dead cells (21%) and early apoptotic cells (6%). Treatment with anti-HER2/neu-IgG3-IFN- α resulted in a further increase in both the number of dead cells (33%) and early apoptotic cells (16%). These results indicated that IFN- α can induce apoptosis in 38C13/HER2 tumor cells, and that targeting IFN- α to tumor cells can markedly increase this effect.

In addition to inducing apoptosis, IFN- α can directly inhibit the proliferation of tumor cells (31). To determine whether both inhibition of proliferation and apoptosis were taking place in treated tumor cells, CFSE-labeled 38C13/HER2 cells were treated with different proteins for 48 h, the live cells were gated, and the level of CFSE was determined by flow cytometry. The CFSE signal in anti-HER2/neu-IgG3-treated cells (Fig. 4D, thin line) overlapped with the PBS-treated cells and was significantly less than that of cells fixed immediately after CFSE labeling (Fig. 4D, dotted line), indicating that anti-HER2/neu-IgG3 did not inhibit the proliferation of the 38C13/HER2. In contrast, IgG3-IFN- α significantly inhibited the proliferation of the surviving 38C13/HER2 cells (Fig. 4D, thick line), and targeting IFN- α to 38C13/HER2 cells by anti-HER2/neu-IgG3-IFN- α potentiated this effect (Fig. 4D, black area). These results indicated that although anti-HER2/neu-IgG3-IFN- α treatment did not result in complete cell death by 48 h, the surviving cells had a reduced ability to proliferate.

IFN- α fusion proteins induce STAT1 activation in tumor cells

Although engagement of the IFN- α receptor can initiate activation of multiple STAT proteins, STAT1 plays an obligate role in mediating IFN- α -dependent signaling (32). To investigate whether IFN- α fusion proteins initiate IFN- α signaling in 38C13/HER2 and that targeting IFN- α to tumor cells augments this effect, the phosphorylation of STAT1 following treatment was examined. As shown in Fig. 5, both anti-HER2/neu-IgG3-IFN- α and IgG3-IFN- α initiated robust STAT1 phosphorylation in 38C13/HER2 with STAT1 phosphorylation increasing 8-fold by 10 min. However, the phosphorylation of STAT1 induced by anti-HER2/neu-IgG3-IFN- α persisted for a longer period of time and greater STAT1 phosphorylation was seen at 30, 60, and 90 min in cells treated with anti-HER2/neu-IgG3-IFN- α . These results indicated that IFN- α fusion proteins can induce IFN- α signaling in 38C13

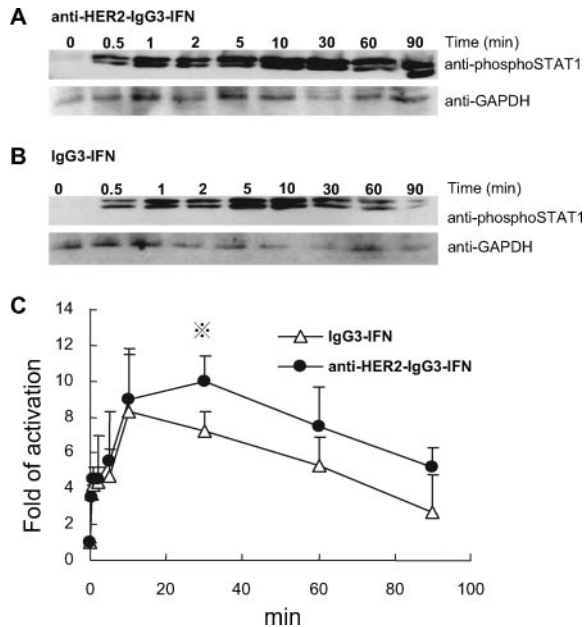


FIGURE 5. IFN- α fusion proteins induced STAT1 activation in 38C13/HER2 cells. In brief, 1×10^7 38C13/HER2 cells were treated with 1000 U/ml of either anti-HER2/*neu*-IgG3-IFN- α (A) or IgG3-IFN- α (B) for the indicated times. The cell lysates were separated by SDS-PAGE and analyzed by Western blot using a polyclonal rabbit anti-phosphoSTAT1. To confirm equal loading of protein samples, blots were probed with a HRP-conjugated rabbit polyclonal Ab against GAPDH. C, The intensity of anti-phosphoSTAT1 was normalized with the intensity of anti-GAPDH for each indicated time point, and the values obtained were divided by the value at time 0 to obtain the fold activation for STAT1. These experiments were performed twice; error bars, SD of the measurements. *, Only point where the two groups differ with a $p < 0.05$.

lymphoma cells and targeting IFN- α to tumor cells augments this effect.

*Anti-HER2/*neu*-IgG3-IFN- α exhibited potent activity against established tumors*

Because anti-HER2/*neu*-IgG3-IFN- α exhibited potent cytotoxicity against 38C13/HER2 tumor cells, we investigated whether anti-HER2/*neu*-IgG3-IFN- α would be effective against established 38C13/HER2 tumors. Syngeneic mice were inoculated s.c. with 1×10^3 38C13/HER2 tumor cells and i.p. treated with 5 μ g (Fig. 6) of the indicated proteins on days 12, 13, and 14 after tumor challenge. The average tumor size on day 12 is 100 mm³ and treatment with PBS or 10 μ g of anti-HER2/*neu*-IgG3 (data not shown) did not

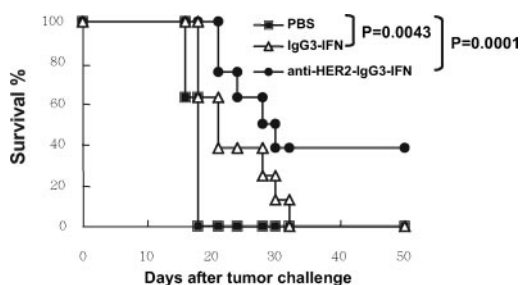


FIGURE 6. IFN- α fusion proteins inhibit the growth of established tumor. C3H/HeN mice were injected s.c. with 1×10^3 38C13/HER2 cells. After 12 days, mice were treated i.p. with 5 μ g of the indicated protein for 3 consecutive days. The tumor volume of each mouse is measured. Animals were sacrificed when the diameter of the s.c. tumor reached 15 mm.

inhibit tumor growth. Treatment with 5 μ g of IgG3-IFN- α showed some effect in inhibiting tumor growth; however, all mice developed bulky tumors and none of them survived 32 days after tumor challenge. In contrast all mice treated with 5 μ g of anti-HER2/*neu*-IgG3-IFN- α had delayed tumor growth, and three of eight mice had complete tumor regression and remained tumor free 50 days after tumor challenge (anti-HER2/*neu*-IgG3-IFN- α vs PBS, $p = 0.0001$; anti-HER2/*neu*-IgG3-IFN- α vs IgG3-IFN- α , $p = 0.063$). Thus, both IgG3-IFN- α and anti-HER2/*neu*-IgG3-IFN- α showed antitumor activity but anti-HER2/*neu*-IgG3-IFN- α was more effective in delaying tumor growth and complete tumor remission was observed only in mice treated with anti-HER2/*neu*-IgG3-IFN- α . When the treatment dose was increased to 10 μ g of the fusion proteins, almost all mice treated with either anti-HER2/*neu*-IgG3-IFN- α or IgG3-IFN- α had complete tumor regression and remained tumor free after 50 days.

The mice that remained tumor free following treatment with three doses of 10 μ g of fusion proteins were rechallenged with 1×10^3 38C13/HER2 tumor cells on day 50. All mice remained tumor free (data not shown). These results suggest that an adaptive immune response with immunologic memory is initiated when larger, established tumors are treated with IFN- α fused to an Ab.

Discussion

Although rIFN- α has shown activity against B cell lymphoma and multiple myeloma, inconsistent efficacy and systemic toxicity have limited its usefulness (33). The present work demonstrates that fusing IFN- α to an Ab improves its efficacy against tumors with further improvement seen when IFN- α is targeted to tumor cells by a tumor-specific Ab. This antitumor efficacy is seen without any apparent toxicity. These studies suggest that fusion of IFN- α with tumor-specific Ab may yield an effective biologic agent for the treatment of B cell lymphoma.

To test the hypothesis that directing IFN- α to tumor sites with Ab would result in improved efficacy, we chose a well-characterized murine B cell lymphoma engineered to express a common TAA, HER2/*neu*, to which Abs are available. Anti-HER2/*neu*-IgG3-IFN- α appears to be more effective in the treatment of the 38C13 B cell lymphoma than previously described immunotherapeutics, although in the present study a foreign Ag introduced by gene transduction was the target. Treatment with three 1- μ g doses of anti-HER2/*neu*-IgG3-IFN- α beginning 1 day after tumor challenge appeared to be as effective in inhibiting tumor growth as treatment with 10 μ g of anti-Id IgG1-IL-2 fusion protein for 5 days beginning 1 day after tumor challenge (34). In addition, anti-HER2/*neu*-IgG3-IFN- α was effective against established tumors (Fig. 6) while anti-Id IgG1-IL-2 had little antitumor activity when treatment was begun either 3 or 7 days after tumor challenge (34). The ability to cure established tumors also suggests that Ab-targeted IFN- α is a more powerful therapeutic agent than GM-CSF (35), CTLA-4 (36), or CD40 ligand (37) fused to the Id Ag since none of these vaccine strategies was effective against established tumors. Therefore, targeting IFN- α to tumor cells could be a promising approach for treating B cell lymphoma.

Targeting IFN- α to tumor cells with a tumor-specific Ab increases the antitumor efficacy of IFN- α . Anti-HER2/*neu*-IgG3-IFN- α is more effective in inhibiting proliferation and inducing apoptosis (Fig. 4) in 38C13/HER2 than IgG3-IFN- α and treatment with either 2.5 or 1 μ g of anti-HER2/*neu*-IgG3-IFN- α was more effective in inhibiting growth of small tumors in vivo than the same doses of IgG3-IFN- α (Fig. 2, A and B). These results suggest that the tumor-specific Ab directs IFN- α to the tumor, thereby improving its therapeutic index with decreased systemic toxicity.

Remarkably, IgG3-IFN- α exhibits a more potent antitumor activity than rIFN- α (Fig. 3A). Although rIFN- α is effective in treatment of a variety of tumors (38–40), prolonged treatment with high doses is required to see effective antitumor activity in part because of the very short half-life of the cytokine. In this study, we demonstrated that fusion of an IgG3 Ab to IFN- α significantly increased its half-life (Fig. 3B), and this increased half-life may contribute to the increased *in vivo* antitumor activity of the fusion protein (Fig. 3A). In addition, the Fc region of the IgG3-IFN- α may help to target IFN- α to the Fc receptors present on B lymphoma cells and consequently increase the antitumor activity. Therefore, fusion of IFN- α to an IgG3 Ab may provide multiple advantages in improving the antitumor efficacy of IFN- α .

Although IFN- α has multiple activities, including activation of the immune response, it appears that direct cytotoxicity plays an important role in the potent antitumor activity of anti-HER2/*neu*-IgG3-IFN- α . Both IFN- α fusion proteins exhibited apoptotic and antiproliferative activities against 38C13/HER2 with tumor targeting significantly increasing these effects (Fig. 4). Although the IFN- α fusion proteins were very effective in treating small tumors (Fig. 2), none of the survivors developed an immune response that protected against second tumor challenge, suggesting that the direct cytotoxicity of the IFN- α fusion proteins was very effective in killing the tumor cells and that the adaptive immunity did not play a role when there was a small tumor burden. Because 38C13 is an extremely malignant B lymphoma cell line and mice injected with as few as 200 cells can develop bulky tumors within 20 days (36), the IFN- α fusion proteins must be very effective in killing most of the inoculated tumor cells to result in long-term survivors. Multiple mechanisms, including down-regulation of NF- κ B (41), induction of apoptosis by activating caspase-3 (42), and up-regulation of both TRAIL and TRAIL receptors (43), have been shown to be involved in IFN- α -mediated cytotoxicity against tumor cells, and we would expect these mechanisms to contribute to the direct cytotoxicity against tumor cells seen with Ab-IFN- α fusion proteins. Consistent with this, we observed STAT1 activation following treatment of tumor cells with the fusion proteins (Fig. 5).

Although IFN- α fusion proteins failed to initiate a memory immune response when mice were treated beginning 1 day after tumor inoculation, IFN- α fusion proteins initiated an immune response that protected against second tumor challenge when mice were treated beginning 12 days after tumor inoculation. Therefore, IFN- α fusion proteins can activate protective adaptive immunity in the presence of a sizable tumor burden. Because IFN- α is capable of activating adaptive immunity via stimulation of DC differentiation and maturation (9), it is possible that the established tumors provide more TAAs for DC activation in the presence of IFN- α . In addition, the foreign Ag human HER2/*neu* may contribute to the antitumor immunity by increasing the immunogenicity of the tumor cells in this model.

CD20, an Ag expressed by B cells, is expressed in most B cell lymphomas (44), and anti-CD20 (rituximab, Genentech;) is one of the most successful cancer therapeutics, having tremendous efficacy against lymphoma with little toxicity (45). Although anti-HER2/*neu* IgG3-IFN- α is very effective against 38C13/HER2, HER2/*neu* is not normally expressed in lymphoma cells and therefore, it probably has limited therapeutic application. However, fusion of IFN- α to anti-CD20 could overcome this drawback and would be expected to yield a fusion protein with even greater antitumor activity by combining the antilymphoma activity of anti-CD20 and the potent immunostimulatory and cytotoxic activity of IFN- α in one protein. Additionally, IFN- α may further up-regulate CD20 expression as was seen in patients with B cell lymphoma following IFN- α treatment (46). We are currently studying the

effects of anti-CD20-IFN- α fusion proteins in murine models of B cell lymphoma.

In summary, we have constructed and characterized a novel fusion protein in which IFN- α was linked to an Ab recognizing a TAA. Our results indicate that fusion of IFN- α to a tumor-specific Ab can dramatically increase the efficacy of IFN- α with antitumor activity observed without any apparent toxicity. Remarkably, the Ab-IFN- α fusion protein was effective against established tumors. Therefore, IFN- α fused to a tumor-specific Ab shows promise for the treatment of B cell lymphoma.

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Disclosures

The authors have no financial conflict of interest.

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