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Anti-CD20-interferon- β fusion protein therapy of murine B cell lymphomas

K. Ryan Trinh¹, Alex Vasuthasawat¹, Kristopher K. Steward², Reiko E. Yamada², John M. Timmerman², and Sherie L. Morrison¹

¹Department of Microbiology, Immunology and Molecular Genetics, David Geffen School of Medicine, University of California, Los Angeles

²Division of Hematology and Oncology, Department of Medicine, David Geffen School of Medicine, University of California, Los Angeles

Abstract

Type I interferons (IFN α/β) are cytokines with a broad spectrum of anti-tumor activities including anti-proliferative, pro-apoptotic, and immunostimulatory effects, and are potentially useful in the treatment of B cell malignancies and other cancers. To improve anti-tumor potency and diminish the systemic side effects of IFN, we recently developed anti-CD20-IFN α fusion proteins with *in vitro* and *in vivo* efficacy against both mouse and human lymphomas expressing CD20. Since IFN β binds more tightly to the IFN α/β receptor (IFNAR) and has more potent anti-tumor activities, we have now constructed an anti-CD20 fusion protein with murine IFN β (mIFN β). Anti-CD20-mIFN β was more potent than recombinant mIFN β and anti-CD20-mIFN α in inhibiting the proliferation of a mouse B cell lymphoma expressing human CD20 (38C13-huCD20). Growth inhibition was accompanied by caspase-independent apoptosis and DNA fragmentation. The efficacy of anti-CD20-mIFN β required the physical linkage of mIFN β to anti-CD20 antibody (Ab). Importantly, anti-CD20-mIFN β was active against tumor cells expressing low levels of IFNAR (38C13-huCD20 IFNAR^{lo}). *In vivo*, established 38C13-huCD20 tumors were largely insensitive to rituximab or a non-targeted mIFN β fusion protein, yet treatment with anti-CD20-mIFN β eradicated 83% of tumors. Anti-CD20-mIFN β was also more potent *in vivo* against 38C13-huCD20 than anti-CD20-mIFN α , curing 75% versus 25% of tumors ($p = 0.001$). Importantly, while anti-CD20-mIFN α could not eradicate 38C13-huCD20 IFNAR^{lo} tumors, anti-CD20-mIFN β treatment prolonged survival ($p = 0.0003$), and some animals remained tumor-free. Thus, Ab fusion proteins targeting mIFN β to tumors show promise as therapeutic agents, especially for use against tumors resistant to the effects of mIFN α .

Keywords

tumor immunity; lymphoma; antibodies; interferon; immunotherapy; rituximab

INTRODUCTION

Although first described for their ability to inhibit viral replication, type I IFNs have multiple actions exhibiting both anti-proliferative effects against and inducing apoptosis in tumor cells (1, 2). They also inhibit angiogenesis (3, 4) and exert multiple effects on the

Corresponding Author: Sherie L. Morrison, Department of Microbiology, Immunology, and Molecular Genetics, Room 241C BSRB, University of California, Los Angeles, Los Angeles, CA 90095-7364, Phone: 310-206-5124, FAX: 310-794-5126, sheriem@microbio.ucla.edu.

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immune response (5, 6). In both humans and mice there is only one form of IFN β , but multiple species of IFN α , with mice having 14 functional and 3 pseudo IFN α genes (7, 8).

Type I IFNs elicit their diverse responses by binding a shared cell surface receptor consisting of the two transmembrane proteins IFN α/β receptor (IFNAR) 1 and 2. Although binding the same receptor, IFN α and IFN β differ significantly in their activity and affinity for these membrane proteins and in the downstream consequences of signaling. Data indicate that IFN first binds to IFNAR2 and subsequently recruits IFNAR1 in a transient fashion (9). Both receptors contribute in an additive manner to the total binding affinity of IFN and it is the stability of this ternary complex that determines both the anti-proliferative and anti-viral activity of the IFN (10). While the anti-proliferative potency was found to correlate with the binding affinity throughout the entire range of affinities examined, the anti-viral potency reached a maximum at binding affinities equivalent to that of wild-type human IFN α 2 (10).

For both humans and mice the isoforms of IFN α differ in their relative tumor anti-proliferative and anti-viral activity. In man the 12 different subtypes of IFN α show a wide range of anti-proliferative activity with IFN α 14 being the strongest. IFN α 14 is nearly 10 times as potent against OVCAR cells as IFN α 2, the IFN α subtype most commonly used in the clinic (11). In this assay human IFN β is approximately 15 times more effective than IFN α 2 (8). In the mouse, mIFN α 11 and mIFN α 12 have the most potent anti-proliferative activity and resemble murine IFN β (mIFN β) in their anti-proliferative activity (8). However, we used mIFN α 1 in our initial fusion protein because the vast majority of the murine IFN α s resemble mIFN α 1 in their anti-proliferative activity. mIFN β is approximately 100-fold more effective than mIFN α 1 in inhibiting proliferation and approximately 10-fold more effective in anti-viral activity (8).

IFNs were the first recombinant proteins to be used in the therapy of cancer (2, 12). Although IFN is effective in the treatment of a variety of malignancies, prolonged treatment with IFN can be problematic because of the associated side effects. The most common adverse events associated with IFN α therapy are flu-like symptoms, fatigue, anorexia, neutropenia, neuropsychiatric symptoms, and injection reactions; these side-effects may become dose-limiting (13). Frequent administration is also required because of the short *in vivo* half-life of IFN with IFN α 2 having a half-life of only about 1 hour (14). PEGylation, in which the protein is covalently linked to linear or branched polyethylene glycols, can prolong the half-life and improve the efficacy of IFN α (4, 15) but the activity of the PEGylated IFN is often compromised (16). Both direct injection into the tumor and gene therapy have been used to deliver high levels of IFN to tumors. Intra-lesional injection of low-dose IFN α was effective for the treatment of patients with primary cutaneous marginal zone B cell lymphomas (17). In mouse models, IFN β gene therapy caused the dramatic regression of human tumors apparently as a result of the direct anti-proliferative or cytotoxic activity of IFN β (18). Interestingly, only a fraction of the cells had to be transduced to achieve tumor regression. These results have suggested that targeting type I IFN to tumor cells *in vivo* might substantially enhance its efficacy and tolerability.

CD20 is a nonglycosylated integral 33–37 kDa transmembrane phosphoprotein expressed on more than 95% of normal and neoplastic B cells. The elevated levels of the CD20 protein in B cell malignancies, its extracellular accessibility and the fact that it is not internalized, downregulated or shed, make it an excellent target for treating B cell cancers. Multiple mechanisms of action have been proposed for the efficacy of anti-CD20 monoclonal Abs (mAbs) in treating lymphoma including the induction of apoptosis, antibody-dependent cell-mediated cytotoxicity (ADCC), phagocytosis, complement-dependent cytotoxicity (CDC),

cross-priming of CD8⁺ T cells (19) and down-regulation of Bcl-X_L with a concomitant increase in chemosensitization (20).

In initial studies we investigated the ability of anti-CD20-IFN α fusion proteins to treat B cell lymphomas in mice (21). Although the mIFN α 1 and human IFN α 2 fusion proteins had reduced IFN α bioactivity *in vitro* compared with native IFN α , CD20 targeting resulted in efficient anti-proliferative and pro-apoptotic effects against an aggressive rituximab-insensitive human CD20⁺ mouse lymphoma (38C13-huCD20) and a human B cell lymphoma (Daudi). Optimal tumor eradication required CD20 targeting. Importantly, potent anti-tumor efficacy was observed in the absence of toxicity. Gene knockdown studies demonstrated that tumor eradication required expression of IFNAR on the tumor cell surface and that anti-CD20 mAb fused with mIFN α had reduced efficacy both *in vitro* and *in vivo* against tumors with decreased expression of IFNAR.

Given the enhanced anti-proliferative activity of mIFN β compared to mIFN α 1, we have now extended these studies by evaluating the efficacy of anti-CD20 fused to mIFN β (anti-CD20-mIFN β) against mouse tumors expressing human CD20. Indeed we show that anti-CD20-mIFN β has more potent anti-tumor activity than anti-CD20-mIFN α . Importantly, anti-CD20-mIFN β is effective both *in vitro* and *in vivo* against a tumor with decreased expression of IFNAR, a tumor that was resistant to the effects of anti-CD20-mIFN α . Thus, fusion proteins targeting IFN β to tumors show great promise as therapeutic agents, especially for use against tumors resistant to the effects of IFN α .

MATERIALS AND METHODS

Cell lines

38C13-huCD20 mouse B cell lymphoma cells, which express human CD20, were previously described (22). Both 38C13 (23) and 38C13-huCD20 were cultured in IMDM (Invitrogen, Carlsbad, CA) supplemented with 5% calf serum (CS) (Atlanta Biologicals, Lawrenceville, GA). The cell line 38C13-huCD20 IFNAR^{lo} was produced by transducing 38C13-huCD20 cells with a lentiviral vector encoding an shRNA targeting the IFNAR1 subunit of IFNAR with the sense sequence 5' – GCGTCTACATTATAGATGACAA – 3' as previously described (21). 38C13-huCD20 IFNAR^{lo} and Chinese Hamster Ovary (CHO) cells were cultured in IMDM supplemented with 5% CS.

Construction of expression vectors

The heavy (V_H) and light (V_L) chain variable regions of the anti-CD20 mAb 2B8(24), the murine parent of rituximab, were cloned into expression vectors for kappa light chain and human gamma heavy chain with the SGGGS peptide linker at the end of C_H3, as previously described (21). mIFN β was cloned using the primers 5' – GGGATCCATCAACTATAAGCAGCTCCAGCTC – 3' and 5' – GGTCTAGAATCAGTTTGGAAAGTTTCTGGTAAGTC – 3' with cDNA obtained from 6.5.2, a mutant of the mouse myeloma cell line J558 (25). After sequence verification, the inserts were cloned as a BamH I/Xba I fragment into pcDNA3.1 in which the *neo* resistance marker had been changed to *his* (unpublished). The anti-CD20 V_H and IgG constant regions were then inserted as EcoR V/BamH I fragments. To produce a non-targeted fusion protein, the V_H of anti-CD20 was replaced with the V_H specific for the hapten dansyl (DNS) (26). Vectors were available for the production of the anti-DNS chimeric kappa light chain.

Protein production and purification

CHO cells were transfected with the heavy and light chain vectors using Lipofectamine PlusTM according to the manufacture's protocol (Invitrogen), and stable transfectants

isolated. For protein production, cells were seeded into roller bottles, and once confluent, cells were expanded to 100–120 mL with IMDM + 1% Fetal Clone (Thermo Fisher, Waltham, MA). The supernatant was removed every 2–3 days and replaced with fresh medium. Cell-free culture supernatants were then passed through a protein A Sepharose 4B fast flow column (Sigma-Aldrich, St. Louis, MO) and the bound protein eluted with 0.1 M citric acid, pH 3.5. Eluted fractions were neutralized immediately with 2 M Tris-HCl pH 8.0. Fractions were run on SDS-PAGE gels and stained with Coomassie blue to verify protein purity and integrity. Concentrations of proteins were determined using the BCA assay (Pierce, Rockford, IL). Recombinant mIFN β reference standard was obtained from the NIH (Bethesda, MD) or purchased from PBL InterferonSource (Piscataway, NJ). Rituximab was obtained from Genentech (South San Francisco, CA).

Determination of Binding to CD20

38C13-huCD20 cells were washed twice with PBS + 1% BSA + 0.1% sodium azide (FACS buffer), resuspended to 3×10^6 cells/ml and 100 μ l cells added to each tube. Rituximab and anti-CD20-mIFN β fusion protein were serially diluted and added to the cells to achieve final concentrations of 0–106 nM. Cells were incubated on ice for 30 minutes, washed twice, then incubated with mouse anti-human kappa-PE (Invitrogen) for 30 minutes on ice. After washing twice, samples were run on a BD FACSCalibur flow cytometer using Cell Quest Pro software and data analyzed using FCS Express (De Novo Software, Los Angeles, CA).

Inhibition of proliferation assay

38C13, 38C13-huCD20, or 38C13-huCD20 IFNAR^{lo} cells (1×10^4 per well) were seeded in 96-well plates and incubated with various treatments at 37°C for 48–72 h. Cell viability was quantified using MTS solution (Promega, Madison, WI) by measuring absorbance at 490 nm using a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Inc., Winooski, VT). Data were analyzed by non-linear regression using Prism (GraphPad Software, Inc., La Jolla, CA) with the log [inhibitor] vs. the response with a variable slope with the IC₅₀ calculated. Data are expressed as the percentage of maximum metabolic activity of untreated cells.

Ki-67 assay

2×10^5 38C13-huCD20 cells were cultured with 20 pM of anti-CD20-mIFN β for 0, 1, 6, 18, 24, 30, or 48 h at 37°C. At each time point cells were pelleted, washed twice with cold PBS, permeabilized with 1 ml cold methanol, washed twice with cold PBS + 3% BSA, and incubated for 1 h at 4°C with rabbit anti-Ki-67 (Abcam, Cambridge, MA) diluted 1:100 in PBS + 3% BSA. Cells were then washed twice, and incubated for 1 h at 4°C with 5 μ l Fc Block (BD Pharmingen, San Jose, CA) and mouse anti-rabbit IgG-FITC (Sigma) diluted to 1:200 in PBS + 3% BSA. After incubating the cells with the secondary Ab, they were washed twice, resuspended in 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), and stored at 4°C in the dark for later analysis by flow cytometry. Controls included unstained cells, cells incubated with Fc Block but stained only with mouse anti-rabbit IgG-FITC, or cells incubated with Fc Block plus rabbit anti-human IgE (MP Biomedicals, Irvine, CA) and mouse anti-rabbit IgG-FITC. Samples were analyzed by flow cytometry and data analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

Cell cycle and DNA fragmentation analysis

38C13-huCD20 cells (2×10^5) were incubated with the indicated reagents for various times, harvested by centrifugation, supernatant removed by aspiration, and cells resuspended in 1 ml of hypotonic DNA staining buffer (1 mg/ml sodium citrate, 100 μ g/ml PI, 20 μ g/ml RNase A, and 0.3% Triton X-100 in dH₂O). Samples were kept on ice protected from light

for 30 minutes or for a maximum of 1 h and analyzed by flow cytometry. Cell cycle analysis was performed using FlowJo with the Watson Model. For DNA fragmentation time course studies, 38C13-huCD20 cells were incubated with 100 pM of anti-CD20-mIFN β for 1, 6, 24, or 48 h, washed and treated with 1X trypsin/EDTA (Invitrogen) for 10 minutes, washed once with IMDM + 5% CS, returned to culture for the remainder of the 48 h culture period before harvest and DNA staining as above.

Apoptosis and metabolic activity assays

2×10^5 38C13-huCD20 or 38C13-huCD20 IFNAR^{lo} cells were incubated with various treatments at 37°C for 48 h. Cells were then stained with Annexin V-FITC and propidium iodide (PI) to distinguish populations of early apoptotic (Annexin V⁺/PI⁻) and dead (Annexin V⁺/PI⁺) cells using Vybrant Apoptosis Kit #2 (Molecular Probes, Carlsbad, CA), and analyzed by flow cytometry. Metabolic activity was quantitated using the LIVE/DEAD Cell Vitality Assay Kit -C₁₂ resazurin/SYTOX Green assay kit (Molecular Probes) per manufacturer's instructions, substituting Annexin V-FITC for SYTOX Green counterstaining where indicated.

Caspase activation assay

38C13-huCD20 cells were seeded in 96-well black walled clear bottom plates at a concentration of 1×10^4 cells/well in IMDM growth medium. Fusion protein or rituximab was added to a final concentration of 100 pM and samples incubated at 37°C. As a positive control, cells were treated with 10 μ M of staurosporine and negative controls included no cells and no treatment. All treatments were performed in triplicate. At each time point, one plate was removed and 100 μ l of caspase substrate (Promega), prepared according to the manufacturer's protocol, was added to wells. Luminescence was read 45 minutes later on a Synergy HT plate reader. Z-DEVD-aminoluciferin was used as substrate for caspase 3/7 activity. Caspase 2 activity was assayed using Z-VDVAD-aminoluciferin as the substrate in the presence of Ac-DEVD-CHO and MG-132 to select for caspase 2 activity. Caspase 8 was assayed using Z-LETD-aminoluciferin as the substrate in the presence of MG-132 to select for caspase 8 activity. Caspase 9 activity was assayed using Z-LEHD-aminoluciferin as substrate in the presence of MG-132 to select for caspase 9 activity. All substrates were purchased from Promega. The pan-caspase inhibitor Z-VAD(OMe)-FMK (BD Pharmingen) was added at 20 μ M and apoptosis analyzed at 24 or 48 h. When apoptosis was analyzed at 48 h a second treatment of 20 μ M Z-VAD(OMe)-FMK was added at 24 h.

Determination of fusion protein half-life

Four mice were injected i.v. with 200 μ g of anti-CD20-mIFN β . Two mice were bled (from retro-orbital sinus) at 5 minutes, 6 h, 48 h, and 101 h; the other two mice were bled at 30 minutes, 24 h, and 101 h. Blood was allowed to clot on ice and the serum removed following centrifugation. Anti-CD20-mIFN β was detected by ELISA using plates coated with anti-mIFN β Ab (clone 7F-D3; Abcam). The standard curve was established using serial 1:2 dilutions into 1% BSA of anti-CD20-mIFN β starting at 0.4 μ g/ml. Controls included serum only, 1% BSA only and wells not coated with Ab. Serial dilutions of previously diluted serum were performed for the experimental serum samples. Plates were incubated at 4°C overnight, washed with PBS + 0.2% Tween 20, and bound fusion protein detected with biotinylated anti-kappa Ab (Immuno Biological Laboratories, Minneapolis, MN) followed by streptavidin-AP (Zymed Laboratories, San Francisco, CA). ELISA plates were developed with 1 mg/ml *p*-nitrophenyl phosphate (Sigma-Aldrich) in 1 M diethanolamine-0.25 mM MgCl₂, pH 9.8 and the absorbance was measured at 410 nm. The concentration of fusion protein remaining in circulation was extrapolated from the standard curve. Data were analyzed using Excel (Microsoft Corporation, Redmond, WA) and the $T_{1/2}$ calculated as $0.693/\lambda$.

Determination of antibody-dependent cellular cytotoxicity (ADCC)

ADCC assays were performed using the lactate dehydrogenase (LDH) release assay method following the manufacturer's protocol (Roche Applied Science, Indianapolis, IN) as previously described (22). Mouse effector cells were collected from C3H spleens by mechanical disruption. After lysis of red blood cells using ammonium chloride buffer (0.15 M NH_4Cl , 10 mM KHCO_3 , 0.1 mM EDTA, pH 7.4), splenocytes were washed and cultured for 48 h in RPMI 1640 supplemented with 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, and 50 μM β -mercaptoethanol (all from Invitrogen; RPMI complete growth medium) containing 10% FCS (Omega Scientific, Tarzana, CA) with 1000 U/ml human rIL-2 (Chiron, Emeryville, CA). Cells were then collected, washed, and counted. 38C13-huCD20 target cells were harvested from culture, washed, counted, and incubated for 30 minutes at 4°C in the presence or absence of 53 nM rituximab or anti-CD20-mIFN β in RPMI complete growth medium containing 5% FCS. After incubation, target cells were seeded in 96-well U-bottom plates, in triplicate, at 1×10^5 cells/well with increasing numbers of effector cells for 5 h at 37°C. Maximum and spontaneous release was measured by adding 0.1% Triton X-100 or medium, respectively, to target cells alone. After incubation, supernatant was collected, and LDH release was measured on a Spectramax 384 Plus microplate reader (Molecular Devices, Sunnyvale, CA). Percentage cytotoxicity was calculated as $100 \times [(\text{effector:target cell mix release} - \text{effector cell control} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})]$.

Determination of complement-dependent cytotoxicity (CDC)

38C13-huCD20 cells were harvested from culture, counted and resuspended to 1.2×10^6 cells/ml in RPMI complete growth medium containing 10% FCS. 250 μl of cells were then added to tubes and rituximab or anti-CD20-mIFN β added to achieve the indicated final concentration ranging from 0–53 nM. Triplicate samples were incubated for 15 minutes on ice to allow Ab binding. After incubation, rabbit serum (Sigma-Aldrich) or heat-inactivated rabbit serum was added as a source of complement to a final concentration of 10%, and samples incubated at 37°C for 3 h. After incubation, cells were washed twice in PBS, stained with PI, and analyzed by flow cytometry to assess viability.

In vivo anti-tumor activity against mouse B cell lymphoma

Six- to eight-week-old female C3Hf/Sed/Kam mice were bred and housed at the UCLA Defined Pathogen Colony according to institutional guidelines. 38C13-huCD20 or 38C13-huCD20 IFNAR^{lo} cells were thawed from common dedicated frozen stocks three days before tumor challenge and split the day before use. For tumor challenge, cells were washed twice in HBSS (Invitrogen) and diluted to the appropriate concentration in HBSS. Challenge inocula consisted of 5×10^3 38C13-huCD20 or 38C13-huCD20 IFNAR^{lo} cells injected subcutaneously (s.c.) above the base of the tail. Experimental groups of 8 mice (except where indicated) were treated with 100 μg of anti-CD20-mIFN α , anti-CD20-mIFN β , anti-DNS-mIFN β , or rituximab intravenously (i.v.) on days 5, 6, and 7 or on days 5, 6, 7 and 14 post tumor challenge. Mice were followed for survival and sacrificed when tumors reached 1.4 cm in diameter as per institutional guidelines. Bidirectional tumor growth measurements were obtained throughout the experiment. All studies were performed in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and were approved by the UCLA Animal Research Committee.

RESULTS

Ab fusions with murine IFN β are more effective than fusions with murine IFN α 1 in inhibiting proliferation of lymphoma cells

Anti-CD20 and anti-DNS fusion proteins were produced with mIFN β joined to the C-terminus of the C_H3 domain of human IgG1 by a SGGGS linker (Fig. 1A). Use of IgG1 fusion proteins permits direct comparison to the IgG1 anti-human CD20 mAb rituximab. The anti-CD20-mIFN β fusion protein bound avidly to cells expressing huCD20 (Fig. 1B), similar to rituximab. Slightly greater binding was observed with the fusion protein, possibly reflecting simultaneous binding of the fusion protein to both CD20 and IFNAR. To assess the ability of the mIFN β moiety within the antibody-mIFN β fusion proteins to inhibit cellular proliferation, 38C13 lymphoma cells were incubated with various concentrations of anti-CD20-mIFN β , anti-DNS-mIFN β , or recombinant mIFN β for 48 h and the metabolic activity of the cells measured using the MTS assay (Fig. 1C). Under these conditions there is no targeting of the fusion proteins and the assay is measuring only the intrinsic anti-proliferative IFN activity of the fusion protein. The fusion proteins containing mIFN β were very effective in inhibiting the proliferation of 38C13, with both fusion proteins exhibiting similar activity and being somewhat more effective on a molar basis than recombinant mIFN β . This contrasts with our previous observations using fusions with mIFN α in which the non-targeted fusion proteins were less effective than recombinant mIFN α in inhibiting cellular proliferation (21).

To determine the contribution of antigen targeting to the ability of the antibody-mIFN β fusion proteins to inhibit cellular proliferation and to compare their efficacy with mIFN α fusion proteins, 38C13-huCD20 lymphoma cells were incubated with various concentrations of anti-CD20-mIFN β , anti-DNS-mIFN β , recombinant mIFN β , anti-CD20-mIFN α , or recombinant mIFN α for 72 h and the metabolic activity of the cells measured using the MTS assay (Fig. 1D). Several things are noteworthy. As has been previously observed (21) anti-CD20-mIFN α was very effective in inhibiting the proliferation of 38C13-huCD20, with anti-CD20-mIFN α (IC₅₀ = 10.3 pM) more effective than recombinant mIFN α (IC₅₀ = 750 pM). Although recombinant mIFN β (IC₅₀ = 70.7 pM) is more potent than recombinant mIFN α , targeted anti-CD20-mIFN α is more potent than non-targeted recombinant mIFN β . Interestingly, the non-targeted anti-DNS-mIFN β (IC₅₀ = 12.7 pM) was approximately as active as the targeted anti-CD20-mIFN α and was more effective than recombinant mIFN β . Targeting the mIFN β fusion protein to CD20 expressed on the cell surface made it even more potent (IC₅₀ = 3.3 pM). The fact that fusion protein was effective in the pM range while rituximab has been reported to have an affinity of 8 nM (<http://www.rxlist.com/rituxan-drug.htm>) probably reflects the increased avidity of the fusion protein, which would bind to cells through both the two Ab binding sites and the two attached IFN β molecules. Overall, anti-CD20-mIFN β was approximately 3-times more potent *in vitro* than anti-CD20-mIFN α , a protein that has been demonstrated to have potent anti-tumor activity both *in vitro* and *in vivo* (21).

Cell killing by IFN α fusion proteins has been shown to require engaging the IFNAR and decreased expression of IFNAR1 has been shown to make 38C13-huCD20 cells relatively insensitive to treatment with anti-CD20-mIFN α (21). In contrast, cells with decreased expression of IFNAR1 (38C13-huCD20 IFNAR^{lo}) remain sensitive to inhibition by anti-CD20-mIFN β (IC₅₀ = 10.7 pM) although they are not as sensitive as the parental cell line with normal IFNAR expression (Fig. 1D). Recombinant mIFN β (IC₅₀ = 420 pM) is also effective against 38C13-huCD20 IFNAR^{lo} but targeted anti-CD20-mIFN β is approximately 40-fold more effective.

Treatment of 38C13-huCD20 with anti-CD20-mIFN β causes DNA fragmentation and alterations in cell cycle

To determine if inhibition of proliferation is associated with changes in the cell cycle, 38C13-huCD20 was treated with varying concentrations of anti-CD20-mIFN β or anti-DNS-mIFN β for 72 h and DNA content analyzed by flow cytometry following staining with PI. Treatment with either anti-CD20-mIFN β or anti-DNS-mIFN β results in dose-dependent loss of cell viability and alterations in the cell cycle with an accumulation of cells in G0/1 and a decrease in cells in S phase (Fig. 2A). However, anti-CD20-mIFN β is far more potent than anti-DNS-mIFN β , with treatment of only 20 pM anti-CD20-mIFN β resulting in a near maximal affect. In contrast, anti-DNS-mIFN β , even at concentrations of 2500 pM, never achieves this level of cell G0/1 accumulation and S phase reduction, clearly demonstrating the importance of targeting for fusion protein efficacy.

The Ki-67 protein is present during all active phases of the cell cycle, but is absent from cells in G0 (27). To determine if cells treated with anti-CD20-mIFN β have exited the cell cycle and ceased dividing, 38C13-huCD20 cells were treated with 20 pM of anti-CD20-mIFN β for varying lengths of time and their expression of Ki-67 determined by flow cytometry (Fig. 2B). Following 6 h of treatment there was no change in Ki-67 expression (data not shown). However, by 24 h decreased Ki-67 expression is clearly evident, and by 48 h virtually all cells had decreased Ki-67 expression, with the majority of the cells expressing none. Thus, treatment with anti-CD20-mIFN β causes tumor cells to exit the cell cycle thereby contributing to inhibition of proliferation and the observed decrease in metabolic activity.

Treatment with 20 pM of anti-CD20-mIFN β causes maximal apoptosis and loss of metabolic activity

To determine if the observed inhibition of proliferation following treatment with anti-CD20-mIFN β is also associated with apoptosis and inhibition of metabolic activity, 38C13-huCD20 cells were treated with 20 pM of anti-CD20-mIFN β for 48 h and examined by flow cytometry (Fig. 3). Following staining with Annexin V-FITC and PI, over 50% were found to have undergone apoptosis as defined by being Annexin V-positive (Fig. 3A). Further increasing the treatment dose to 2500 pM did not increase the percentage of Annexin V-positive cells. However, decreasing the treatment dose below 20 pM did result in a decrease in the percentage of Annexin V-positive cells (data not shown). Non-targeted anti-DNS-mIFN β was much less effective in causing apoptosis, with a concentration of 2500 pM of anti-DNS-mIFN β failing to achieve the same degree of apoptosis as 20 pM of anti-CD20-mIFN β with only 41.9% of the cells Annexin V-positive.

To examine the relationship between alterations in membrane structure and metabolic activity, cells were treated with anti-CD20-mIFN β for 48 h and examined following staining with Annexin V-FITC and resazurin. Annexin V staining of exposed phosphatidylserine indicates change in membrane structure and C₁₂-resazurin is reduced to the red-fluorescent C₁₂-resorufin by metabolically active cells. Staining with Annexin V correlated with loss of metabolic activity as Annexin V-positive cells failed to reduce C₁₂-resazurin (Fig. 3B). However, a population of metabolically active cells remained even when the treatment dose was increased to 2500 pM of anti-CD20-mIFN β . Similar results were observed when cells were stained with SYTOX green, a nucleic acid stain that crosses compromised plasma membranes and C₁₂-resazurin, with cells with compromised membranes being metabolically inactive (data not shown). Thus, anti-CD20-mIFN β treatment causes alterations in the plasma membrane as evidenced by both Annexin V and SYTOX green staining and these alterations correlated with a reduction in metabolic activity, and maximum effects seen following treatment with only 20 pM of anti-CD20-mIFN β . Similar results were obtained

after treatment with anti-DNS-mIFN β except that much larger doses were required to achieve the maximum effect (data not shown).

Covalent linkage of anti-CD20 mAb with mIFN β is required for DNA fragmentation, alterations in cell cycle and apoptosis

To determine if mIFN β must be physically linked to anti-CD20 mAb for efficacy, 38C13-huCD20 cells were incubated for 48 h with 20 pM of rituximab, 40 pM mIFN β , 20 pM anti-DNS-mIFN β , 20 pM anti-CD20-mIFN β , or 20 pM rituximab + 40 pM mIFN β and then DNA content analyzed by flow cytometry (Fig. 4A). Alterations in cell cycle distribution were observed only following treatment with 20 pM anti-CD20-mIFN β and not following treatment with the same amount of mIFN β or mIFN β + rituximab (Fig. 4B). Anti-DNS-mIFN β also was ineffective at this dose, again demonstrating the importance of targeting mIFN β to the CD20 antigen expressed on the cell surface for anti-tumor efficacy.

To determine if mIFN β fused to anti-CD20 mAb was more effective in inducing apoptosis than mIFN β present at the same time as anti-CD20 mAb but not physically linked to it, 38C13-huCD20 cells were incubated with 20 pM of rituximab, 40 pM mIFN β , 20 pM anti-CD20-mIFN β , 20 pM anti-DNS-mIFN β , or 20 pM rituximab + 40 pM mIFN β for 48 h, and apoptosis measured by flow cytometry following staining with Annexin V-FITC and PI (Fig. 4C). Effective apoptosis of 38C13-huCD20 cells was only seen following treatment with anti-CD20-mIFN β . Approximately 34% of the cells were Annexin V⁺ following treatment with anti-CD20-mIFN β while only 10–13 % were Annexin V⁺ following all of the other treatments. Thus anti-CD20-mIFN β was effective in inducing apoptosis in 38C13-huCD20 with physical linkage of mIFN β to anti-CD20 mAb necessary for efficacy. Untargeted anti-DNS-mIFN β was not effective in causing apoptosis, demonstrating that it is cell surface targeting, not just linkage of mIFN β in the fusion protein which results in its efficacy.

Time course of commitment to undergo DNA fragmentation

An important question is how long must the fusion protein be bound to IFNAR for the anti-tumor effects to be irreversible. When 100 pM of anti-CD20-mIFN β was added to cells, excess non-bound fusion protein washed away, and cells examined for DNA content 48 h later, extensive DNA fragmentation was seen (data not shown). However, when bound protein was removed by treating the cells with trypsin, DNA fragmentation was observed if the fusion protein had been present for 24 h but not if it had been present for only 6 h (Fig. 5A). Having the fusion protein present for 48 h did not result in a further increase in the extent of DNA fragmentation observed. Therefore, sometime between 6 and 24 h the cells become committed to undergo DNA fragmentation and cell death.

Caspase activation and the anti-tumor activity of anti-CD20-mIFN β

To determine if caspase activation was associated with the observed DNA fragmentation, 38C13-huCD20 cells were incubated with 100 pM of anti-CD20-mIFN β , 100 pM anti-DNS-mIFN β , or 100 pM rituximab for varying lengths of times and caspase activation measured. Treatment with 10 μ M of staurosporine served as a positive control. Little activation of caspase 2, 8, or 9 was observed following treatment with anti-CD20-mIFN β (data not shown). Modest activation of the executioner caspases 3 and 7 was seen after 24 h of treatment but not after 6 h, consistent with the time course for the observed DNA fragmentation (Fig. 5B).

Caspase activation does not play a significant role in anti-CD20-mIFN β -induced DNA fragmentation or apoptosis

Although only modest caspase activation was observed, it was important to determine if this played a significant role in the activity of anti-CD20-mIFN β . However, inhibition of caspase activity using 50 μ M of the pan-caspase inhibitor Z-VAD(OMe)-FMK had little to no effect on the amount of DNA fragmentation and cell cycle alterations observed following treatment with 20 pM of anti-CD20-mIFN β for 24 (data not shown) or 48 h (Fig. 6A,B). Similar results were obtained when the efficacy of Z-VAD(OMe)-FMK on anti-CD20-mIFN β induced apoptosis was assessed by Annexin V-FITC and PI staining (Fig. 6C). Complete inhibition of caspase activity by Z-VAD(OMe)-FMK on staurosporine- and fusion protein-treated cells was confirmed using the luminescence caspase activity assays (data not shown). Thus, it appears that most of the cell cycle alterations and apoptosis observed following treatment with anti-CD20-mIFN β took place through caspase-independent pathways.

Effector functions of anti-CD20-mIFN β

In vivo efficacy of fusion protein therapy might reflect many different mechanisms including the immunologic effector functions of the Ab within the fusion protein. Therefore, the ability of the fusion protein to carry out ADCC and CDC was determined. Against 38C13-huCD20 cells, anti-CD20-mIFN β was even more effective than rituximab in carrying out ADCC (Fig. 7A) ($p = 0.01$ at all concentrations). This improved efficacy may reflect the fact that anti-CD20-mIFN β appears to bind more strongly to 38C13-huCD20 than does rituximab (see Fig. 1B). In contrast to what was observed with ADCC, anti-CD20-mIFN β was not able to mediate CDC, a property of the unmodified mAb (rituximab) (Fig. 7B).

Anti-CD20-mIFN β has more potent *in vivo* efficacy than anti-CD20-mIFN α against huCD20-expressing lymphomas, including those with decreased IFNAR expression

We chose to determine the *in vivo* efficacy of anti-CD20-mIFN β using 38C13-huCD20, which was previously used to evaluate the efficacy of anti-CD20-mIFN α (21). 38C13-huCD20 expresses physiologic levels of human CD20 and is a model of rituximab-insensitive lymphoma when injected s.c. into syngeneic C3H mice (22). Free recombinant IFN β has a very short *in vivo* half-life of 3–5 h (28). However, similar to what had been observed with mIFN α fusion proteins (29), following i.v. injection and distribution from the circulation into the tissues, anti-CD20-mIFN β cleared from the serum with a half-life of approximately 8 h (data not shown). To rule out any adverse effects of fusion protein on normal host leukocyte populations, mice were treated daily for 3 days with PBS or 100 μ g of anti-CD20-mIFN β , and spleens and lymph nodes harvested 24 hours later for quantitation of B cells, T cells, and myeloid cells. No toxic effects against these leukocyte populations were observed (data not shown).

To compare the *in vivo* anti-tumor efficacy of anti-CD20-mIFN β with rituximab and to assess the importance of fusion protein targeting, mice injected s.c. with 5×10^3 38C13-huCD20 tumor cells were treated i.v. on days 5, 6, 7 and 14 post tumor challenge with 100 μ g of rituximab, anti-DNS-mIFN β , or anti-CD20-mIFN β fusion proteins, followed for tumor growth, and sacrificed when tumors reached 1.4 cm in diameter. In this stringent setting, rituximab increased median survival by only 2 days with no animals cured ($p = 0.0035$ vs. PBS). Similarly, the untargeted fusion protein anti-DNS-mIFN β prolonged median survival by only 4 days but did not result in tumor eradication ($p = 0.0003$ vs. PBS) (Fig. 8A). Only anti-CD20-mIFN β achieved tumor eradication with 83% of the treated animals cured ($p = 0.0003$ vs. rituximab and anti-DNS-mIFN β).

To directly compare the relative *in vivo* efficacy of anti-CD20-mIFN α and anti-CD20-mIFN β fusion proteins, mice injected s.c. with 5×10^3 tumor cells were treated with 100 μ g

of anti-CD20-mIFN α or anti-CD20-mIFN β fusion protein i.v. on days 5, 6, and 7 post tumor challenge, followed for tumor growth and sacrificed when tumors reached 1.4 cm in diameter. All mice treated with PBS succumbed to tumor by day 14 (Fig. 8B). As observed earlier, anti-CD20-mIFN α was partially effective against the tumor with the death of all of the mice delayed compared to the PBS control and 25% of the mice surviving tumor-free ($p < 0.0001$). However, anti-CD20-mIFN β was even more effective, achieving 75% tumor eradication ($p = 0.023$ compared to anti-CD20-mIFN α) (Fig. 8B). Importantly, using these doses, animals did not display any signs of acute IFN-mediated systemic toxicity such as lethargy, ruffled fur, or weight loss.

In previous studies anti-CD20-mIFN α failed to provide protection against the growth of a tumor (38C13-huCD20 IFNAR^{lo}) with decreased cell surface expression of the IFNAR resulting from the use of shRNA to target IFNAR1 (21). 38C13-huCD20 IFNAR^{lo} remains a very aggressive tumor with 100% of PBS treated mice sacrificed by 15 days (Fig. 8C). While treatment with 100 μ g of anti-CD20-mIFN α on days 5, 6, and 7 was able to delay tumor growth by one week ($p < 0.0001$ vs. PBS), all animals succumbed to tumor by day 22. However, anti-CD20-mIFN β was significantly more effective against this resistant tumor, with the survival of all mice prolonged compared to mice treated with anti-CD20-mIFN α , and 25% of the mice remaining tumor-free ($p = 0.0003$ compared to anti-CD20-mIFN α). Thus, fusion proteins targeting IFN β to tumor cells may be efficacious therapeutics for the treatment of tumors that are not effectively treated with IFN α .

DISCUSSION

Anti-proliferative effects of IFN have been associated with enhanced stability of the IFN/IFNAR complex. mIFN β has higher affinity for IFNAR than does mIFN α 1. Therefore one goal of the current study was to determine if antibody-targeted mIFN β would be a more effective anti-cancer therapeutic than antibody-targeted mIFN α 1. Indeed we found that fusions with mIFN β had very potent IFN bioactivity. In contrast to what we had observed with mIFN α 1 (21), fusion of mIFN β to the carboxy-terminus of the heavy chain does not impede its anti-proliferative activity (Fig. 1). In fact, when its ability to inhibit the proliferation of 38C13 cells was analyzed, anti-DNS-mIFN β and anti-CD20-mIFN β were both more effective than recombinant mIFN β . Although it is generally accepted that the type I IFNs function as monomers, early studies had indicated that the functional unit for IFN β is a dimer (30). In the fusion protein two mIFN β molecules are present at the end of C_H3 and this close proximity may enhance their function. The structure of mIFN β may also influence its improved activity in the fusion proteins. mIFN β contains three predicted N-glycosylation sites, all of which are used (31); mIFN α 1 contains only one. mIFN β also has two deletions in the AB loop proposed to interact with the IFNAR2 subunit of the receptor and lacks the disulfide bridge between the AB loop and helix E which is conserved among all other type I IFNs (32). Thus, it is likely that the interaction of mIFN β with the IFNAR differs locally from that of mouse and human IFN α and human IFN β .

Different type I IFNs react differently with the two receptor subunits resulting in variable downstream effects (reviewed in (33)). IFNAR1 is associated with TYK2 and IFNAR2 with JAK1. Interestingly, mIFN β was found to stimulate apoptosis in pro-B cells from STAT1 deficient mice, but not in pro-B cells from TYK2 deficient mice that had normal activation of STAT1 and STAT2 but were deficient in STAT3 phosphorylation. Inhibition of STAT3 activation in wild-type pro-B cells also blocked apoptosis. mIFN β but not mIFN α induced programmed cell death in primary murine IL-7-dependent bone marrow derived pro-B cells with TYK2-mediated tyrosine phosphorylation of STAT3 required for mIFN β to stimulate apoptosis (34).

We found that antibody-mIFN β fusion protein treatment causes caspase-independent cell death, as evidenced by DNA fragmentation, exit from the cell cycle, and loss of metabolic activity correlating with compromised membrane integrity. DNA fragmentation is seen in 38C13-huCD20 cells undergoing apoptosis with a sub-G1 apoptotic peak characteristic of DNA fragmentation observed (See Figs. 5 and 6). DNA fragmentation during apoptosis is carried out by CAD, a caspase-activated DNase. CAD cannot be synthesized by itself but its inhibitor, ICAD, must be present in the cytoplasm to assist in the correct and productive folding of the CAD polypeptides. When cells are induced to undergo apoptosis, caspase 3 cleaves ICAD to dissociate the CAD:ICAD complex allowing CAD to cleave chromosomal DNA (35). However, cells can be killed by apoptotic stimuli without DNA fragmentation, as MCF-7 breast carcinoma cells that lack active caspase 3 remain sensitive to TNF- or staurosporine-induced apoptosis, although no DNA fragmentation was observed (36). In our studies active caspase 3 is observed at 24 h, a time-point at which DNA fragmentation is observed (Fig. 5). However, treatment with the pan-caspase inhibitor Z-VAD(OMe)-FMK had little to no effect on apoptosis or DNA fragmentation following treatment with anti-CD20-mIFN β , suggesting that their occurrence is caspase-independent (Fig. 6).

The observed efficacy of antibody-targeted IFN may also relate to the requirement for extended exposure to anti-CD20-mIFN β for cells to commit to apoptosis. Extended signaling through mIFN β bound to the receptor is necessary for the cells to commit to DNA fragmentation and apoptosis, as removal of IFN bound for up to 6 h resulted in no DNA fragmentation (Fig. 5A). IFN β -induced apoptosis in melanoma cells was also found to be delayed and became apparent by Annexin V staining only after ~40 h of treatment (37). IFN α -induced apoptosis of Daudi cells was also seen only 48 h after stimulation with a further increase in proportion up to 72 h (38). This contrasts with what is seen with other apoptotic agents such as camptothecin, staurosporine or Fas Ab that induce apoptosis within a few hours of treatment. There was also a delay in cells exiting the cell cycle following treatment with anti-CD20-mIFN β as evidenced by a decrease in expression of Ki-67 (Fig. 2B).

It should be appreciated that the anti-CD20-mIFN β fusion protein is more than just the sum of its parts. Anti-CD20-mIFN β is more effective than anti-CD20 mAb plus mIFN β in causing both DNA fragmentation and apoptosis in treated cells (Fig. 4). It is not just the fusion per se that is responsible for the enhanced activity since anti-DNS-mIFN β is less active than anti-CD20-mIFN β in both the *in vitro* assays and in treating tumors *in vivo*. Targeting of IFN by binding to CD20 on the cell surface plays a critical role in the activity and efficacy of anti-CD20-mIFN β . Targeted mIFN β is not identical to recombinant mIFN β in its activity and efficacy.

In addition to directly causing tumor cell death, antibody-IFN fusion proteins may also interact with the host immune effector system. Interestingly, anti-CD20-mIFN β has unimpaired and possibly enhanced ability to carry out ADCC (Fig. 7A). The binding sites for the Fc γ Rs used for ADCC are in the hinge and the hinge proximal region of C_H2 (39, 40). One Fc γ R binds to one Fc interacting with the two C_H2 domains in an asymmetric manner. When IgG is bound to a Fc γ R the two Fab arms are modeled to be free to interact with bulky antigens. Fusion with mIFN β does not appear to affect this binding site.

Unexpectedly, we found that anti-CD20-mIFN β was unable to carry out CDC (Fig. 7B). This contrasts with what we have observed with anti-CD20-human IFN α , which we have found to have enhanced ability to carry out CDC against human lymphoma cells (41). Although a crystal structure of C1q, the first component of the classical complement cascade, bound to Fc has not been published, site directed mutagenesis has implicated amino acids at positions 234, 235, 270, 322, 326, 329, 331, and 333 on an exposed face within C_H2

as playing an important role in complement activation (42–48). The presence of mIFN β in the fusion protein may either alter the conformation of the binding site or impede access to the site, thus limiting CDC. Although the anti-CD20-mIFN β fusion protein has a half-life of approximately 8 h, that is still far shorter than the extended half-life in mice of approximately 200 h reported for human IgG1 (49). One important determinant of the half-life of IgG is its binding to FcRn at a site located at the C_H2/C_H3 interface in proximity to the C1q binding site and the ability of the fusion protein to bind FcRn may also be impaired. An additional possibility is that the attached mIFN β binds to IFN receptors and that this binding results in the rapid clearance of the fusion protein.

Antibody-mIFN β fusion proteins are more effective than antibody-mIFN α fusion proteins in treating tumors in a syngeneic mouse model of B cell lymphoma. Comparison of the *in vivo* efficacy of anti-CD20-mIFN β to identical doses of anti-CD20-mIFN α using the 38C13-huCD20 tumor model showed that the mIFN β fusion protein was more effective, with 75% of the mice remaining tumor-free compared to only 25% of the mice treated with anti-CD20-mIFN α (Fig. 8B). Especially important is that anti-CD20-mIFN β was also effective in preventing the growth of 38C13-huCD20 IFNAR^{lo} tumors with 25% of the mice remaining tumor-free while none of the mice treated with anti-CD20-mIFN α survived (Fig. 8C). As discussed above, IFN β has a higher affinity than IFN α for the IFNAR and its greater *in vivo* efficacy likely reflects this improved ability to activate the IFN signaling pathway especially in cells with decreased expression of IFNAR. Thus, fusion proteins targeting IFN β to tumor cells may provide effective therapeutics for the treatment of cancers that are not effectively treated with IFN α .

Numerous studies have documented the potent biologic activities of type I IFNs against B cell malignancies. However, to date their utility has been restricted by dose-limiting toxicities as more effective treatment was found at higher IFN doses (50). Targeting IFN directly to tumors by fusing it to an Ab recognizing a tumor associated antigen appears to be an effective way to overcome these limitations. Our earlier studies have shown that anti-CD20-IFN α fusion proteins possess a markedly improved therapeutic index compared to recombinant IFN α and can eradicate both mouse and human lymphomas *in vivo* with a small fraction of the IFN α required for an effective systemic dose (21). An alternative approach of targeting IFN α to CD20 expressing cells using a hexavalent anti-CD20 antibody also found strong anti-lymphoma effects in pre-clinical mouse models (51). Our current studies suggest that targeting of a more potent type I IFN such as IFN β using an Ab recognizing a tumor associated antigen may produce an even more effective cancer therapeutic that is active against tumors resistant to the effects of IFN α . Therefore, we propose that IFN β fusion proteins warrant further evaluation as cancer therapeutics.

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Figure 1. Construction and characterization of Ab fusion proteins containing mouse IFN β

A. Diagram of the fusion proteins in which mIFN β was joined to the C-terminus of human γ 1 heavy chains specific for either CD20 or dansyl (DNS). The heavy chains were expressed with the appropriate light chain to generate the antigen-specific fusion proteins. B. The anti-CD20-mIFN β fusion protein binds to CD20-expressing 38C13-huCD20 cells with affinity comparable to rituximab. Cells were incubated on ice with varying equimolar concentrations of rituximab or anti-CD20-mIFN β , bound Ab detected with anti-human kappa-PE, and analyzed by flow cytometry. Data are expressed as mean fluorescence intensity (MFI) vs. protein concentration. C. The fusion proteins are more effective than mIFN β in inhibiting the growth of 38C13 lymphoma cells. 38C13 cells were incubated with differing concentrations of mIFN β , anti-CD20-mIFN β , or anti-DNS-mIFN β for 48 h. The metabolic activity of the remaining cells was then measured using the MTS assay. Data are expressed as the percent metabolic activity in the treated cells compared with untreated cells. Data shown are the mean of quadruplicate samples $\pm$ SD. The IFN concentration assumes 2 moles of IFN for each mole of fusion protein. D. IFN β fusion proteins are more effective than IFN α fusion proteins in inhibiting the growth of 38C13-huCD20 (left panel) and 38C13-huCD20 IFNAR^{lo} (right panel) with CD20-targeting making them even more effective. 38C13-huCD20 and 38C13-huCD20 IFNAR^{lo} cells were incubated with differing concentrations of the indicated proteins for 72 h and cellular metabolic activity measured and displayed as described above.

Figure 2. Treatment of 38C13-huCD20 with anti-CD20-mIFN β or anti-DNS-mIFN β causes DNA fragmentation and alterations in cell cycle

A. 38C13-huCD20 cells treated with varying concentrations of anti-CD20-mIFN β or anti-DNS-mIFN β for 72 h were stained with PI and analyzed by flow cytometry to determine the proportion in G0/1, S, or G2 phases of the cell cycle. B. 38C13-huCD20 cells treated with 20 pM anti-CD20-mIFN β for varying lengths of time were stained with rabbit anti-Ki-67 followed by mouse anti-rabbit IgG-FITC and analyzed by flow cytometry (solid line). Controls include unstained cells (faint dotted line), untreated cells stained only with mouse anti-rabbit IgG-FITC (shaded peak), and untreated cells stained with rabbit anti-Ki-67 and mouse anti-rabbit IgG-FITC (dashed line).

Figure 3. Anti-CD20-mIFN β is effective in causing apoptosis and alterations in metabolic activity in 38C13-huCD20 cells

A. 38C13-huCD20 cells were incubated with 20 or 2500 pM anti-CD20-mIFN β or anti-DNS-mIFN β or left untreated for 48 h and then examined by flow cytometry following staining with Annexin V-FITC and PI. B. Cells treated as indicated for 48 h were analyzed by flow cytometry for membrane alterations and metabolic activity following staining with Annexin V-FITC and resazurin. The percentage of cells in each quadrant is indicated.

Figure 4. Physical linkage of mIFN β with anti-CD20 is required for effective DNA fragmentation, alterations in cell cycle and apoptosis

A. 38C13-huCD20 cells were incubated with medium alone, 20 pM rituximab, 20 pM anti-DNS-mIFN β , 20 pM anti-CD20-mIFN β , 40 pM mIFN β , or 40 pM mIFN β + 20 pM rituximab for 48 h, stained with PI and analyzed by flow cytometry. B. Cell cycle distribution of treated cells from A. C. 38C13-huCD20 cells were incubated as above for 48 h, stained with Annexin V-FITC and PI and analyzed by flow cytometry. The percentage of cells in each quadrant is indicated.

Figure 5. Time course of commitment to DNA fragmentation and caspase activation following treatment with anti-CD20-mIFN β

A. 38C13-huCD20 cells were treated with 100 pM of anti-CD20-mIFN β for the indicated time and then bound fusion protein removed by trypsin treatment. DNA content was analyzed by flow cytometry at 48 h following cell permeabilization and staining with PI. Untreated cells are indicated by the filled peak and treated cells are indicated by the solid line. B. Caspase activity was analyzed at the indicated times following treatment with 100 pM of anti-CD20-mIFN β , anti-DNS-mIFN β , or rituximab. Treatment with 10 μ M of staurosporine served as a positive control. Data are shown as the mean + SD of the relative caspase activity (RLU) of triplicate samples.

Figure 6. Caspase inhibition does not influence anti-CD20-mIFN β induced changes in cell cycle or apoptosis

A. 38C13-huCD20 cells were treated with medium alone, 20 pM of anti-CD20-mIFN β , 50 μ M of the pan-caspase inhibitor Z-VAD(OMe)-FMK, or 50 μ M of Z-VAD(OMe)-FMK + 20 pM of anti-CD20-mIFN β for 48 h, permeabilized, stained with PI and analyzed by flow cytometry. B. Cell cycle distribution of treated cells from A. C. 38C13-huCD20 cells were treated with medium alone, 20 μ M of Z-VAD(OMe)-FMK, 20 pM anti-CD20-mIFN β , or 20 μ M of Z-VAD(OMe)-FMK + 20 pM anti-CD20-mIFN β for 48 h and analyzed by flow cytometry following staining with Annexin V-FITC and PI. The percentage of cells in each quadrant is indicated.

Figure 7. Anti-CD20-mIFN β is effective in carrying out ADCC but not CDC

A. ADCC was assessed by LDH release following incubation of 38C13-huCD20 target cells for 5 h at 37°C with 53 nM of anti-CD20-mIFN β or rituximab and IL-2-stimulated splenocytes from C3H mice at different effector:target ratios. Percentage cytotoxicity was calculated as $100 \times [(\text{effector:target cell release} - \text{effector cell control} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})]$. Data are expressed as mean $\pm$ SD for triplicate samples. B. To determine CDC, 38C13-huCD20 cells were incubated in triplicate with rituximab or anti-CD20-mIFN β at concentrations ranging from 0–53 nM for 15 minutes on ice. Rabbit serum, as a source of complement, was added to a final concentration of 10% and samples incubated at 37°C for 3 h. Following washing, cells were stained with PI and analyzed by flow cytometry to assess viability. Data are expressed as the mean $\pm$ SD of the percentage of PI-positive events.

Figure 8. Targeted mIFN β is effective against syngeneic huCD20-expressing lymphomas *in vivo*

A. Anti-CD20-mIFN β is more effective than anti-DNS-mIFN β and rituximab in treating established 38C13-huCD20 tumors. Survival and tumor growth curves for mice injected s.c. with 5×10^3 tumor cells on day 0 and treated i.v. with 100 μ g of anti-CD20-mIFN β , anti-DNS-mIFN β , or rituximab on days 5, 6, 7 and 14 post tumor challenge. Groups included 8 mice except for anti-CD20-mIFN β , where $n=6$. B. Anti-CD20-mIFN β is more effective than anti-CD20-mIFN α in treating mice bearing 38C13-huCD20 tumors. Survival and tumor growth curves for mice injected s.c. with 5×10^3 tumor cells on day 0 and treated i.v. with 100 μ g of anti-CD20-mIFN α or anti-CD20-mIFN β on days 5, 6, and 7 post tumor challenge. C. Anti-CD20-mIFN β is more effective than anti-CD20-mIFN α in treating mice bearing 38C13-huCD20 IFNAR 10 tumors. Survival and tumor growth curves for mice injected s.c. with 5×10^3 tumor cells on day 0 and treated i.v. with 100 μ g of anti-CD20-mIFN α or anti-CD20-mIFN β on days 5, 6, and 7 post tumor challenge. Arrows indicate days of treatment. Tumor growth curves for the individual mice in each group are included to the right of each survival curve. The numbers at the bottom of the tumor growth curves indicate the number of mice without tumor at day 60.