Production and purification of DNL modules comprising C2-2b-2b

The construction of the mammalian expression vector as well as the subsequent generation of the production clones and the purification of C_H3-AD2-IgG modules have been reported previously.[1-3] The C_H3-AD2-IgG-hL243 expression vector was generated by replacement of a Sac II/Eag I restriction fragment from the hL243 IgG_1-pdHL2 vector with a Sac II/Eag I fragment that was excised form C_H3-AD2-IgG-v-mab-pdHL2.[1] The expressed recombinant fusion protein has the AD2 peptide linked to the carboxyl terminus of the C_H3 domain of hL243 IgG_1 via a 15 amino acid long flexible linker peptide. Co-expression of the heavy chain-AD2 and light chain polypeptides results in the formation of an IgG structure equipped with two AD2 peptides. The expression vector was transfected into Sp/ESF cells (an engineered cell line of Sp2/0) by electroporation. The pdHL2 vector contains the gene for dihydrofolate reductase, thus allowing clonal selection, as well as gene amplification with methotrexate (MTX). Stable clones were isolated from 96-well plates selected with media containing 0.2 μM MTX. Clones were screened for C_H3-AD2-IgG-hL243 productivity via a sandwich ELISA. The module was produced in roller bottle culture with serum-free media.

The DDD-module, IFNα2b-DDD2, was generated by recombinant fusion of the DDD2 peptide to the carboxyl terminus of human IFNα2b via an 18 amino acid long flexible linker peptide.[3] As is the case for all DDD-modules, the expressed fusion protein spontaneously forms a stable homodimer. Using a full-length human IFNα2b cDNA clone as a template, cDNA was amplified by PCR resulting in a sequence comprising

XbaI---Signal peptide---IFNα2b---6 His---BamHI, in which XbaI and BamHI are restriction sites, the signal peptide is native to IFNα2b, and 6 His is a hexahistidine tag. The PCR amplimer was cloned into a DDD2-pdHL2 mammalian expression vector via Xba I and Bam HI restriction sites to generate the expression vector IFNα2b-DDD2-pdHL2. The resulting secreted protein consists of IFNα2b fused at its carboxyl terminus to a polypeptide with a 63-amino-acid sequence. The IFNα2b-DDD2-pdHL2 vector was stably transfected into Sp/ESF myeloma cells by electroporation. Selection and production media was supplemented with 0.2 μM MTX. Transfectant clones were selected in 96-well tissue culture plates and screened for IFNα2b expression with a human interferon alpha ELISA kit (PBL Interferon Source, Piscataway, NJ). The IFNα2b-DDD2 module was produced in fed-batch bioreactor culture.

C_H3-AD2-IgG-hL243 and IFNα2b-DDD2 were purified from the culture broths by affinity chromatography using MAbSelect (GE Healthcare) and His-Select HF Nickel Affinity Gel (Sigma), respectively, as described previously.[3]

The purity of the DNL modules was assessed by SDS-PAGE (Figure S1). Analysis under non-reducing conditions shows that, prior to the DNL reaction, IFNα2b-DDD2 (lane 5) exists as disulfide-linked dimer. This phenomenon, which is always seen with DDD-modules, is beneficial, as it protects the reactive sulfhydryl groups from irreversible oxidation. In comparison, C_H3-AD2-IgG-hL243 (lane 6) exists as both a monomer and a disulfide-linked dimer, and is reduced to monomer during the DNL reaction. SE-HPLC analyses agreed with the non-reducing SDS-PAGE results, indicating monomeric species as well as dimeric modules that were converted to monomeric forms upon reduction. The sulfhydryl groups are protected in both forms by participation in

disulfide bonds between AD2 cysteine residues. Reducing SDS-PAGE demonstrated that each module was purified to near homogeneity and identified the component polypeptides comprising each module. For C_H3-AD2-IgG-hL243, heavy chain-AD2 and kappa light chains were identified. One major and one minor band (slightly higher mobility) were resolved for IFNα2b-DDD2, which were determined to be non-glycosylated and O-glycosylated species, respectively.

Generation of C2-2b-2b by DNL

Two molar equivalents of IFN-α2b-DDD2 were combined with C_H3-AD2-IgG-hL243 to generate the C2-2b-2b. Following an overnight docking step under mild reducing conditions (1 mM reduced glutathione) at room temperature, oxidized glutathione was added (2 mM) to facilitate disulfide bond formation (locking). C2-2b-2b was purified to near homogeneity using Protein A affinity chromatography.

Results

Non-reducing SDS-PAGE resolved C2-2b-2b as a cluster of high relative mobility (M_r) bands, similar to 20-2b-2b (Figure S1A). Purity was demonstrated by reducing SDS-PAGE, which resolved the three polypeptides (hL243 HC-AD2, hL243 kappa light chain & IFNα2b-DDD2) comprising C2-2b-2b (Figure S1B).

SE-HPLC analysis of C2-2b-2b resolved a predominant protein peak with a retention time (7.89 min) consistent with its calculated mass, as well as a higher molecular weight peak of C2-2b-2b dimer formed via self-association of IFNα2b (Figure S2A). C2-2b-2b bound quantitatively with anti-IFNα and hL243 anti-idiotype (WT)

mAbs, resulting in the formation of high molecular weight immune complexes and the disappearance of the C2-2b-2b peak (Figure S2B). The size of the immune complex comprising C2-2b-2b/anti-IFNα (5.52 min) is larger than that of C2-2b-2b/WT (6.22 min), which is consistent with C2-2b-2b binding two molecules of anti-IFNα (to the four IFNα2b) versus one molecule of WT (to hL243 IgG). The C_H3-AD2-IgG-hL243 module and 20-2b-2b and did not react with anti-IFNα and WT, respectively.

LC/MS analysis

Following reduction of C2-2b-2b, its five component polypeptides were resolved by RP-HPLC and individual ESI-TOF deconvoluted mass spectra were generated for each peak (Figure S2 and Table S1). Native, but not bacterially-expressed recombinant IFNα2, is O-glycosylated at Thr-106.[4] We previously determined that ~15% of the polypeptides comprising the IFNα2b-DDD2 module are O-glycosylated and can be resolved from the non-glycosylated polypeptides by RP-HPLC and SDS-PAGE, and LC/MS analysis indicated an O-linked glycan having the structure NeuGc-NeuGc-Gal-GalNAc.[5] LC/MS analysis of C2-2b-2b identified both the O-glycosylated and non-glycosylated species of IFNα2b-DDD2 with mass accuracies of <12 ppm (Figure S2A). LC/MS identified the hL243 kappa polypeptide (Figure S2B) as a single, unmodified species, with an observed mass matching the calculated mass (9 ppm). Two major glycoforms of hL243 HC-AD2 were identified as having masses of 53,801.42 (77%) and 53,965.24 (23%), indicating G0F and G1F N-glycans, respectively, which are typically associated with IgG (Figure S2C). The analysis also confirmed that the amino terminus of

the HC-AD2 is modified to pyroglutamate, as predicted for polypeptides having an amino terminal glutamine.

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Table S1. liquid chromatography/mass spectrometry analysis of reduced C2-2b-2b

Polypeptide	M_{obs}	M_{calc}	Δ(ppm)	Δ(mDa)	Modifications
IFNα2b-DDD2	26075.14	26074.94	7.7	200	None
	27055.25	27055.56	11.5	310	NeuGc-NeuGc-Gal-GalNAc- (+979.852)
hL243 Kappa	23376.02	23376.23	-9.0	-210	None
hL243-HC-AD2	53801.42	53802.92	-28.0	-1500	pQ (-18); G0F (+1445.36)
	53965.24	53966.07	-55.4	-830	pQ (-18); G1F (+1607.50)

M_{obs} indicates observed LC/MS mass; $M_{calc,}$ calculated mass including indicated modifications; Δ(ppm), difference between M_{calc} and M_{obs} in parts per million; Δ(mDa)**,** difference between M_{calc} and M_{obs} in millidaltons; pQ, pyroglutamate from amino terminal glutamine; G0F and G1F, N-linked glycans.

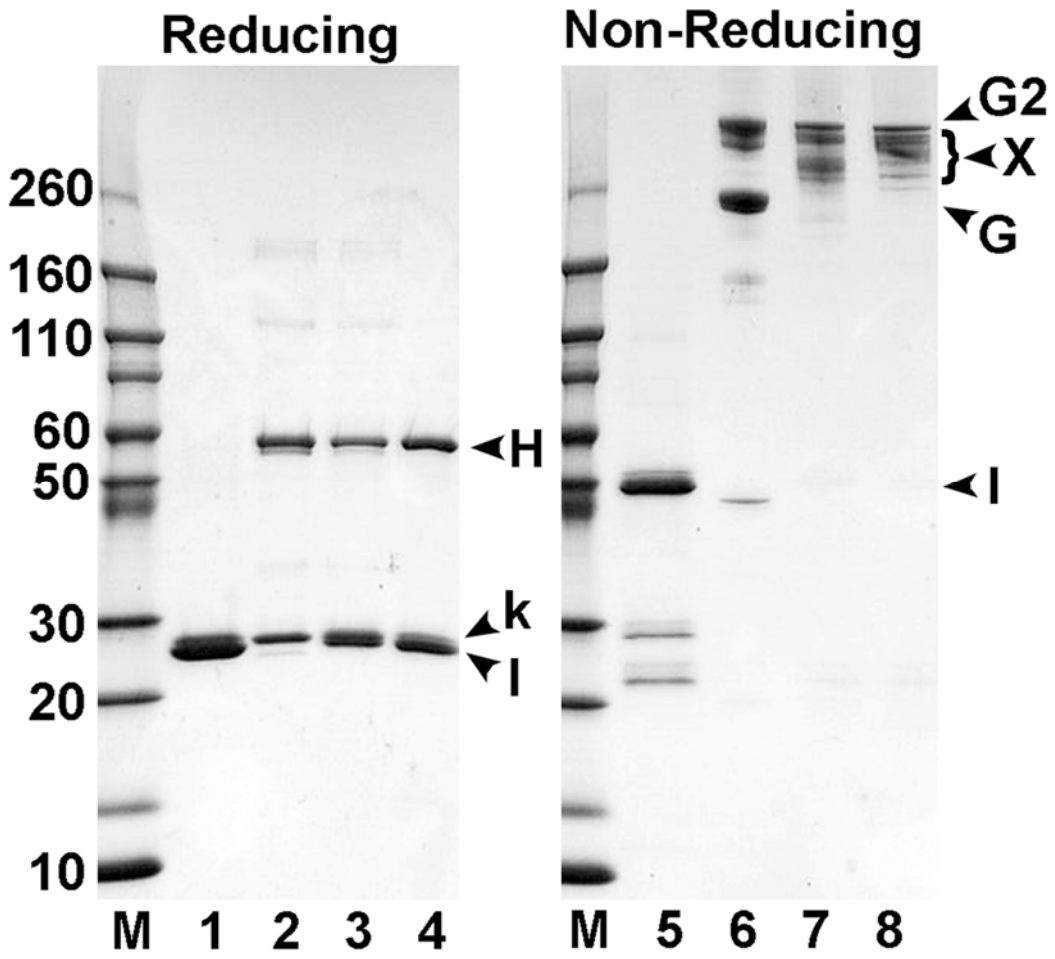

Figure S1. SDS-PAGE analysis of C2-2b-2b. Coomassie blue-stained SDS-PAGE of IFNα2b-DDD2 (lane 1), C_H3-AD2-IgG-hL243 (lane 2), C2-2b-2b (lane 3) and 20-2b-2b (lane 4) under reducing (left) and non-reducing (right) conditions. The positions of M_r standards (lane M) are indicated on the left. The positions of IFNα2b-DDD2 (I), heavy chain-AD2 (H) kappa light chain (k), C_H3-AD2-IgG monomers (G), and dimers (G2) and mAb-IFNα (X) are indicated with arrow heads.

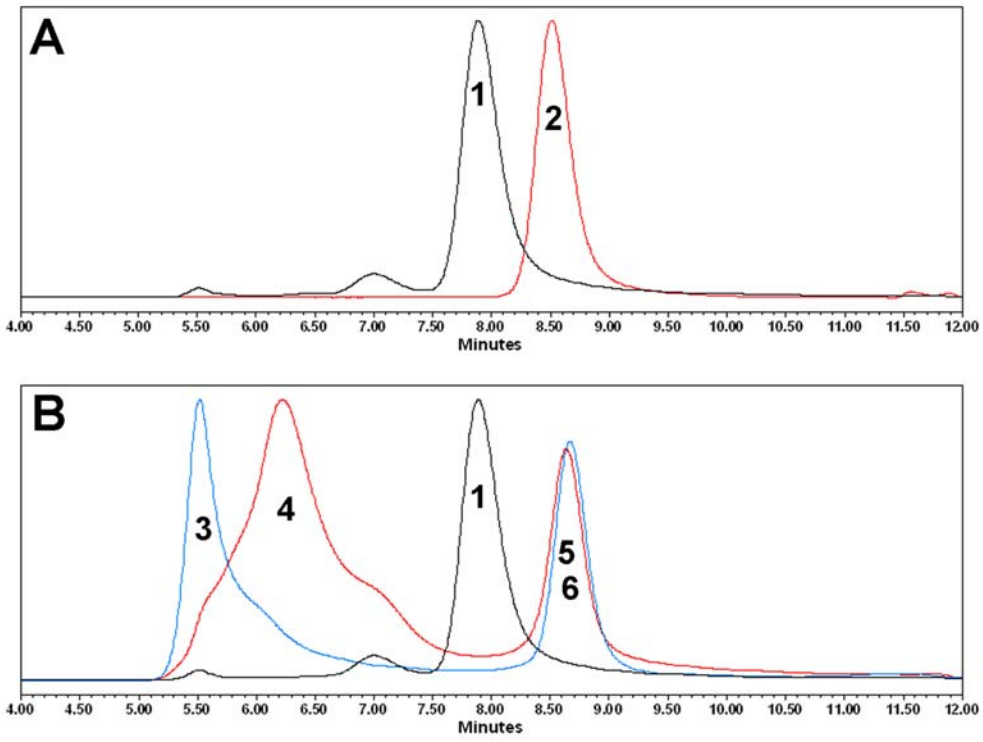

Figure S2. Size exclusion HPLC and immunoreactivity. (A) SE-HPLC analysis of C2-2b-2b (black trace) and C_H3-AD2-IgG-hL243 (red trace). (B) SE-HPLC showing C2-2b-2b (black trace) and its immunoreactivity with WT (red trace) and anti-IFNα2 (blue trace). Peaks representing C2-2b-2b (1), C_H3-AD2-IgG-hL243 (2), C2-2b-2b/anti-IFNα immune complex (3), C2-2b-2b/WT immune complex (4), excess WT (5), and excess anti-IFNα (6) are indicated.

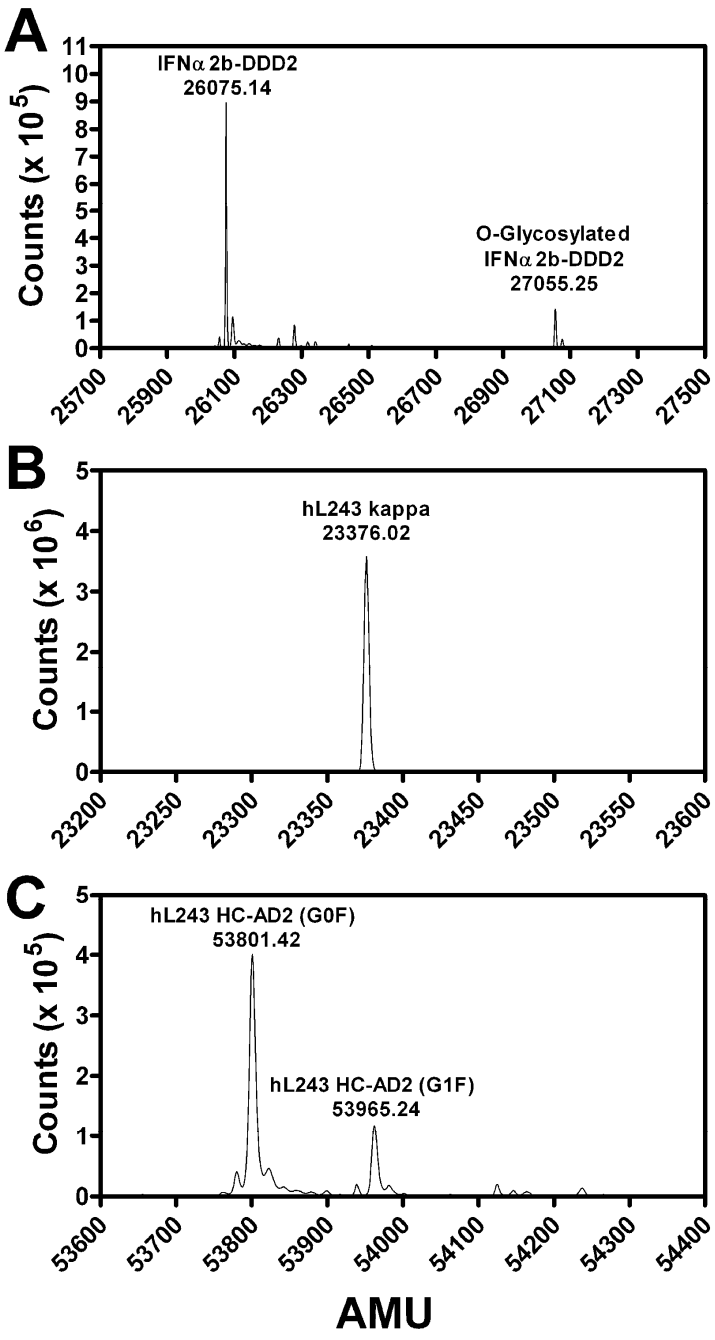

Figure S3. LC/MS analysis of C2-2b-2b. Deconvoluted masses shown graphically as atomic mass units (AMU) *vs*. abundance. Following reduction of C2-2b-2b with 50 mM TCEP, the IFNα2b-DDD2 (A), hL243 kappa chain (B) and hL243 heavy chain-AD2 (C) polypeptides were resolved by RP-HPLC and the individual peaks were analyzed by ESI-TOF MS. The identified polypeptide species and observed mass are indicated above each peak.

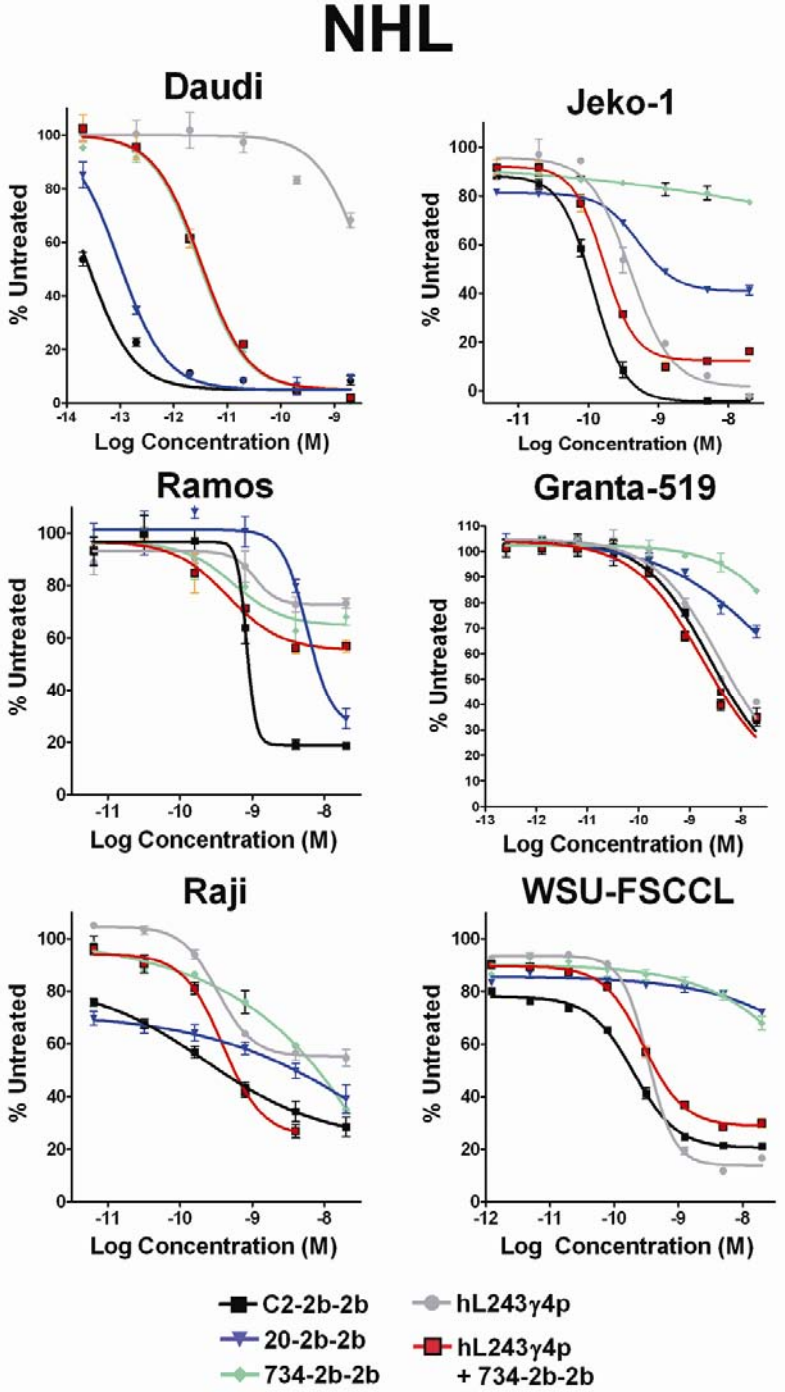

Figure S4. ***In-vitro*** **cytotoxicity of non-Hodgin lymphoma cell lines**. Cells were cultured in 48-well plates in the presence of increasing concentrations of the indicated constructs or combination and the relative viable cell densities were measured with MTS after the density of untreated cells increased by a minimum of 10-fold (3-7 days). The percent of the signal obtained from untreated cells was plotted *vs*. the log of the molar concentration. Dose-response curves and IC_{50} values were generated using Prism software. Error bars, SD.

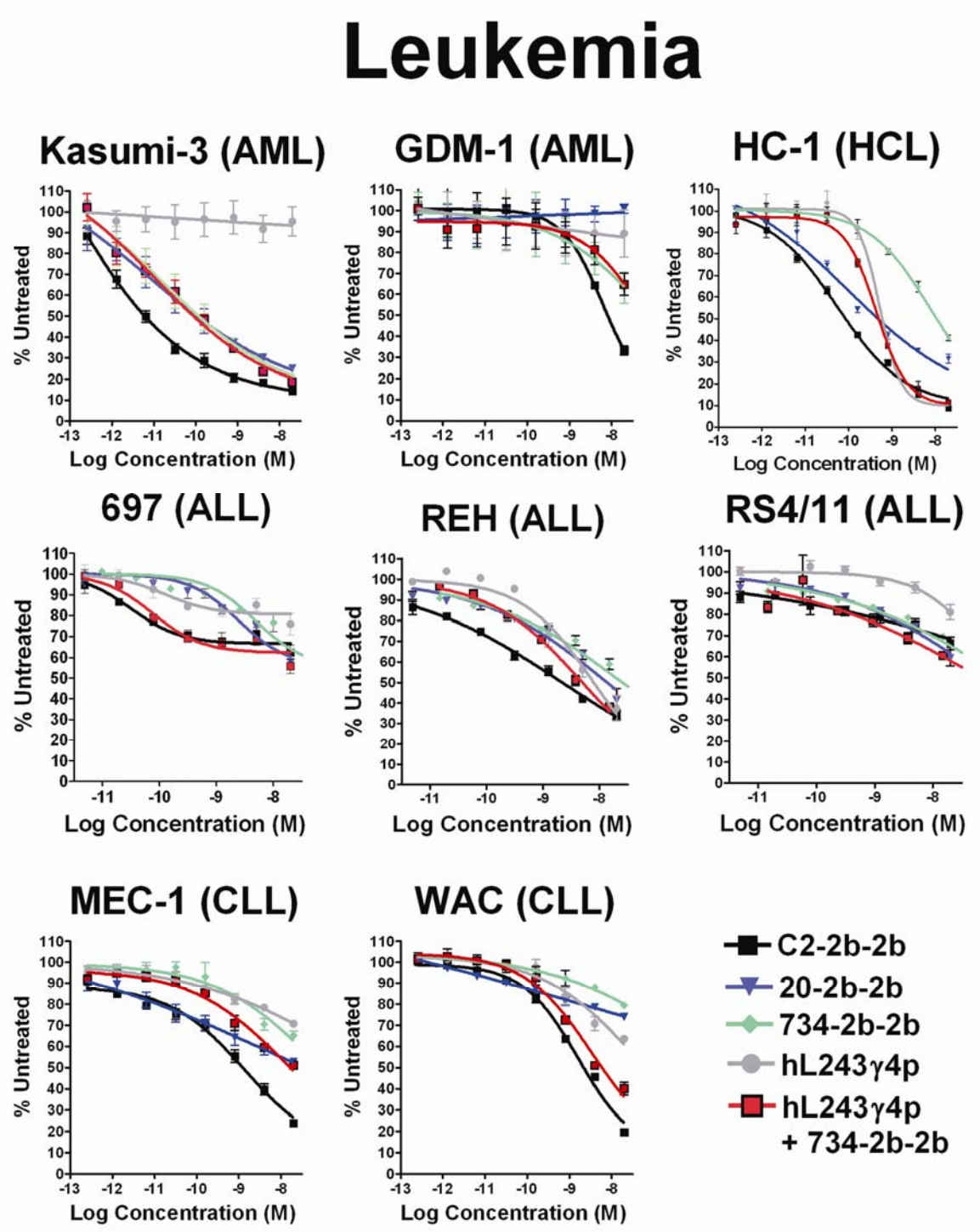

Figure S5. *In-vitro* cytotoxicity of leukemia cell lines. Cells were cultured in the presence of increasing concentrations of the indicated constructs or combination and the relative viable cell densities were measured with MTS after the density of untreated cells increased by a minimum of 10-fold (3-7 days). The percent of the signal obtained from untreated cells was plotted *vs*. the log of the molar concentration. Dose-response curves and IC_{50} values were generated using Prism software. Error bars, SD.

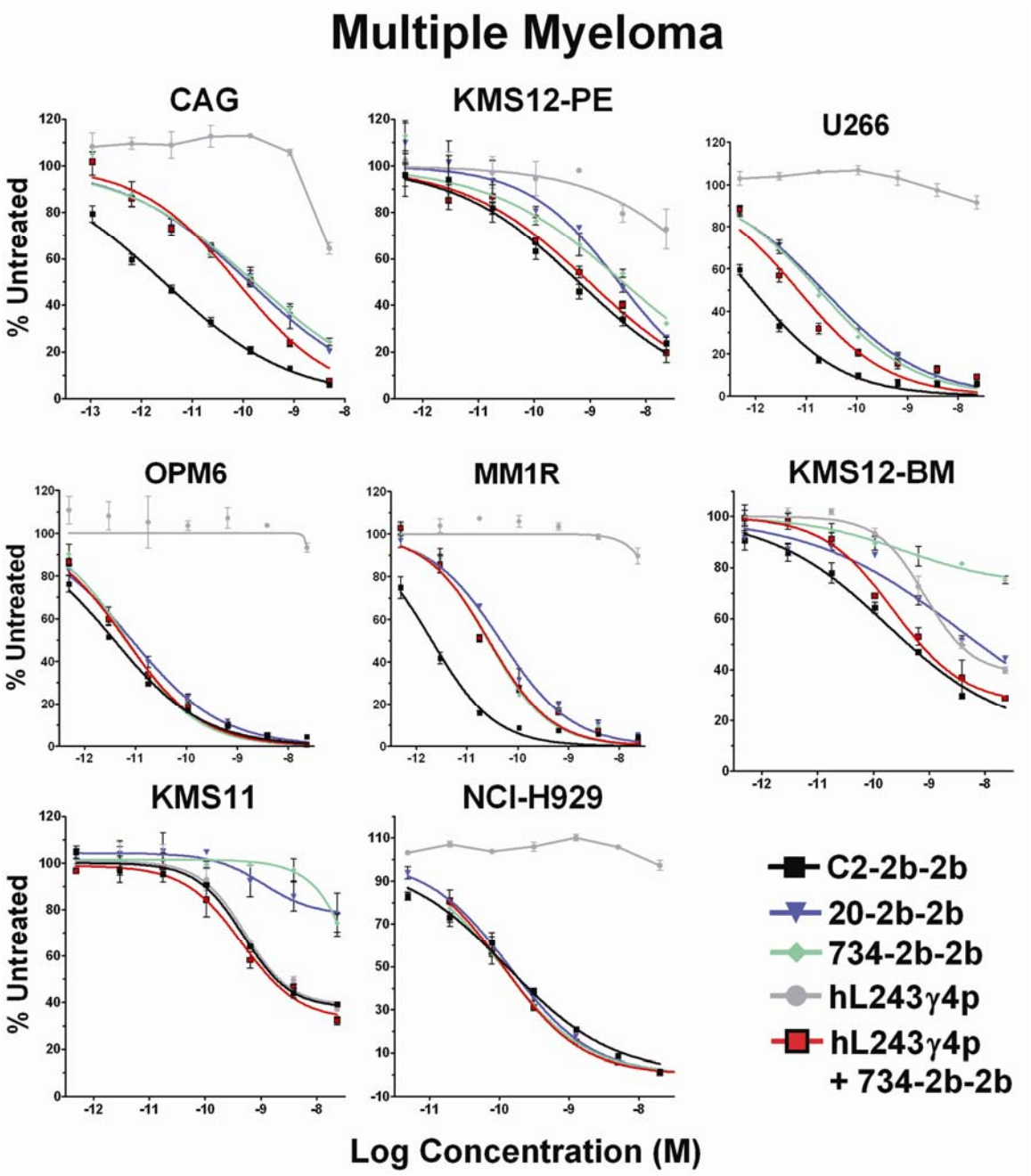

Figure S6. ***In-vitro*** **cytotoxicity of multiple myeloma cell lines**. Cells were cultured in the presence of increasing concentrations of the indicated constructs or combination and the relative viable cell densities were measured with MTS after the density of untreated cells increased by a minimum of 10-fold (3-7 days). The percent of the signal obtained from untreated cells was plotted *vs*. the log of the molar concentration. Dose-response curves and IC_{50} values were generated using Prism software. Error bars, SD.

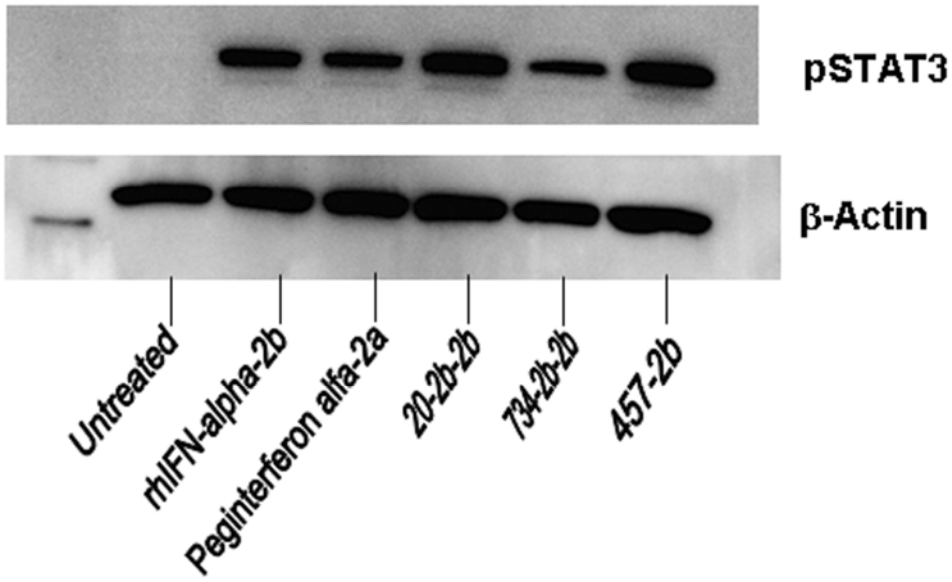

Figure S7. Induction of STAT3 phosphorylation with IFNα. Daudi cells (5×10^7 cells) were exposed to 100 U/mL of rhIFNα2b, Peginterferonalfa-2a, 20-2b-2b, 734-2b-2b, or α2b-457 for 30 minutes. Loaded 10 μg total protein/lane.