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(71) Applicant(s)
IBC Pharmaceuticals, Inc.

(72) Inventor(s)
Chang, Chien-Hsing;Goldenberg, David M.

(74) Agent / Attorney
Davies Collison Cave, PO Box 2219 BUSINESS CENTRE, MILTON, QLD, 4064

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(71) Applicant *(for all designated States except US)*: **IBC PHARMACEUTICALS, INC.** [US/US]; 300 American Road, Morris Plains, New Jersey 07950 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **CHANG, Chien-Hsing** [US/US]; 300 American Road, Morris Plains, New Jersey 07950 (US). **GOLDENBERG, David, M.** [US/US]; 300 American Road, Morris Plains, New Jersey 07950 (US).

(74) Agent: **NAKASHIMA, Richard, A.**; 300 American Road, Morris Plains, New Jersey 07950 (US).

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(54) Title: BISPECIFIC IMMUNOCYTOKINE DOCK-AND-LOCK (DNL) COMPLEXES AND THERAPEUTIC USE THEREOF

(57) Abstract: The present invention concerns methods and compositions for forming cytokine-antibody complexes using dock-and-lock technology. In preferred embodiments, the bispecific immunocytokine DNL construct comprises an IgG antibody attached to a Fab antibody fragment and a cytokine, wherein the IgG and the Fab bind to different target antigens which may be expressed on the same target cell. The bispecific immunocytokine DNL construct exhibits improved pharmacokinetics, with a longer serum half-life and significantly greater efficacy compared to cytokine alone, antibody alone, unconjugated cytokine plus antibody or even other types of cytokine-antibody DNL constructs. In a most preferred embodiment the construct comprises an anti-CD20 IgG antibody conjugated to an anti-HLA-DR Fab and IFNα2b, although other combinations of antibodies, antibody fragments and cytokines may be used to form the subject DNL complexes.

BISPECIFIC IMMUNOCYTOKINE DOCK-AND-LOCK (DNL) COMPLEXES AND THERAPEUTIC USE THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

[01] This application claims priority to U.S. Patent Applications Serial Nos. 12/754,740, filed April 6, 2010; 12/754,140, filed April 5, 2010; 12/752,649, filed April 1, 2010; 12/731,781, filed March 25, 2010; 12/644,146, filed December 22, 2009; and U.S. Provisional Patent Application No. 61/238,424, filed August 31, 2009. Each priority application is incorporated herein by reference in its entirety.

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SEQUENCE LISTING

[02.1] The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on September 14, 2010, is named IBC128WO.txt and is 39,795 bytes in size.

FIELD

[03] The present invention relates to compositions and methods of use of bispecific antibody immunocytokine DNL constructs, comprising first and second antibodies or antigen-binding antibody fragments and one or more copies of a cytokine. More generally, the present invention relates to compositions and methods of use of any DNL construct in which three different effector moieties are joined together using the DDD (dimerization and docking domain) and AD (anchoring domain) conjugation technique described below. The first and second antibodies or fragments thereof preferably bind to two different target antigens. Administration of the bispecific immunocytokine DNL construct, comprising a therapeutic cytokine, provides for highly effective delivery of the cytokine to target cells, tissues or organs, while allowing improved pharmacokinetics, dosing schedule and/or efficacy. The bispecific immunocytokine constructs show greater potency against target cells than the parent antibodies alone, the cytokine alone, a non-conjugated combination of antibodies and cytokine or cytokine conjugated to control antibodies. In a more preferred embodiment, the DNL construct may comprise interferon-α2b linked to an anti-CD20 IgG and anti-HLA-DR Fab. In a most preferred embodiment, the anti-CD20 IgG is veltuzumab and the anti-HLA-DR Fab is derived from a humanized L243 antibody. The DNL complex exhibits high toxicity for human lymphoma cells, multiple myeloma cells and other hematopoietic cancers *in vitro* and *in vivo*. However, the skilled artisan will realize that the subject DNL complexes may comprise any combination of antibodies or antibody fragments,

with specificity against target antigens that may be expressed by any tumor, autoimmune disease cell or other diseased cell. Similarly, the subject DNL complexes may be utilized for delivery of any therapeutic cytokine for treatment of a wide variety of diseases, such as cancer, immune dysfunction or autoimmune disease. The skilled artisan will futher realize that the subject bispecific complexes are not limited to delivery of cytokines, but may provide highly efficacious delivery of any therapeutic protein, peptide or other therapeutic effector moiety known in the art.

BACKGROUND

[04] In the United States, there were 65,980 new cases of non-Hodgkin lymphoma (NHL) and 19,500 deaths from this disease in 2009 (Jemal et al., CA Cancer J Clin 2009;59:225-49). Approximately half of NHL patients fail first-line therapy and are rarely cured (McLaughlin et al., J Clin Oncol 1998;16:2825-33). In addition to NHL, there were 20,580 new cases and 10,580 deaths from multiple myeloma (MM) (Jemal et al., CA Cancer J Clin 2009;59:225-49). The clinical activity of interferon-alpha (IFNα) is established in NHL therapy (Armitage et al., Bone Marrow Transplant 2006;38:701-2; Ann Oncol 2000;11:359-61), and the addition of IFNα to rituximab immunotherapy has shown some clinical advantage (Davis et al., Clin Cancer Res 2000;6:2644-52; Kimby et al., Leuk Lymphoma 2008;49:102-12). Available data suggest that progression-free survival of MM patients is improved with IFNα, but the benefit is small and its use remains controversial because of toxicity (Gisslinger and Kees, Wien Klin Wochenschr 2003;115:451-61).

[05] Interferon-α (IFNα) has been reported to have anti-tumor activity in both animal models of cancer (Ferrantini et al., 1994, J Immunol 153:4604-15) and human cancer patients (Gutterman et al., 1980, Ann Intern Med 93:399-406). IFNα can exert a variety of direct anti-tumor effects, including down-regulation of oncogenes, up-regulation of tumor suppressors, enhancement of immune recognition via increased expression of tumor surface MHC class I proteins, potentiation of apoptosis, and sensitization to chemotherapeutic agents (Gutterman et al., 1994, PNAS USA 91:1198-205; Matarrese et al., 2002, Am J Pathol 160:1507-20; Mecchia et al., 2000, Gene Ther 7:167-79; Sabaawy et al., 1999, Int J Oncol 14:1143-51; Takaoka et al, 2003, Nature 424:516-23).

[06] For some tumors, IFNα can have a direct and potent anti-proliferative effect through activation of STAT1 (Grimley et al., 1998 Blood 91:3017-27). Indirectly, IFNα can inhibit angiogenesis (Sidky and Borden, 1987, Cancer Res 47:5155-61) and stimulate host immune

cells, which may be vital to the overall antitumor response but has been largely under-appreciated (Belardelli et al., 1996, Immunol Today 17:369-72). IFNα has a pleiotropic influence on immune responses through effects on myeloid cells (Raefsky et al, 1985, J Immunol 135:2507-12; Luft et al, 1998, J Immunol 161:1947-53), T-cells (Carrero et al, 2006, J Exp Med 203:933-40; Pilling et al., 1999, Eur J Immuol 29:1041-50), and B-cells (Le et al, 2001, Immunity 14:461-70). As an important modulator of the innate immune system, IFNα induces the rapid differentiation and activation of dendritic cells (Belardelli et al, 2004, Cancer Res 64:6827-30; Paquette et al., 1998, J Leukoc biol 64:358-67; Santini et al., 2000, J Exp med 191:1777-88) and enhances the cytotoxicity, migration, cytokine production and antibody-dependent cellular cytotoxicity (ADCC) of NK cells (Biron et al., 1999, Annu Rev Immunol 17:189-220; Brunda et al. 1984, Cancer Res 44:597-601).

[07] The promise of IFNα as a cancer therapeutic has been hindered primarily due to its short circulating half-life and systemic toxicity. PEGylated forms of IFNα2 display increased circulation time, which augments their biological efficacy (Harris and Chess, 2003, Nat Rev Drug Discov 2:214-21; Osborn et al., 2002, J Pharmacol Exp Ther 303:540-8). Fusion of IFNα to a monoclonal antibody (MAb) can provide similar benefits as PEGylation, including reduced renal clearance, improved solubility and stability, and markedly increased circulating half-life. The immediate clinical benefit of this is the requirement for less frequent and lower doses, allowing prolonged therapeutic concentrations.

[08] Targeting of IFNα to tumors using MAbs to a tumor-associated antigen (TAA) can significantly increase its tumor accretion and retention while limiting its systemic concentration, thereby increasing the therapeutic index. Increased tumor concentrations of IFNα can augment its direct antiproliferative, apoptotic and anti-angiogenic activity, as well as prime and focus an antitumor immune response. Indeed, studies in mice using syngeneic murine IFNα-secreting transgenic tumors demonstrated an enhanced immune response elicited by a localized concentration of IFNα (Ferrantini et al., 2007, Biochimie 89:884-93).

[09] CD20 is an attractive candidate TAA for the therapy of B-cell lymphomas using MAb-IFNα. Anti-CD20 immunotherapy with rituximab is one of the most successful therapies against lymphoma, with relatively low toxicity (McLaughlin et al., 1998, J Clin Oncol 16:2825-33). Since rituximab is a chimeric antibody that can show immunogenicity in some patient populations and has considerably long infusion times for the initial administration (Cheson et al., 2008, NEJM 359:613-26), a better candidate for CD20-

targeting is the humanized MAb, veltuzumab (Stein et al., 2004, Clin Cancer Res 10:2868-78).

[010] Combination therapies with rituximab and IFNα currently under clinical evaluation have shown improved efficacy over rituximab alone (Kimby et al., 2008, Leuk Lymphoma 49:102-12; Salles et al., 2008, Blood 112:4824-31). These studies demonstrate some advantages of this combination as well as the drawbacks associated with IFNα. In addition to weekly infusions with rituximab, patients are typically administered IFNα three times/week for months and suffer the flu-like symptoms that are common side effects associated with IFNα therapy and which limit the tolerable dose. An antibody-IFNα conjugate could allow the less frequent administration of a single agent at a lower dose, limit or eliminate side effects, and may result in far superior efficacy. However, lymphomas and leukemias that express little or no CD20 are expected to be resistant to therapy with an immunoconjugated anti-CD20-IFNα construct. A need exists in the field for bispecific immunocytokine constructs that could target IFN-α or other therapeutic cytokines to two or more different tumor-associated antigens, such as CD20 and HLA-DR, to provide a more effective therapeutic against a wide variety of hematopoietic and other malignancies

[011] The human leukocyte antigen–DR (HLA-DR) is one of three isotypes of the major histocompatibilty complex (MHC) class II antigens. HLA-DR is highly expressed on a variety of hematologic malignancies and some solid cancers and has been actively pursued for antibody-based lymphoma therapy (Brown et al., 2001, Clin Lymphoma 2:188-90; DeNardo et al., 2005, Clin Cancer Res 11:7075s-9s; Stein et al., 2006, Bloood 108:2736-44). Preliminary studies indicate that anti-HLA-DR mAbs are markedly more potent than other naked mAbs of current clinical interest in *in vitro* and *in vivo* experiments in lymphomas, leukemias, and multiple myeloma (Stein et al., unpublished results). HLA-DR is also expressed on a subset of normal immune cells, including B cells, monocytes/macrophages, Langerhans cells, dendritic cells, and activated T cells (Dechant et al., 2003, Semin Oncol 30:465-75).

SUMMARY

[012] The present invention concerns compositions and methods of use of dock-and-lock (DNL) constructs (complexes) comprising three or more different effector moieties, such as antibodies, antibody fragments and cytokines. However, the skilled artisan will be aware that the DNL constructs are not so limited and the effector moieties of use may comprise any

protein, peptide or other molecule that may be attached to a DDD or AD moiety. Effector moieties of use in DNL constructs include but are not limited to proteins, peptides, antibodies, antibody fragments, immunomodulators, cytokines, hormones, enzymes, antisense oligonucleotides such as siRNA, toxins such as ribonucleases, xenoantigens, polyethylene glycol (PEG) and other polymers, anti-angiogenic agents, cytotoxic agents, pro-apoptosis agents and other known therapeutic agents.

[013] Preferred embodiments concern DNL constructs comprising three different effector moieties - first and second antibodies or antibody fragments and one or more copies of a cytokine. In the most preferred embodiment, the DNL construct comprises an anti-CD20 antibody, such as veltuzumab, an anti-HLA-DR antibody fragment, such as hL243, and a cytokine, such as IFN-α2b. Such DNL constructs are highly efficacious for therapy of hematopoietic and other tumors that express CD20, HLA-DR, or both. Although each component of the multifunctional complex (veltuzumab, anti-HLA-DR Fab, and IFN-α2b) has anti-tumor activity independently, the combined construct shows greater efficacy than any individual component, or the sum of the individual components administered in unconjugated form.

[014] In particular embodiments, the DNL construct may comprise a humanized anti-HLA-DR antibody or fragment thereof, such as an hL243 antibody comprising the heavy chain CDR sequences CDR1 (NYGMN, SEQ ID NO:1), CDR2 (WINTYTREPTYADDFKG, SEQ ID NO:2) and CDR3 (DITAVVPTGFDY, SEQ ID NO:3) and the light chain CDR sequences CDR1 (RASENIYSNLA, SEQ ID NO:4), CDR2 (AASNLAD, SEQ ID NO:5), and CDR3 (QHFWTTPWA, SEQ ID NO:6), attached to human antibody framework (FR) and constant region sequences (see, e.g., U.S. Patent No. 7,612,180, the Examples section of which is incorporated herein by reference). In preferred embodiments, a humanized L243 antibody may further comprise one or more of framework residues 27, 38, 46, 68 and 91 substituted from the murine L243 (mL243) heavy chain and/or one or more of framework residues 37, 39, 48 and 49 substituted from the mL243 light chain. The mL243 may be obtained at the American Type Culture Collection, Rockville, MD, (see Accession number ATCC HB55).

[015] In other particular embodiments, the DNL construct may comprise a humanized anti-CD20 antibody or fragment thereof, such as veltuzumab, comprising light chain variable region CDR1 (RASSSVSYIH, SEQ ID NO:7); CDR2 (ATSNLAS, SEQ ID NO:8); and CDR3 (QQWTSNPPT, SEQ ID NO:9); and heavy chain variable region CDR1 (SYNMH, SEQ ID NO:10); CDR2 (AIYPGNGDTSYNQKFKG, SEQ ID NO:11); and CDR3

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(STYYGGDWYFDV (SEQ ID NO: 95) or VVYYSNSYWYFDV, SEQ ID NO:12) (see, e.g., U.S. Patent No. 7,435,803, the Examples section of which is incorporated herein by reference.

[016] In more particular embodiments, the DNL construct may comprise a human IFN-α2b amino acid sequence. Clones comprising such sequences are commercially available from a variety of sources, such as a full length human IFNα2b cDNA clone (Ultimate ORF human clone cat# HORF01Clone ID IOH35221, Invitrogen, Carlsbad, CA).

[017] In various embodiments, the DNL constructs may comprise one or more antibodies or fragments thereof which bind to an antigen antigen other than CD20 and/or HLA-DR. In preferred embodiments, the antigen(s) may be selected from the group consisting of carbonic anhydrase IX, CCCL19, CCCL21, CSAp, CD1, CD1a, CD2, CD3, CD4, CD5, CD8, CD11A, CD14, CD15, CD16, CD18, CD19, IGF-1R, CD20, CD21, CD22, CD23, CD25, CD29, CD30, CD32b, CD33, CD37, CD38, CD40, CD40L, CD45, CD46, CD52, CD54, CD55, CD59, CD64, CD66a-e, CD67, CD70, CD74, CD79a, CD80, CD83, CD95, CD126, CD133, CD138, CD147, CD154, AFP, PSMA, CEACAM5, CEACAM-6, B7, ED-B of fibronectin, Factor H, FHL-1, Flt-3, folate receptor, GROB, HMGB-1, hypoxia inducible factor (HIF), HM1.24, insulin-like growth factor-1 (ILGF-1), IFN-γ, IFN-α, IFN-β, IL-2, IL-4R, IL-6R, IL-13R, IL-15R, IL-17R, IL-18R, IL-6, IL-8, IL-12, IL-15, IL-17, IL-18, IL-25, IP-10, MAGE, mCRP, MCP-1, MIP-1A, MIP-1B, MIF, MUC1, MUC2, MUC3, MUC4, MUC5, PAM4 antigen, NCA-95, NCA-90, Ia, HM1.24, EGP-1, EGP-2, HLA-DR, tenascin, Le(y), RANTES, T101, TAC, Tn antigen, Thomson-Friedenreich antigens, tumor necrosis antigens, TNF-α, TRAIL receptor (R1 and R2), VEGFR, EGFR, PlGF, complement factors C3, C3a, C3b, C5a, C5, and an oncogene product.

[018] Exemplary antibodies that may be utilized include, but are not limited to, hR1 (anti-IGF-1R, U.S. Patent Application Serial No. 12/722,645, filed 3/12/10) hPAM4 (anti-mucin, U.S. Patent No. 7,282,567), hA20 (anti-CD20, U.S. Patent No. 7,251,164), hA19 (anti-CD19, U.S. Patent No. 7,109,304), hIMMU31 (anti-AFP, U.S. Patent No. 7,300,655), hLL1 (anti-CD74, U.S. Patent No. 7,312,318), hLL2 (anti-CD22, U.S. Patent No. 7,074,403), hMu-9 (anti-CSAp, U.S. Patent No. 7,387,773), hL243 (anti-HLA-DR, U.S. Patent No. 7,612,180), hMN-14 (anti-CEACAM5, U.S. Patent No. 6,676,924), hMN-15 (anti-CEACAM6, U.S. Patent No. 7,541,440), hRS7 (anti-EGP-1, U.S. Patent No. 7,238,785) and hMN-3 (anti-CEACAM6, U.S. Patent Application Serial No. 7,541,440) the Examples section of each cited patent or application incorporated herein by reference. The skilled artisan will realize that this list is not limiting and that any known antibody may be used, as discussed in more

detail below.

[019] Exemplary cytokines that may be incorporated into the DNL constructs include but are not limited to MIF (macrophage migration inhibitory factor), HMGB-1 (high mobility group box protein 1), TNF-α, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, IL-17, IL-18, IL-19, IL-23, IL-24, CCL19, CCL21, IL-8, MCP-1, RANTES, MIP-1A, MIP-1B, ENA-78, MCP-1, IP-10, Gro-ß, Eotaxin, interferon-α, -β, -λ, G-CSF, GM-CSF, SCF, PDGF, MSF, Flt-3 ligand, erythropoietin, thrombopoietin, CNTF, leptin, oncostatin M, VEGF, EGF, FGF, PlGF, insulin, hGH, calcitonin, Factor VIII, IGF, somatostatin, tissue plasminogen activator and LIF. The sequences of the human forms of each of the recited cytokines is known in the art (see for example NCBI database) and clones encoding many of the exemplary cytokines are commercially available from Invitrogen, the American Type Culture Collection and other sources known in the art.

[020] Various embodiments may concern use of the subject DNL constructs to treat or diagnose a disease, including but not limited to non-Hodgkin's lymphomas, B cell acute and chronic lymphoid leukemias, Burkitt lymphoma, Hodgkin's lymphoma, hairy cell leukemia, acute and chronic myeloid leukemias, T cell lymphomas and leukemias, multiple myeloma, glioma, Waldenstrom's macroglobulinemia, carcinomas, melanomas, sarcomas, gliomas, and skin cancers. The carcinomas may be selected from the group consisting of carcinomas of the oral cavity, gastrointestinal tract, pulmonary tract, lung, breast, ovary, prostate, uterus, endometrium, cervix, urinary bladder, pancreas, bone, liver, gall bladder, kidney, skin, and testes. In addition, the subject DNL constructs may be used to treat an autoimmune disease, for example acute idiopathic thrombocytopenic purpura, chronic idiopathic thrombocytopenic purpura, dermatomyositis, Sydenham's chorea, myasthenia gravis, systemic lupus erythematosus, lupus nephritis, rheumatic fever, polyglandular syndromes, bullous pemphigoid, diabetes mellitus, Henoch-Schonlein purpura, post-streptococcal nephritis, erythema nodosum, Takayasu's arteritis, Addison's disease, rheumatoid arthritis, multiple sclerosis, sarcoidosis, ulcerative colitis, erythema multiforme, IgA nephropathy, polyarteritis nodosa, ankylosing spondylitis, Goodpasture's syndrome, thromboangitis obliterans, Sjogren's syndrome, primary biliary cirrhosis, Hashimoto's thyroiditis, thyrotoxicosis, scleroderma, chronic active hepatitis, polymyositis/dermatomyositis, polychondritis, pemphigus vulgaris, Wegener's granulomatosis, membranous nephropathy, amyotrophic lateral sclerosis, tabes dorsalis, giant cell arteritis/polymyalgia, pernicious anemia, rapidly progressive glomerulonephritis, psoriasis, or fibrosing alveolitis. In certain embodiments,

the subject antibodies may be used to treat leukemia, such as chronic lymphocytic leukemia, acute lymphocytic leukemia, chronic myeloid leukemia or acute myeloid leukemia.

[021] In one embodiment, a pharmaceutical composition of the present invention may be use to treat a subject having a metabolic disease, such amyloidosis, or a neurodegenerative disease, such as Alzheimer's disease. In addition, a pharmaceutical composition of the present invention may be use to treat a subject having an immune-dysregulatory disorder.

BRIEF DESCRIPTION OF THE DRAWINGS

[022] The following drawings form part of the present specification and are included to further demonstrate certain embodiments of the present invention. The embodiments may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[023] **FIG. 1** shows *in vitro* IFNα activity in a cytokine-MAb DNL construct compared to PEGylated or native IFNα. Specific activities (IU/pmol) measured as described in the Examples. The activity of known concentrations of each test article was extrapolated from a rhIFNα2b standard curve. Cultures were grown in the presence of increasing concentrations of 20-2b (●), 734-2b (■), v-mab (○), v-mab + 734-2b (□), PEGASYS (▼), PEG-Intron (▲) or 1R-2b (▽) and the relative viable cell densities were measured with MTS. The % of the signal obtained from untreated cells was plotted vs. the log of the molar concentration. Dose-response curves and EC_{50} values were generated using Prism software. Error bars, SD. **(A)** cell-based reporter gene assay. **(B)** viral protection assay with EMC virus and A549 cells. **(C)** *In vitro* lymphoma proliferation assays using Daudi cells. **(D)** *In vitro* lymphoma proliferation assays using Jeko-1 cells.

[024] **FIG. 2** shows the results of pharmacokinetic analyses in Swiss-Webster mice. Mice were administered 20-2b, α2b-413, PEGINTRON or PEGASYS and serum samples were analyzed for IFNα2b concentration by ELISA over 96 hours. Serum elimination curves are shown. Serum half-life ($T_{1/2}$) elimination rates and mean residence times (MRT) are summarized in the inserted table.

[025] **FIG. 3** **(A)** illustrates ADCC effector functions of 20-2b. Daudi or Raji cells were incubated with 20-2b, 22-2b, v-mab, epratuzumab (e-mab), or h734 at 5 μg/ml in the presence of freshly isolated PBMCs for 4 h before quantification of cell lysis. **(B)** shows CDC effector functions of 20-2B. Daudi cells were incubated with serial dilutions of 20-2b

(●), 734-2b (■) or v-mab (○) in the presence of human complement. The % complement control (number of viable cells in the test sample compared to cells treated with complement only) was plotted vs. the log of the nM concentration. Error bars, SD.

[026] **FIG. 4** shows enhanced depletion of NHL cells from whole blood by 20-2b. Fresh heparinized human blood was mixed with either Daudi or Ramos and incubated with 20-2b (●), v-mab (○), 734-2b (■) or v-mab + 734-2b (□) at 0.01, 0.1 or 1 nM for two days. The effect of the indicated treatments on lymphoma and peripheral blood lymphocytes was evaluated using flow cytometry. Error bars, SD.

[027] **FIG. 5 (A)** illustrates survival curves showing therapeutic efficacy of 20-2b in a disseminated Burkitt's lymphoma (Daudi) xenograft model. Female C.B. 17 SCID mice were administered Daudi cells i.v. on day 0. Treatments consisted of 20-2b (●), 734-2b (■), v-mab (○), PEGASYS (▼) or saline (X) given as a single s.c. doses. Days of treatment are indicated with arrows. Survival curves were analyzed using Prism software. In an Early Daudi model. Groups of 10 mice were given a single dose of 0.7 pmol (solid line) or 0.07 pmol (dashed line) on day 1. **(B)** shows a similar study to FIG. 6(A), but in an Advanced Daudi model. Groups of 10 mice were given a single dose of 0.7 pmol (solid line), 7 pmol (dashed line) or 70 pmol (gray line) on day 7.

[028] **FIG. 6 (A)** presents survival curves showing therapeutic efficacy of 20-2b in disseminated Burkitt's lymphoma (Raji and NAMALWA) xenograft models. Female C.B. 17 SCID mice were administered NHL cells i.v. on day 0. Treatments consisted of 20-2b (●), 734-2b (■), v-mab (○) or saline (X) given as s.c. doses. Days of treatment are indicated with arrows. Survival curves were analyzed using Prism software. In an Advanced Raji model, groups of 10 received 250 pmol doses on days 5, 7, 9, 12, 14 and 16. **(B)** shows a similar study to FIG. 7(A), but in an Early NAMALWA model. Groups of 6 received 250 pmol doses of 20-2b or 734-2b on days 1, 3, 5, 8, 10 and 12 or 3.5 nmol doses of v-mab on days 1, 5, 9, 13, 17, 21 and 25.

[029] **FIG. 7** shows the results of a cell-based assay for EPO activity using TF1 cells that were treated with EPO standard, 734-EPO, or EPO-DDD2 for 72 hours. Dose response curves and EC_{50} values were generated using Graph Pad Prism software.

[030] **FIG. 8.** Biological activity of 20-C2-2b. *A* and *B*, indirect immunofluorescence showing binding of MAbs and MAb-IFNα to live NHL cells (Raji or RL). Cells were

incubated at 4°C for 1 h in the presence of 5 nM **(A)** or 0.2 – 50 nM **(B)** of the indicated construct prior to probing with PE-conjugated goat-anti-human Fc. *MFI*, mean fluorescence intensity; error bars, 95% CI. **(C)** IFNα2 specific activities determined using a cell-based reporter gene assay shown as IU/pmol of the whole molecule and IU/pmol of IFNα2b.

[031] FIG. 9. Apoptosis in NHL and MM cells. Cells were treated for 48 h before quantification of the % annexin-V-positive cells by flow cytometry. **(A)** For Daudi: v-mab and hL243γ4p were 10 pM; 20-C2-2b, 20-2b-2b and V+L243+2b (a mixture of v-mab, hL243γ4p and 734-2b-2b) were 1 pM. For Jeko-1, all treatments were at 0.5 nM. **(B)** CAG was treated at 1, 0.1 and 0.01 nM. **(C)** KMS12-BM was treated at 20 and 2 nM. V+L243, mixture of v-mab and hL243γ4p; L243+2b, mixture of hL243γ4p and 734-2b-2b.

[032] FIG. 10. Characterization of multiple myeloma cell lines. **(A)** Antigen densities of HLA-DR and CD20 on selected myeloma lines. After 30 min incubation with hL243γ4p, v-mab or hMN-14 (isotype control MAb), cells were probed with PE-Goat anti-human IgG (Fab) and analyzed by flow cytometry. **(B)** Relative sensitivity of myeloma lines to IFNα2. Cells were incubated in the presence or absence of 3 nM peginterferon alfa-2b for 4 days prior to quantification of viable cells with MTS.

[033] FIG. 11. *In vitro* cytotoxicity of multiple myeloma. Indicated cell lines were cultured in the presence of increasing concentrations of the indicated constructs or combinations and the relative viable cell densities were measured with MTS. The % of the signal obtained from untreated cells was plotted *vs.* the log of the molar concentration. Dose-response curves and EC_{50} values were generated using Prism software. Error bars, SD.

[034] FIG. 12. Enhanced depletion of NHL cells from whole blood. Fresh heparinized human blood was mixed with Daudi and incubated with 1 nM of the indicated Mab-IFNα or MAb for two days. The effect on Daudi, B cells, T cells, and monocytes was evaluated by flow cytometry. Error bars, SD.

DETAILED DESCRIPTION

Definitions

[035] Unless otherwise specified, "a" or "an" means "one or more".

[036] As used herein, the terms "and" and "or" may be used to mean either the conjunctive

or disjunctive. That is, both terms should be understood as equivalent to "and/or" unless otherwise stated.

[037] A "therapeutic agent" is an atom, molecule, or compound that is useful in the treatment of a disease. Examples of therapeutic agents include antibodies, antibody fragments, peptides, drugs, toxins, enzymes, nucleases, hormones, immunomodulators, antisense oligonucleotides, small interfering RNA (siRNA), chelators, boron compounds, photoactive agents, dyes, and radioisotopes.

[038] A "diagnostic agent" is an atom, molecule, or compound that is useful in diagnosing a disease. Useful diagnostic agents include, but are not limited to, radioisotopes, dyes (such as with the biotin-streptavidin complex), contrast agents, fluorescent compounds or molecules, and enhancing agents (e.g., paramagnetic ions) for magnetic resonance imaging (MRI).

[039] An "antibody" as used herein refers to a full-length (ie, naturally occurring or formed by normal immunoglobulin gene fragment recombinatorial processes) immunoglobulin molecule *(eg,* an IgG antibody) or an immunologically active (ie, specifically binding) portion of an immunoglobulin molecule, like an antibody fragment. An "antibody" includes monoclonal, polyclonal, bispecific, multispecific, murine, chimeric, humanized and human antibodies.

[040] A "naked antibody" is an antibody or antigen binding fragment thereof that is not attached to a therapeutic or diagnostic agent. The Fc portion of an intact naked antibody can provide effector functions, such as complement fixation and ADCC (see, *e.g.,* Markrides, *Pharmacol Rev* 50:59-87, 1998). Other mechanisms by which naked antibodies induce cell death may include apoptosis. (Vaswani and Hamilton, *Ann Allergy Asthma Immunol* 81: 105-119,1998.)

[041] An "antibody fragment" is a portion of an intact antibody such as $F(ab')_2$, Fab', Fab, Fv, sFv, scFv and the like. Regardless of structure, an antibody fragment binds with the same antigen that is recognized by the full-length antibody. For example, antibody fragments include isolated fragments consisting of the variable regions, such as the "Fv" fragments consisting of the variable regions of the heavy and light chains or recombinant single chain polypeptide molecules in which light and heavy variable regions are connected by a peptide linker ("scFv proteins"). "Single-chain antibodies", often abbreviated as "scFv" consist of a polypeptide chain that comprises both a V_H and a V_L domain which interact to form an antigen- binding site. The V_H and V_L domains are usually linked by a peptide of 1 to 25 amino acid residues. Antibody fragments also include diabodies, triabodies and single domain antibodies (dAb).

[042] An antibody or immunoconjugate preparation, or a composition described herein, is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient subject. In particular embodiments, an antibody preparation is physiologically significant if its presence invokes an antitumor response or mitigates the signs and symptoms of an autoimmune disease state. A physiologically significant effect could also be the evocation of a humoral and/or cellular immune response in the recipient subject leading to growth inhibition or death of target cells.

Dock and Lock (DNL) method

[043] The "dock-and-lock" (DNL) method exploits specific protein/protein interactions that occur between the regulatory (R) subunits of cAMP-dependent protein kinase (PKA) and the anchoring domain (AD) of A-kinase anchoring proteins (AKAPs) (Baillie *et al.*, FEBS Letters. 2005; **579:** 3264. Wong and Scott, Nat. Rev. Mol. Cell Biol. 2004; **5:** 959). PKA, which plays a central role in one of the best studied signal transduction pathways triggered by the binding of the second messenger cAMP to the R subunits, was first isolated from rabbit skeletal muscle in 1968 (Walsh *et al.*, J. Biol. Chem. 1968;243:3763). The structure of the holoenzyme consists of two catalytic subunits held in an inactive form by the R subunits (Taylor, J. Biol. Chem. 1989;264:8443). Isozymes of PKA are found with two types of R subunits (RI and RII), and each type has α and β isoforms (Scott, Pharmacol. Ther. 1991;50:123). The R subunits have been isolated only as stable dimers and the dimerization domain has been shown to consist of the first 44 amino-terminal residues (Newlon *et al.*, Nat. Struct. Biol. 1999;6:222). Binding of cAMP to the R subunits leads to the release of active catalytic subunits for a broad spectrum of serine/threonine kinase activities, which are oriented toward selected substrates through the compartmentalization of PKA via its docking with AKAPs (Scott *et al.*, J. Biol. Chem. 1990;265;21561)

[044] Since the first AKAP, microtubule-associated protein-2, was characterized in 1984 (Lohmann *et al.*, Proc. Natl. Acad. Sci USA. 1984;81:6723), more than 50 AKAPs that localize to various suB cellular sites, including plasma membrane, actin cytoskeleton, nucleus, mitochondria, and endoplasmic reticulum, have been identified with diverse structures in species ranging from yeast to humans (Wong and Scott, Nat. Rev. Mol. Cell Biol. 2004;5:959). The AD of AKAPs for PKA is an amphipathic helix of 14-18 residues (Carr *et al.*, J. Biol. Chem. 1991;266:14188). The amino acid sequences of the AD are quite varied among individual AKAPs, with the binding affinities reported for RII dimers ranging

from 2 to 90 nM (Alto *et al.*, Proc. Natl. Acad. Sci. USA. 2003;100:4445). Interestingly, AKAPs will only bind to dimeric R subunits. For human RIIα, the AD binds to a hydrophobic surface formed by the 23 amino-terminal residues (Colledge and Scott, Trends Cell Biol. 1999; 6:216). Thus, the dimerization domain and AKAP binding domain of human RIIα are both located within the same N-terminal 44 amino acid sequence (Newlon *et al.*, Nat. Struct. Biol. 1999;6:222; Newlon *et al.*, EMBO J. 2001;20:1651), which is termed the DDD herein.

DDD of Human RIIα and AD of AKAPs as Linker Modules

[045] We have developed a platform technology to utilize the DDD of human RIIα and the AD of AKAP proteins as an excellent pair of linker modules for docking any combination of entities, referred to hereafter as **A** and **B**, into a noncovalent complex, which could be further locked into a stably tethered structure through the introduction of cysteine residues into both the DDD and AD at strategic positions to facilitate the formation of disulfide bonds. The general methodology of the "dock-and-lock" approach is as follows. Entity **A** is constructed by linking a DDD sequence to a precursor of **A**, resulting in a first component hereafter referred to as **a**. Because the DDD sequence would effect the spontaneous formation of a dimer, **A** would thus be composed of $\mathbf{a_2}$. Entity **B** is constructed by linking an AD sequence to a precursor of **B**, resulting in a second component hereafter referred to as **b**. The dimeric motif of DDD contained in $\mathbf{a_2}$ will create a docking site for binding to the AD sequence contained in **b**, thus facilitating a ready association of $\mathbf{a_2}$ and **b** to form a trimeric complex composed of $\mathbf{a_2b}$. This binding event is made irreversible with a subsequent reaction to covalently secure the two entities via disulfide bridges, which occurs very efficiently based on the principle of effective local concentration because the initial binding interactions should bring the reactive thiol groups placed onto both the DDD and AD into proximity (Chmura *et al.*, Proc. Natl. Acad. Sci. USA. 2001;98:8480) to ligate site-specifically. Although in certain embodiments the $\mathbf{a_2}$ subunit may contain two identical effector moieties, in preferred embodiments described below the $\mathbf{a_2}$ subunit may comprise two different effector moieties, each attached to an identical DDD sequence. Thus, the trimeric $\mathbf{a_2b}$ complex may comprise three different effector moieties.

[046] In preferred embodiments, the immunocytokine DNL constructs may be based on a variation of the $\mathbf{a_2b}$ structure, in which a first and a second effector are attached to DDD moieties and a third effector is attached to an AD moiety. Each AD moiety is capable of

binding to two DDD moieties in the form of a dimer. By attaching the DDD and AD away from the functional groups of the precursors, such site-specific ligations are also expected to preserve the original activities of the precursors. This approach is modular in nature and potentially can be applied to link, site-specifically and covalently, a wide range of substances. The DNL method was disclosed in U.S. Patent Nos. 7,550,143; 7,521,056; 76,534,866; 7,527,787 and 7,666,400, the Examples section of each incorporated herein by reference.

[047] In preferred embodiments, the effector moiety is a protein or peptide, more preferably an antibody, antibody fragment or cytokine, which can be linked to a DDD or AD moiety to form a fusion protein or peptide. A variety of methods are known for making fusion proteins, including nucleic acid synthesis, hybridization and/or amplification to produce a synthetic double-stranded nucleic acid encoding a fusion protein of interest. Such double-stranded nucleic acids may be inserted into expression vectors for fusion protein production by standard molecular biology techniques (see, e.g. Sambrook et al., Molecular Cloning, A laboratory manual, 2nd Ed, 1989). In such preferred embodiments, the AD and/or DDD moiety may be attached to either the N-terminal or C-terminal end of an effector protein or peptide. However, the skilled artisan will realize that the site of attachment of an AD or DDD moiety to an effector moiety may vary, depending on the chemical nature of the effector moiety and the part(s) of the effector moiety involved in its physiological activity. In a most preferred embodiment, attachment of AD or DDD moieties to an antibody or antibody fragment occurs at the C-terminal end of the heavy chain subunit, at the opposite end of the molecule from the antingen-binding site. However, as discussed below, N-terminal attachment to antibodies or antibody fragments may also be utilized while retaining antigen-binding activity. Site-specific attachment of a variety of effector moieties may be performed using techniques known in the art, such as the use of bivalent cross-linking reagents and/or other chemical conjugation techniques.

DDD and AD Sequence Variants

[048] In certain embodiments, the AD and DDD sequences incorporated into the immunocytokine DNL complex comprise the amino acid sequences of DDD1 and AD1 below. In more preferred embodiments, the AD and DDD sequences comprise the amino acid sequences of DDD2 and AD2, which are designed to promote disulfide bond formation between the DDD and AD moieties.

DDD1

SHIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID

NO:13)

DDD2

CGHIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:14)

AD1

QIEYLAKQIVDNAIQQA (SEQ ID NO:15)

AD2

CGQIEYLAKQIVDNAIQQAGC (SEQ ID NO:16)

[049] The skilled artisan will realize that DDD1 and DDD2 comprise the DDD sequence of the human RIIα form of protein kinase A. However, in alternative embodiments, the DDD and AD moieties may be based on the DDD sequence of the human RIIα form of protein kinase A and a corresponding AKAP sequence, as exemplified in DDD3, DDD3C and AD3 below.

DDD3

SLRECELYVQKHNIQALLKDSIVQLCTARPERPMAFLREYFERLEKEEAK (SEQ ID NO:17)

DDD3C

MSCGGSLRECELYVQKHNIQALLKDSIVQLCTARPERPMAFLREYFERLEKEEAK (SEQ ID NO:18)

AD3

CGFEELAWKIAKMIWSDVFQQGC (SEQ ID NO:19)

[050] Still other alternative DDD moieties based on the known human RIβ and RIIβ amino acid sequences may be designed and utilized (see, e.g., NCBI Accession Nos. NP_001158233 and NP_002727, sequences below).

Human PKA RIβ Amino Acid Sequence

MASPPACPSE EDESLKGCEL YVQLHGIQQV LKDCIVHLCI SKPERPMKFL REHFEKLEKE ENRQILARQK SNSQSDSHDE EVSPTPPNPV VKARRRRGGV

SAEVYTEEDA VSYVRKVIPK DYKTMTALAK AISKNVLFAH LDDNERSDIF DAMFPVTHIA GETVIQQGNE GDNFYVVDQG EVDVYVNGEW VTNISEGGSF GELALIYGTP RAATVKAKTD LKLWGIDRDS YRRILMGSTL RKRKMYEEFL SKVSILESLE KWERLTVADA LEPVQFEDGE KIVVQGEPGD DFYIITEGTA SVLQRRSPNE EYVEVGRLGP SDYFGEIALL LNRPRAATVV ARGPLKCVKL DRPRFERVLG PCSEILKRNI QRYNSFISLT V (SEQ ID NO:20)

Human PKA RIß Amino Acid Sequence

MSIEIPAGLT ELLQGFTVEV LRHQPADLLE FALQHFTRLQ QENERKGTAR FGHEGRTWGD LGAAAGGGTP SKGVNFAEEP MQSDSEDGEE EEAAPADAGA FNAPVINRFT RRASVCAEAY NPDEEEDDAE SRIIHPKTDD QRNRLQEACK DILLFKNLDP EQMSQVLDAM FEKLVKDGEH VIDQGDDGDN FYVIDRGTFD IYVKCDGVGR CVGNYDNRGS FGELALMYNT PRAATITATS PGALWGLDRV TFRRIIVKNN AKKRKMYESF IESLPFLKSL EFSERLKVVD VIGTKVYNDG EQIIAQGDSA DSFFIVESGE VKITMKRKGK SEVEENGAVE IARCSRGQYF GELALVTNKP RAASAHAIGT VKCLAMDVQA FERLLGPCME IMKRNIATYE EQLVALFGTN MDIVEPTA (SEQ ID NO:21)

[051] In other alternative embodiments, different sequence variants of AD and/or DDD moieties may be utilized in construction of the bispecific immunocytokine DNL complexes. The structure-function relationships of the AD and DDD domains have been the subject of investigation. (See, e.g., Burns-Hamuro et al., 2005, Protein Sci 14:2982-92; Carr et al., 2001, J Biol Chem 276:17332-38; Alto et al., 2003, Proc Natl Acad Sci USA 100:4445-50; Hundsrucker et al., 2006, Biochem J 396:297-306; Stokka et al., 2006, Biochem J 400:493-99; Gold et al., 2006, Mol Cell 24:383-95; Kinderman et al., 2006, Mol Cell 24:397-408, the entire text of each of which is incorporated herein by reference.)

[052] For example, Kinderman et al. (2006) examined the crystal structure of the AD-DDD binding interaction and concluded that the human DDD sequence contained a number of conserved amino acid residues that were important in either dimer formation or AKAP binding, underlined in the sequence below. (See Figure 1 of Kinderman et al., 2006, incorporated herein by reference.) The skilled artisan will realize that in designing sequence variants of the DDD sequence, one would desirably avoid changing any of the underlined

residues, while conservative amino acid substitutions might be made for residues that are less critical for dimerization and AKAP binding. Thus, a potential alternative DDD sequence of use for construction of DNL complexes is shown in the following sequence, wherein "X" represents a conservative amino acid substitution. Conservative amino acid substitutions are discussed in more detail below, but could involve for example substitution of an aspartate residue for a glutamate residue, or a leucine or valine residue for an isoleucine residue, etc. Such conservative amino acid substitutions are well known in the art.

Human DDD sequence from protein kinase A

SHIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:13)

XXIXIXXXLXXLLXXYXVXVLXXXXXXLVXFXVXYFXXLXXXXX (SEQ ID NO:22)

[053] Alto et al. (2003) performed a bioinformatic analysis of the AD sequence of various AKAP proteins to design an RII selective AD sequence called AKAP-IS shown below, with a binding constant for DDD of 0.4 nM. The AKAP-IS sequence was designed as a peptide antagonist of AKAP binding to PKA. Residues in the AKAP-IS sequence where substitutions tended to decrease binding to DDD are underlined in the sequence below. Therefore, the skilled artisan will realize that variants which may function for DNL constructs are indicated by the following sequence, where "X" is a conservative amino acid substitution.

AKAP-IS sequence

QIEYLAKQIVDNAIQQA (SEQ ID NO:15)

XXXXXAXXIVXXAIXXX (SEQ ID NO:23)

[054] Similarly, Gold (2006) utilized crystallography and peptide screening to develop a SuperAKAP-IS sequence shown below, exhibiting a five order of magnitude higher selectivity for the RII isoform of PKA compared with the RI isoform. Underlined residues indicate the positions of amino acid substitutions, relative to the AKAP-IS sequence, that increased binding to the DDD moiety of RIIα. In this sequence, the N-terminal Q residue is numbered as residue number 4 and the C-terminal A residue is residue number 20. Residues where substitutions could be made to affect the affinity for RIIα were residues 8, 11, 15, 16, 18, 19 and 20 (Gold et al., 2006). It is contemplated that in certain alternative embodiments, the SuperAKAP-IS sequence may be substituted for the AKAP-IS AD moiety sequence to

prepare bispecific immunocytokine DNL constructs. Other alternative sequences that might be substituted for the AKAP-IS AD sequence are shown below. Substitutions relative to the AKAP-IS sequence are underlined. It is anticipated that, as with the AKAP-IS sequence, the AD moiety may also include the additional N-terminal residues cysteine and glycine and C-terminal residues glycine and cysteine.

SuperAKAP-IS

QIEYVAKQIVDYAIHQA (SEQ ID NO:24)

Alternative AKAP sequences

QIEYKAKQIVDHAIHQA (SEQ ID NO:25)

QIEYHAKQIVDHAIHQA (SEQ ID NO:26)

QIEYVAKQIVDHAIHQA (SEQ ID NO:27)

[055] Figure 2 of Gold et al. disclosed additional DDD-binding sequences from a variety of AKAP proteins, shown below.

RII-Specific AKAPs

AKAP-KL

PLEYQAGLLVQNAIQQAI (SEQ ID NO:28)

AKAP79

LLIETASSLVKNAIQLSI (SEQ ID NO:29)

AKAP-Lbc

LIEEAASRIVDAVIEQVK (SEQ ID NO:30)

RI-Specific AKAPs

AKAPce

ALYQFADRFSELVISEAL (SEQ ID NO:31)

RIAD

LEQVANQLADQIIKEAT (SEQ ID NO:32)

PV38

FEELAWKIAKMIWSDVF (SEQ ID NO:33)

Dual-Specificity AKAPs

AKAP7

ELVRLSKRLVENAVLKAV (SEQ ID NO:34)

MAP2D

TAEEVSARIVQVVTAEAV (SEQ ID NO:35)

DAKAP1

QIKQAAFQLISQVILEAT (SEQ ID NO:36)

DAKAP2

LAWKIAKMIVSDVMQQ (SEQ ID NO:37)

[056] Stokka et al. (2006) also developed peptide competitors of AKAP binding to PKA, shown below. The peptide antagonists were designated as Ht31, RIAD and PV-38. The Ht-31 peptide exhibited a greater affinity for the RII isoform of PKA, while the RIAD and PV-38 showed higher affinity for RI.

Ht31

DLIEEAASRIVDAVIEQVKAAGAY (SEQ ID NO:38)

RIAD

LEQYANQLADQIIKEATE (SEQ ID NO:39)

PV-38

FEELAWKIAKMIWSDVFQQC (SEQ ID NO:40)

[057] Hundsrucker et al. (2006) developed still other peptide competitors for AKAP binding to PKA, with a binding constant as low as 0.4 nM to the DDD of the RII form of PKA. The sequences of various AKAP antagonistic peptides is provided in Table 1 of Hundsrucker et al., reproduced below.

Table 1 AKAP Peptide sequences

AKAPIS represents a synthetic RII subunit-binding peptide. All other peptides are derived from the RII-binding domains of the indicated AKAPs.

	Peptide Sequence
AKAPIS	QIEYLAKQIVDNAIQQA (SEQ ID NO:15)
AKAPIS-P	QIEYLAKQIPDNAIQQA (SEQ ID NO:41)
Ht31	KGADLIEEAASRIVDAVIEQVKAAG (SEQ ID NO:42)
Ht31-P	KGADLIEEAASRIPDAPIEQVKAAG (SEQ ID NO:43)
AKAP7δ-wt-pep	PEDAELVRLSKRLVENAVLKAVQQY (SEQ ID NO:44)
AKAP7δ-L304T-pep	PEDAELVRTSKRLVENAVLKAVQQY (SEQ ID NO:45)
AKAP7δ-L308D-pep	PEDAELVRLSKRDVENAVLKAVQQY (SEQ ID NO:46)
AKAP7δ-P-pep	PEDAELVRLSKRLPENAVLKAVQQY (SEQ ID NO:47)
AKAP7δ-PP-pep	PEDAELVRLSKRLPENAPLKAVQQY (SEQ ID NO:48)

AKAP7δ-L314E-pep	PEDAELVRLSKRLVENAVEKAVQQY (SEQ ID NO:49)
AKAP1-pep	EEGLDRNEEIKRAAFQIISQVISEA (SEQ ID NO:50)
AKAP2-pep	LVDDPLEYQAGLLVQNAIQQAIAEQ (SEQ ID NO:51)
AKAP5-pep	QYETLLIETASSLVKNAIQLSIEQL (SEQ ID NO:52)
AKAP9-pep	LEKQYQEQLEEEVAKVIVSMSIAFA (SEQ ID NO:53)
AKAP10-pep	NTDEAQEELAWKIAKMIVSDIMQQA (SEQ ID NO:54)
AKAP11-pep	VNLDKKAVLAEKIVAEAIEKAEREL (SEQ ID NO:55)
AKAP12-pep	NGILELETKSSKLVQNIIQTAVDQF (SEQ ID NO:56)
AKAP14-pep	TQDKNYEDELTQVALALVEDVINYA (SEQ ID NO:57)
Rab32-pep	ETSAKDNINIEEAARFLVEKILVNH (SEQ ID NO:58)

[058] Residues that were highly conserved among the AD domains of different AKAP proteins are indicated below by underlining with reference to the AKAP IS sequence below. The residues are the same as observed by Alto et al. (2003), with the addition of the C-terminal alanine residue. (See FIG. 4 of Hundsrucker et al. (2006), incorporated herein by reference.) The sequences of peptide antagonists with particularly high affinities for the RII DDD sequence were those of AKAP-IS, AKAP7δ-wt-pep, AKAP7δ-L304T-pep and AKAP7δ-L308D-pep.

AKAP-IS

QIEYLAKQIVDNAIQQA (SEQ ID NO:15)

[059] Carr et al. (2001) examined the degree of sequence homology between different AKAP-binding DDD sequences from human and non-human proteins and identified residues in the DDD sequences that appeared to be the most highly conserved among different DDD moieties. These are indicated below by underlining with reference to the human PKA RIIα DDD sequence. Residues that were particularly conserved are further indicated by italics. The residues overlap with, but are not identical to those suggested by Kinderman et al. (2006) to be important for binding to AKAP proteins. Thus, a potential DDD sequence is indicated below, wherein "X" represents a conservative amino acid substitution.

SHIQ*IP*P*GL*T*ELLQGYT*V*EVLR*Q*Q*P*P*DLVEFA*VE*YF*TR*L*REA*R*A (SEQ ID NO:13)

XHIX*IP*X*GL*X*ELLQGYT*X*EVLR*X*QP*X*DLVEFA*XX*YF*XX*L*XE*X*R*X (SEQ ID NO:59)

[060] The skilled artisan will realize that in general, those amino acid residues that are highly conserved in the DDD and AD sequences from different proteins are ones that it may

be preferred to remain constant in making amino acid substitutions, while residues that are less highly conserved may be more easily varied to produce sequence variants of the AD and/or DDD sequences described herein.

[061] In addition to sequence variants of the DDD and/or AD moieties, in certain embodiments it may be preferred to introduce sequence variations in the antibody moiety or the linker peptide sequence joining the antibody with the AD sequence. In one illustrative example, three possible variants of fusion protein sequences, are shown below.

(L)
QKSLSLSPGLGSGGGGSGGCG (SEQ ID NO:60)
(A)
QKSLSLSPGAGSGGGGSGGCG (SEQ ID NO:61)
(-)
QKSLSLSPGGSGGGGSGGCG (SEQ ID NO:62)

Amino Acid Substitutions

[062] In certain embodiments, the disclosed methods and compositions may involve production and use of proteins or peptides with one or more substituted amino acid residues. The structural, physical and/or therapeutic characteristics of native, chimeric, humanized or human antibodies, or AD or DDD sequences may be optimized by replacing one or more amino acid residues. For example, it is well known in the art that the functional characteristics of humanized antibodies may be improved by substituting a limited number of human framework region (FR) amino acids with the corresponding FR amino acids of the parent murine antibody. This is particularly true when the framework region amino acid residues are in close proximity to the CDR residues.

[063] In other cases, the therapeutic properties of an antibody, such as binding affinity for the target antigen, the dissociation- or off-rate of the antibody from its target antigen, or even the effectiveness of induction of CDC (complement-dependent cytotoxicity) or ADCC (antibody dependent cellular cytotoxicity) by the antibody, may be optimized by a limited number of amino acid substitutions.

[064] In alternative embodiments, the DDD and/or AD sequences used to make the subject DNL constructs may be further optimized, for example to increase the DDD-AD binding affinity. Potential sequence variations in DDD or AD sequences are discussed above.

[065] The skilled artisan will be aware that, in general, amino acid substitutions typically involve the replacement of an amino acid with another amino acid of relatively similar properties (i.e., conservative amino acid substitutions). The properties of the various amino

acids and effect of amino acid substitution on protein structure and function have been the subject of extensive study and knowledge in the art.

[066] For example, the hydropathic index of amino acids may be considered (Kyte & Doolittle, 1982, J. Mol. Biol., 157:105-132). The relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules. Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics (Kyte & Doolittle, 1982), these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5). In making conservative substitutions, the use of amino acids whose hydropathic indices are within ± 2 is preferred, within ± 1 are more preferred, and within ± 0.5 are even more preferred.

[067] Amino acid substitution may also take into account the hydrophilicity of the amino acid residue (e.g., U.S. Pat. No. 4,554,101). Hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0); glutamate (+3.0); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 .+-.1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4). Replacement of amino acids with others of similar hydrophilicity is preferred.

[068] Other considerations include the size of the amino acid side chain. For example, it would generally not be preferred to replace an amino acid with a compact side chain, such as glycine or serine, with an amino acid with a bulky side chain, e.g., tryptophan or tyrosine. The effect of various amino acid residues on protein secondary structure is also a consideration. Through empirical study, the effect of different amino acid residues on the tendency of protein domains to adopt an alpha-helical, beta-sheet or reverse turn secondary structure has been determined and is known in the art (see, e.g., Chou & Fasman, 1974, Biochemistry, 13:222-245; 1978, Ann. Rev. Biochem., 47: 251-276; 1979, Biophys. J., 26:367-384).

[069] Based on such considerations and extensive empirical study, tables of conservative amino acid substitutions have been constructed and are known in the art. For example:

arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine. Alternatively: Ala (A) leu, ile, val; Arg (R) gln, asn, lys; Asn (N) his, asp, lys, arg, gln; Asp (D) asn, glu; Cys (C) ala, ser; Gln (Q) glu, asn; Glu (E) gln, asp; Gly (G) ala; His (H) asn, gln, lys, arg; Ile (I) val, met, ala, phe, leu; Leu (L) val, met, ala, phe, ile; Lys (K) gln, asn, arg; Met (M) phe, ile, leu; Phe (F) leu, val, ile, ala, tyr; Pro (P) ala; Ser (S), thr; Thr (T) ser; Trp (W) phe, tyr; Tyr (Y) trp, phe, thr, ser; Val (V) ile, leu, met, phe, ala.

[070] Other considerations for amino acid substitutions include whether or not the residue is located in the interior of a protein or is solvent exposed. For interior residues, conservative substitutions would include: Asp and Asn; Ser and Thr; Ser and Ala; Thr and Ala; Ala and Gly; Ile and Val; Val and Leu; Leu and Ile; Leu and Met; Phe and Tyr; Tyr and Trp. (See, e.g., PROWL website at rockefeller.edu) For solvent exposed residues, conservative substitutions would include: Asp and Asn; Asp and Glu; Glu and Gln; Glu and Ala; Gly and Asn; Ala and Pro; Ala and Gly; Ala and Ser; Ala and Lys; Ser and Thr; Lys and Arg; Val and Leu; Leu and Ile; Ile and Val; Phe and Tyr. (Id.) Various matrices have been constructed to assist in selection of amino acid substitutions, such as the PAM250 scoring matrix, Dayhoff matrix, Grantham matrix, McLachlan matrix, Doolittle matrix, Henikoff matrix, Miyata matrix, Fitch matrix, Jones matrix, Rao matrix, Levin matrix and Risler matrix (*Idem.*)

[071] In determining amino acid substitutions, one may also consider the existence of intermolecular or intramolecular bonds, such as formation of ionic bonds (salt bridges) between positively charged residues (e.g., His, Arg, Lys) and negatively charged residues (e.g., Asp, Glu) or disulfide bonds between nearby cysteine residues.

[072] Methods of substituting any amino acid for any other amino acid in an encoded protein sequence are well known and a matter of routine experimentation for the skilled artisan, for example by the technique of site-directed mutagenesis or by synthesis and assembly of oligonucleotides encoding an amino acid substitution and splicing into an expression vector construct.

Cytokines and Other Immunomodulators

[073] In certain preferred embodiments, an effector moiety may be an immunomodulator. An immunomodulator is an agent that when present, alters, suppresses or stimulates the body's immune system. Immunomodulators of use may include a cytokine, a stem cell growth factor, a lymphotoxin, a hematopoietic factor, a colony stimulating factor (CSF), an interferon (IFN),

erythropoietin, thrombopoietin and a combination thereof. Specifically useful are lymphotoxins such as tumor necrosis factor (TNF), hematopoietic factors, such as interleukin (IL), colony stimulating factor, such as granulocyte-colony stimulating factor (G-CSF) or granulocyte macrophage-colony stimulating factor (GM-CSF), interferon, such as interferons-α, -β or -γ, and stem cell growth factor, such as that designated "S1 factor".

[074] In more preferred embodiments, the effector moieties are cytokines, such as lymphokines, monokines, growth factors and traditional polypeptide hormones. Included among the cytokines are growth hormones such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); placenta growth factor (PlGF), hepatic growth factor; prostaglandin, fibroblast growth factor; prolactin; placental lactogen, OB protein; tumor necrosis factor-α and - ß; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-ß; platelet-growth factor; transforming growth factors (TGFs) such as TGF- α and TGF-ß; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α, -β, and -γ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); interleukins (ILs) such as IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12; IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-21, IL-25, LIF, kit-ligand or FLT-3, angiostatin, thrombospondin, endostatin, tumor necrosis factor (TNF, such as TNF-α) and LT.

[075] The amino acid sequences of protein or peptide immunomodulators, such as cytokines, are well known in the art and any such known sequences may be used in the practice of the instant invention. The skilled artisan is aware of numerous sources of public information on cytokine sequence. For example, the NCBI database contains both protein and encoding nucleic acid sequences for a large number of cytokines and immunomodulators, such as erythropoietin (GenBank NM 000799), IL-1 beta (GenPept AAH08678), GM-CSF (GenPept AAA52578), TNF-α (GenPept CAA26669), interferon-alpha (GenPept AAA52716.1), interferon-alpha 2b (GenPept AAP20099.1) and virtually any of the peptide or protein immunomodulators listed above. It is a matter of routine for the skilled artisan to identify an appropriate amino acid and/or nucleic acid sequence for essentially any protein or peptide effector moiety of interest.

Antibodies and Antibody Fragments

[076] Techniques for preparing monoclonal antibodies against virtually any target antigen are well known in the art. *See*, for example, Kohler and Milstein, *Nature 256*: 495 (1975), and Coligan *et al.* (eds.), CURRENT PROTOCOLS IN IMMUNOLOGY, VOL. 1, pages 2.5.1-2.6.7 (John Wiley & Sons 1991). Briefly, monoclonal antibodies can be obtained by injecting mice with a composition comprising an antigen, removing the spleen to obtain B-lymphocytes, fusing the B-lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones which produce antibodies to the antigen, culturing the clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures.

[077] MAbs can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography. See, for example, Coligan at pages 2.7.1-2.7.12 and pages 2.9.1-2.9.3. Also, see Baines *et al.*, "Purification of Immunoglobulin G (IgG)," in METHODS IN MOLECULAR BIOLOGY, VOL. 10, pages 79-104 (The Humana Press, Inc. 1992).

[078] After the initial raising of antibodies to the immunogen, the antibodies can be sequenced and subsequently prepared by recombinant techniques. Humanization and chimerization of murine antibodies and antibody fragments are well known to those skilled in the art. The use of antibody components derived from humanized, chimeric or human antibodies obviates potential problems associated with the immunogenicity of murine constant regions.

Chimeric Antibodies

[079] A chimeric antibody is a recombinant protein in which the variable regions of a human antibody have been replaced by the variable regions of, for example, a mouse antibody, including the complementarity-determining regions (CDRs) of the mouse antibody. Chimeric antibodies exhibit decreased immunogenicity and increased stability when administered to a subject. General techniques for cloning murine immunoglobulin variable domains are disclosed, for example, in Orlandi *et al., Proc. Nat'l Acad. Sci. USA 86*: 3833 (1989). Techniques for constructing chimeric antibodies are well known to those of skill in the art. As an example, Leung *et al., Hybridoma 13:*469 (1994), produced an LL2 chimera by combining DNA sequences encoding the V_κ and V_H domains of murine LL2, an anti-CD22 monoclonal antibody, with respective human κ and IgG_1 constant region domains.

Humanized Antibodies

[080] Techniques for producing humanized MAbs are well known in the art (see, e.g., Jones *et al., Nature 321*: 522 (1986), Riechmann *et al., Nature 332*: 323 (1988), Verhoeyen *et al., Science 239*: 1534 (1988), Carter *et al., Proc. Nat'l Acad. Sci. USA 89*: 4285 (1992), Sandhu, *Crit. Rev. Biotech. 12*: 437 (1992), and Singer *et al., J. Immun. 150*: 2844 (1993)). A chimeric or murine monoclonal antibody may be humanized by transferring the mouse CDRs from the heavy and light variable chains of the mouse immunoglobulin into the corresponding variable domains of a human antibody. The mouse framework regions (FR) in the chimeric monoclonal antibody are also replaced with human FR sequences. As simply transferring mouse CDRs into human FRs often results in a reduction or even loss of antibody affinity, additional modification might be required in order to restore the original affinity of the murine antibody. This can be accomplished by the replacement of one or more human residues in the FR regions with their murine counterparts to obtain an antibody that possesses good binding affinity to its epitope. See, for example, Tempest *et al.*, *Biotechnology* 9:266 (1991) and Verhoeyen *et al.*, *Science* 239: 1534 (1988). Generally, those human FR amino acid residues that differ from their murine counterparts and are located close to or touching one or more CDR amino acid residues would be candidates for substitution.

Human Antibodies

[081] Methods for producing fully human antibodies using either combinatorial approaches or transgenic animals transformed with human immunoglobulin loci are known in the art (*e.g.,* Mancini et al., 2004, *New Microbiol.* 27:315-28; Conrad and Scheller, 2005, *Comb. Chem. High Throughput Screen.* 8:117-26; Brekke and Loset, 2003, *Curr. Opin. Phamacol.* 3:544-50). A fully human antibody also can be constructed by genetic or chromosomal transfection methods, as well as phage display technology, all of which are known in the art. See for example, McCafferty *et al., Nature* 348:552-553 (1990). Such fully human antibodies are expected to exhibit even fewer side effects than chimeric or humanized antibodies and to function *in vivo* as essentially endogenous human antibodies. In certain embodiments, the claimed methods and procedures may utilize human antibodies produced by such techniques.

[082] In one alternative, the phage display technique may be used to generate human antibodies (*e.g.,* Dantas-Barbosa et al., 2005, *Genet. Mol. Res.* 4:126-40). Human antibodies may be generated from normal humans or from humans that exhibit a particular disease state, such as cancer (Dantas-Barbosa et al., 2005). The advantage to constructing human

antibodies from a diseased individual is that the circulating antibody repertoire may be biased towards antibodies against disease-associated antigens.

[083] In one non-limiting example of this methodology, Dantas-Barbosa et al. (2005) constructed a phage display library of human Fab antibody fragments from osteosarcoma patients. Generally, total RNA was obtained from circulating blood lymphocytes (*Id.*). Recombinant Fab were cloned from the μ, γ and κ chain antibody repertoires and inserted into a phage display library (*Id.*). RNAs were converted to cDNAs and used to make Fab cDNA libraries using specific primers against the heavy and light chain immunoglobulin sequences (Marks et al., 1991, *J. Mol. Biol.* 222:581-97). Library construction was performed according to Andris-Widhopf et al. (2000, In: *Phage Display Laboratory Manual*, Barbas et al. (eds), 1st edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY pp. 9.1 to 9.22). The final Fab fragments were digested with restriction endonucleases and inserted into the bacteriophage genome to make the phage display library. Such libraries may be screened by standard phage display methods, as known in the art (see, e.g., Pasqualini and Ruoslahti, 1996, Nature 380:364-366; Pasqualini, 1999, The Quart. J. Nucl. Med. 43:159-162).

[084] Phage display can be performed in a variety of formats, for their review, see e.g. Johnson and Chiswell, *Current Opinion in Structural Biology* 3:5564-571 (1993). Human antibodies may also be generated by *in vitro* activated B cells. See U.S. Patent Nos. 5,567,610 and 5,229,275, incorporated herein by reference in their entirety. The skilled artisan will realize that these techniques are exemplary and any known method for making and screening human antibodies or antibody fragments may be utilized.

[085] In another alternative, transgenic animals that have been genetically engineered to produce human antibodies may be used to generate antibodies against essentially any immunogenic target, using standard immunization protocols. Methods for obtaining human antibodies from transgenic mice are disclosed by Green *et al., Nature Genet. 7*:13 (1994), Lonberg *et al., Nature 368*:856 (1994), and Taylor *et al., Int. Immun. 6*:579 (1994). A non-limiting example of such a system is the XenoMouse® (*e.g.*, Green et al., 1999, *J. Immunol. Methods* 231:11-23) from Abgenix (Fremont, CA). In the XenoMouse® and similar animals, the mouse antibody genes have been inactivated and replaced by functional human antibody genes, while the remainder of the mouse immune system remains intact.

[086] The XenoMouse® was transformed with germline-configured YACs (yeast artificial chromosomes) that contained portions of the human IgH and Igkappa loci, including the

majority of the variable region sequences, along accessory genes and regulatory sequences. The human variable region repertoire may be used to generate antibody producing B cells, which may be processed into hybridomas by known techniques. A XenoMouse® immunized with a target antigen will produce human antibodies by the normal immune response, which may be harvested and/or produced by standard techniques discussed above. A variety of strains of XenoMouse® are available, each of which is capable of producing a different class of antibody. Transgenically produced human antibodies have been shown to have therapeutic potential, while retaining the pharmacokinetic properties of normal human antibodies (Green et al., 1999). The skilled artisan will realize that the claimed compositions and methods are not limited to use of the XenoMouse® system but may utilize any transgenic animal that has been genetically engineered to produce human antibodies.

Antibody Fragments

[087] Antibody fragments which recognize specific epitopes can be generated by known techniques. Antibody fragments are antigen binding portions of an antibody, such as $F(ab')_2$, Fab', $F(ab)_2$, Fab, Fv, sFv and the like. $F(ab')_2$ fragments can be produced by pepsin digestion of the antibody molecule and Fab′ fragments can be generated by reducing disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab′ expression libraries can be constructed (Huse *et al.*, 1989, *Science*, 246:1274-1281) to allow rapid and easy identification of monoclonal Fab′ fragments with the desired specificity. $F(ab)_2$ fragments may be generated by papain digestion of an antibody and Fab fragments obtained by disulfide reduction.

[088] A single chain Fv molecule (scFv) comprises a VL domain and a VH domain. The VL and VH domains associate to form a target binding site. These two domains are further covalently linked by a peptide linker (L). Methods for making scFv molecules and designing suitable peptide linkers are described in US Patent No. 4,704,692, US Patent No. 4,946,778, R. Raag and M. Whitlow, "*Single Chain Fvs.*" FASEB Vol 9:73-80 (1995) and R.E. Bird and B.W. Walker, "*Single Chain Antibody Variable Regions*," TIBTECH, Vol 9: 132-137 (1991).

[089] Techniques for producing single domain antibodies (DABs) are also known in the art, as disclosed for example in Cossins et al. (2006, Prot Express Purif 51:253-259), incorporated herein by reference.

[090] An antibody fragment can be prepared by proteolytic hydrolysis of the full length antibody or by expression in *E. coli* or another host of the DNA coding for the fragment. An antibody fragment can be obtained by pepsin or papain digestion of full length antibodies by conventional methods. These methods are described, for example, by Goldenberg, U.S. Patent Nos. 4,036,945 and 4,331,647 and references contained therein. Also, see Nisonoff *et*

al., Arch Biochem. Biophys. 89: 230 (1960); Porter, *Biochem. J. 73*: 119 (1959), Edelman *et al.,* in METHODS IN ENZYMOLOGY VOL. 1, page 422 (Academic Press 1967), and Coligan at pages 2.8.1-2.8.10 and 2.10.-2.10.4.

Known Antibodies

[091] Antibodies of use may be commercially obtained from a wide variety of known sources. For example, a variety of antibody secreting hybridoma lines are available from the American Type Culture Collection (ATCC, Manassas, VA). A large number of antibodies against various disease targets, including but not limited to tumor-associated antigens, have been deposited at the ATCC and/or have published variable region sequences and are available for use in the claimed methods and compositions. See, e.g., U.S. Patent Nos. 7,312,318; 7,282,567; 7,151,164; 7,074,403; 7,060,802; 7,056,509; 7,049,060; 7,045,132; 7,041,803; 7,041,802; 7,041,293; 7,038,018; 7,037,498; 7,012,133; 7,001,598; 6,998,468; 6,994,976; 6,994,852; 6,989,241; 6,974,863; 6,965,018; 6,964,854; 6,962,981; 6,962,813; 6,956,107; 6,951,924; 6,949,244; 6,946,129; 6,943,020; 6,939,547; 6,921,645; 6,921,645; 6,921,533; 6,919,433; 6,919,078; 6,916,475; 6,905,681; 6,899,879; 6,893,625; 6,887,468; 6,887,466; 6,884,594; 6,881,405; 6,878,812; 6,875,580; 6,872,568; 6,867,006; 6,864,062; 6,861,511; 6,861,227; 6,861,226; 6,838,282; 6,835,549; 6,835,370; 6,824,780; 6,824,778; 6,812,206; 6,793,924; 6,783,758; 6,770,450; 6,767,711; 6,764,688; 6,764,681; 6,764,679; 6,743,898; 6,733,981; 6,730,307; 6,720,15; 6,716,966; 6,709,653; 6,693,176; 6,692,908; 6,689,607; 6,689,362; 6,689,355; 6,682,737; 6,682,736; 6,682,734; 6,673,344; 6,653,104; 6,652,852; 6,635,482; 6,630,144; 6,610,833; 6,610,294; 6,605,441; 6,605,279; 6,596,852; 6,592,868; 6,576,745; 6,572;856; 6,566,076; 6,562,618; 6,545,130; 6,544,749; 6,534,058; 6,528,625; 6,528,269; 6,521,227; 6,518,404; 6,511,665; 6,491,915; 6,488,930; 6,482,598; 6,482,408; 6,479,247; 6,468,531; 6,468,529; 6,465,173; 6,461,823; 6,458,356; 6,455,044; 6,455,040, 6,451,310; 6,444,206' 6,441,143; 6,432,404; 6,432,402; 6,419,928; 6,413,726; 6,406,694; 6,403,770; 6,403,091; 6,395,276; 6,395,274; 6,387,350; 6,383,759; 6,383,484; 6,376,654; 6,372,215; 6,359,126; 6,355,481; 6,355,444; 6,355,245; 6,355,244; 6,346,246; 6,344,198; 6,340,571; 6,340,459; 6,331,175; 6,306,393; 6,254,868; 6,187,287; 6,183,744; 6,129,914; 6,120,767; 6,096,289; 6,077,499; 5,922,302; 5,874,540; 5,814,440; 5,798,229; 5,789,554; 5,776,456; 5,736,119; 5,716,595; 5,677,136; 5,587,459; 5,443,953, 5,525,338. These are exemplary only and a wide variety of other antibodies and their hybridomas are known in the art. The skilled artisan will realize that antibody sequences or antibody-secreting hybridomas against almost any disease-associated antigen may be obtained by a

simple search of the ATCC, NCBI and/or USPTO databases for antibodies against a selected disease-associated target of interest. The antigen binding domains of the cloned antibodies may be amplified, excised, ligated into an expression vector, transfected into an adapted host cell and used for protein production, using standard techniques well known in the art.

Immunoconjugates

[092] In certain embodiments, the antibodies or fragments thereof may be conjugated to one or more therapeutic or diagnostic agents. The therapeutic agents do not need to be the same but can be different, e.g. a drug and a radioisotope. For example, ^{131}I can be incorporated into a tyrosine of an antibody or fusion protein and a drug attached to an epsilon amino group of a lysine residue. Therapeutic and diagnostic agents also can be attached, for example to reduced SH groups and/or to carbohydrate side chains. Many methods for making covalent or non-covalent conjugates of therapeutic or diagnostic agents with antibodies or fusion proteins are known in the art and any such known method may be utilized.

[093] A therapeutic or diagnostic agent can be attached at the hinge region of a reduced antibody component via disulfide bond formation. Alternatively, such agents can be attached using a heterobifunctional cross-linker, such as *N*-succinyl 3-(2-pyridyldithio)propionate (SPDP). Yu *et al., Int. J. Cancer 56*: 244 (1994). General techniques for such conjugation are well-known in the art. See, for example, Wong, CHEMISTRY OF PROTEIN CONJUGATION AND CROSS-LINKING (CRC Press 1991); Upeslacis *et al.*, "Modification of Antibodies by Chemical Methods," in MONOCLONAL ANTIBODIES: PRINCIPLES AND APPLICATIONS, Birch *et al.* (eds.), pages 187-230 (Wiley-Liss, Inc. 1995); Price, "Production and Characterization of Synthetic Peptide-Derived Antibodies," in MONOCLONAL ANTIBODIES: PRODUCTION, ENGINEERING AND CLINICAL APPLICATION, Ritter *et al.* (eds.), pages 60-84 (Cambridge University Press 1995). Alternatively, the therapeutic or diagnostic agent can be conjugated via a carbohydrate moiety in the Fc region of the antibody. The carbohydrate group can be used to increase the loading of the same agent that is bound to a thiol group, or the carbohydrate moiety can be used to bind a different therapeutic or diagnostic agent.

[094] Methods for conjugating peptides to antibody components via an antibody carbohydrate moiety are well-known to those of skill in the art. See, for example, Shih *et al., Int. J. Cancer 41*: 832 (1988); Shih *et al., Int. J. Cancer 46*: 1101 (1990); and Shih *et al.*, U.S. Patent No. 5,057,313, incorporated herein in their entirety by reference. The general method involves reacting an antibody component having an oxidized carbohydrate portion with a

carrier polymer that has at least one free amine function. This reaction results in an initial Schiff base (imine) linkage, which can be stabilized by reduction to a secondary amine to form the final conjugate.

[095] The Fc region may be absent if the antibody used as the antibody component of the immunoconjugate is an antibody fragment. However, it is possible to introduce a carbohydrate moiety into the light chain variable region of a full length antibody or antibody fragment. See, for example, Leung *et al., J. Immunol. 154*: 5919 (1995); Hansen *et al.*, U.S. Patent No. 5,443,953 (1995), Leung *et al.*, U.S. patent No. 6,254,868, incorporated herein by reference in their entirety. The engineered carbohydrate moiety is used to attach the therapeutic or diagnostic agent.

[096] In some embodiments, a chelating agent may be attached to an antibody, antibody fragment or fusion protein and used to chelate a therapeutic or diagnostic agent, such as a radionuclide. Exemplary chelators include but are not limited to DTPA (such as Mx-DTPA), DOTA, TETA, NETA or NOTA. Methods of conjugation and use of chelating agents to attach metals or other ligands to proteins are well known in the art (see, e.g., U.S. Patent Application Serial No. 12/112,289, incorporated herein by reference in its entirety).

[097] In certain embodiments, radioactive metals or paramagnetic ions may be attached to proteins or peptides by reaction with a reagent having a long tail, to which may be attached a multiplicity of chelating groups for binding ions. Such a tail can be a polymer such as a polylysine, polysaccharide, or other derivatized or derivatizable chains having pendant groups to which can be bound chelating groups such as, *e.g.*, ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), porphyrins, polyamines, crown ethers, bis-thiosemicarbazones, polyoximes, and like groups known to be useful for this purpose.

[098] Chelates may be directly linked to antibodies or peptides, for example as disclosed in U.S. Patent 4,824,659, incorporated herein in its entirety by reference. Particularly useful metal-chelate combinations include 2-benzyl-DTPA and its monomethyl and cyclohexyl analogs, used with diagnostic isotopes in the general energy range of 60 to 4,000 keV, such as ^{125}I, ^{131}I, ^{123}I, ^{124}I, ^{62}Cu, ^{64}Cu, ^{18}F, ^{111}In, ^{67}Ga, ^{68}Ga, ^{99m}Tc, ^{94m}Tc, ^{11}C, ^{13}N, ^{15}O, ^{76}Br, for radioimaging. The same chelates, when complexed with non-radioactive metals, such as manganese, iron and gadolinium are useful for MRI. Macrocyclic chelates such as NOTA, DOTA, and TETA are of use with a variety of metals and radiometals, most particularly with radionuclides of gallium, yttrium and copper, respectively. Such metal-chelate complexes can be made very stable by tailoring the ring size to the metal of interest. Other ring-type

chelates such as macrocyclic polyethers, which are of interest for stably binding nuclides, such as ^{223}Ra for RAIT are encompassed.

[099] More recently, methods of ^{18}F-labeling of use in PET scanning techniques have been disclosed, for example by reaction of F-18 with a metal or other atom, such as aluminum. The ^{18}F-Al conjugate may be complexed with chelating groups, such as DOTA, NOTA or NETA that are attached directly to antibodies or used to label targetable constructs in pre-targeting methods. Such F-18 labeling techniques are disclosed in U.S. Patent No. 7,563,433, the Examples section of which is incorporated herein by reference.

Therapeutic Agents

[0100] In alternative embodiments, therapeutic agents such as cytotoxic agents, anti-angiogenic agents, pro-apoptotic agents, antibiotics, hormones, hormone antagonists, chemokines, drugs, prodrugs, toxins, enzymes or other agents may be used, either conjugated to the subject DNL complexes or separately administered before, simultaneously with, or after the antibody. Drugs of use may possess a pharmaceutical property selected from the group consisting of antimitotic, antikinase, alkylating, antimetabolite, antibiotic, alkaloid, anti-angiogenic, pro-apoptotic agents and combinations thereof.

[0101] Exemplary drugs of use may include 5-fluorouracil, aplidin, azaribine, anastrozole, anthracyclines, bendamustine, bleomycin, bortezomib, bryostatin-1, busulfan, calicheamycin, camptothecin, carboplatin, 10-hydroxycamptothecin, carmustine, celebrex, chlorambucil, cisplatin (CDDP), Cox-2 inhibitors, irinotecan (CPT-11), SN-38, carboplatin, cladribine, camptothecans, cyclophosphamide, cytarabine, dacarbazine, docetaxel, dactinomycin, daunorubicin, doxorubicin, 2-pyrrolinodoxorubicine (2P-DOX), cyano-morpholino doxorubicin, doxorubicin glucuronide, epirubicin glucuronide, estramustine, epipodophyllotoxin, estrogen receptor binding agents, etoposide (VP16), etoposide glucuronide, etoposide phosphate, floxuridine (FUdR), 3',5'-O-dioleoyl-FudR (FUdR-dO), fludarabine, flutamide, farnesyl-protein transferase inhibitors, gemcitabine, hydroxyurea, idarubicin, ifosfamide, L-asparaginase, lenolidamide, leucovorin, lomustine, mechlorethamine, melphalan, mercaptopurine, 6-mercaptopurine, methotrexate, mitoxantrone, mithramycin, mitomycin, mitotane, navelbine, nitrosurea, plicomycin, procarbazine, paclitaxel, pentostatin, PSI-341, raloxifene, semustine, streptozocin, tamoxifen, taxol, temazolomide (an aqueous form of DTIC), transplatinum, thalidomide, thioguanine, thiotepa, teniposide, topotecan, uracil mustard, vinorelbine, vinblastine, vincristine and vinca alkaloids.

[0102] Toxins of use may include ricin, abrin, alpha toxin, saporin, ribonuclease (RNase), e.g., onconase, DNase I, *Staphylococcal* enterotoxin-A, pokeweed antiviral protein, gelonin, diphtheria toxin, *Pseudomonas* exotoxin, and *Pseudomonas* endotoxin.

[0103] Chemokines of use may include RANTES, MCAF, MIP1-alpha, MIP1-Beta and IP-10.

[0104] In certain embodiments, anti-angiogenic agents, such as angiostatin, baculostatin, canstatin, maspin, anti-VEGF antibodies, anti-PlGF peptides and antibodies, anti-vascular growth factor antibodies, anti-Flk-1 antibodies, anti-Flt-1 antibodies and peptides, anti-Kras antibodies, anti-cMET antibodies, anti-MIF (macrophage migration-inhibitory factor) antibodies, laminin peptides, fibronectin peptides, plasminogen activator inhibitors, tissue metalloproteinase inhibitors, interferons, interleukin-12, IP-10, Gro-ß, thrombospondin, 2-methoxyoestradiol, proliferin-related protein, carboxiamidotriazole, CM101, Marimastat, pentosan polysulphate, angiopoietin-2, interferon-alpha, herbimycin A, PNU145156E, 16K prolactin fragment, Linomide (roquinimex), thalidomide, pentoxifylline, genistein, TNP-470, endostatin, paclitaxel, accutin, angiostatin, cidofovir, vincristine, bleomycin, AGM-1470, platelet factor 4 or minocycline may be of use.

[0105] Immunomodulators of use may be selected from a cytokine, a stem cell growth factor, a lymphotoxin, a hematopoietic factor, a colony stimulating factor (CSF), an interferon (IFN), erythropoietin, thrombopoietin and a combination thereof. Specifically useful are lymphotoxins such as tumor necrosis factor (TNF), hematopoietic factors, such as interleukin (IL), colony stimulating factor, such as granulocyte-colony stimulating factor (G-CSF) or granulocyte macrophage-colony stimulating factor (GM-CSF), interferon, such as interferons-α, -β or -γ, and stem cell growth factor, such as that designated "S1 factor". Included among the cytokines are growth hormones such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; prostaglandin, fibroblast growth factor; prolactin; placental lactogen, OB protein; tumor necrosis factor-α and - ß; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-ß; platelet-growth factor; transforming growth factors (TGFs) such as TGF- α and TGF- ß; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α, -β, and

-γ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); interleukins (ILs) such as IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12; IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-21, IL-25, LIF, kit-ligand or FLT-3, angiostatin, thrombospondin, endostatin, tumor necrosis factor and LT.

[0106] Radionuclides of use include, but are not limited to- ^{111}In, ^{177}Lu, ^{212}Bi, ^{213}Bi, ^{211}At, ^{62}Cu, ^{67}Cu, ^{90}Y, ^{125}I, ^{131}I, ^{32}P, ^{33}P, ^{47}Sc, ^{111}Ag, ^{67}Ga, ^{142}Pr, ^{153}Sm, ^{161}Tb, ^{166}Dy, ^{166}Ho, ^{186}Re, ^{188}Re, ^{189}Re, ^{212}Pb, ^{223}Ra, ^{225}Ac, ^{59}Fe, ^{75}Se, ^{77}As, ^{89}Sr, ^{99}Mo, ^{105}Rh, ^{109}Pd, ^{143}Pr, ^{149}Pm, ^{169}Er, ^{194}Ir, ^{198}Au, ^{199}Au, and ^{211}Pb. The therapeutic radionuclide preferably has a decay-energy in the range of 20 to 6,000 keV, preferably in the ranges 60 to 200 keV for an Auger emitter, 100-2,500 keV for a beta emitter, and 4,000-6,000 keV for an alpha emitter. Maximum decay energies of useful beta-particle-emitting nuclides are preferably 20-5,000 keV, more preferably 100-4,000 keV, and most preferably 500-2,500 keV. Also preferred are radionuclides that substantially decay with Auger-emitting particles. For example, Co-58, Ga-67, Br-80m, Tc-99m, Rh-103m, Pt-109, In-111, Sb-119, 1-125, Ho-161, Os-189m and Ir-192. Decay energies of useful beta-particle-emitting nuclides are preferably <1,000 keV, more preferably <100 keV, and most preferably <70 keV. Also preferred are radionuclides that substantially decay with generation of alpha-particles. Such radionuclides include, but are not limited to: Dy-152, At-211, Bi-212, Ra-223, Rn-219, Po-215, Bi-211, Ac-225, Fr-221, At-217, Bi-213 and Fm-255. Decay energies of useful alpha-particle-emitting radionuclides are preferably 2,000-10,000 keV, more preferably 3,000-8,000 keV, and most preferably 4,000-7,000 keV. Additional potential radioisotopes of use include ^{11}C, ^{13}N, ^{15}O, ^{75}Br, ^{198}Au, ^{224}Ac, ^{126}I, ^{133}I, ^{77}Br, ^{113m}In, ^{95}Ru, ^{97}Ru, ^{103}Ru, ^{105}Ru, ^{107}Hg, ^{203}Hg, ^{121m}Te, ^{122m}Te, ^{125m}Te, ^{165}Tm, ^{167}Tm, ^{168}Tm, ^{197}Pt, ^{109}Pd, ^{105}Rh, ^{142}Pr, ^{143}Pr, ^{161}Tb, ^{166}Ho, ^{199}Au, ^{57}Co, ^{58}Co, ^{51}Cr, ^{59}Fe, ^{75}Se, ^{201}Tl, ^{225}Ac, ^{76}Br, ^{169}Yb, and the like. Some useful diagnostic nuclides may include ^{18}F, ^{52}Fe, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{86}Y, ^{89}Zr, ^{94}Tc, ^{94m}Tc, ^{99m}Tc, or ^{111}In.

[0107] Therapeutic agents may include a photoactive agent or dye. Fluorescent compositions, such as fluorochrome, and other chromogens, or dyes, such as porphyrins sensitive to visible light, have been used to detect and to treat lesions by directing the suitable light to the lesion. In therapy, this has been termed photoradiation, phototherapy, or photodynamic therapy. See Jori et al. (eds.), PHOTODYNAMIC THERAPY OF TUMORS AND OTHER DISEASES (Libreria Progetto 1985); van den Bergh, Chem. Britain (1986), 22:430. Moreover, monoclonal antibodies have been coupled with photoactivated dyes for

achieving phototherapy. See Mew et al., J. Immunol. (1983),130:1473; idem., Cancer Res. (1985), 45:4380; Oseroff et al., Proc. Natl. Acad. Sci. USA (1986), 83:8744; idem., Photochem. Photobiol. (1987), 46:83; Hasan et al., Prog. Clin. Biol. Res. (1989), 288:471; Tatsuta et al., Lasers Surg. Med. (1989), 9:422; Pelegrin et al., Cancer (1991), 67:2529.

[0108] Other useful therapeutic agents may comprise oligonucleotides, especially antisense oligonucleotides that preferably are directed against oncogenes and oncogene products, such as bcl-2 or p53. A preferred form of therapeutic oligonucleotide is siRNA.

Diagnostic Agents

[0109] Diagnostic agents are preferably selected from the group consisting of a radionuclide, a radiological contrast agent, a paramagnetic ion, a metal, a fluorescent label, a chemiluminescent label, an ultrasound contrast agent and a photoactive agent. Such diagnostic agents are well known and any such known diagnostic agent may be used. Non-limiting examples of diagnostic agents may include a radionuclide such as ^{110}In, ^{111}In, ^{177}Lu, ^{18}F, ^{52}Fe, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{86}Y, ^{90}Y, ^{89}Zr, ^{94m}Tc, ^{94}Tc, ^{99m}Tc, ^{120}I, ^{123}I, ^{124}I, ^{125}I, ^{131}I, $^{154\text{-}158}$Gd, ^{32}P, ^{11}C, ^{13}N, ^{15}O, ^{186}Re, ^{188}Re, ^{51}Mn, ^{52m}Mn, ^{55}Co, ^{72}As, ^{75}Br, ^{76}Br, ^{82m}Rb, ^{83}Sr, or other gamma-, beta-, or positron-emitters. Paramagnetic ions of use may include chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) or erbium (III). Metal contrast agents may include lanthanum (III), gold (III), lead (II) or bismuth (III). Ultrasound contrast agents may comprise liposomes, such as gas filled liposomes. Radiopaque diagnostic agents may be selected from compounds, barium compounds, gallium compounds, and thallium compounds. A wide variety of fluorescent labels are known in the art, including but not limited to fluorescein isothiocyanate, rhodamine, phycoerytherin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine. Chemiluminescent labels of use may include luminol, isoluminol, an aromatic acridinium ester, an imidazole, an acridinium salt or an oxalate ester.

Methods of Therapeutic Treatment

[0110] Various embodiments concern methods of treating a cancer in a subject, such as a mammal, including humans, domestic or companion pets, such as dogs and cats, comprising administering to the subject a therapeutically effective amount of a bispecific immunocytokine DNL construct.

[0111] In one embodiment, immunological diseases which may be treated with the subject

DNL constructs may include, for example, joint diseases such as ankylosing spondylitis, juvenile rheumatoid arthritis, rheumatoid arthritis; neurological disease such as multiple sclerosis and myasthenia gravis; pancreatic disease such as diabetes, especially juvenile onset diabetes; gastrointestinal tract disease such as chronic active hepatitis, celiac disease, ulcerative colitis, Crohn's disease, pernicious anemia; skin diseases such as psoriasis or scleroderma; allergic diseases such as asthma and in transplantation related conditions such as graft versus host disease and allograft rejection.

[0112] The administration of the bispecific immunocytokine DNL constructs can be supplemented by administering concurrently or sequentially a therapeutically effective amount of another antibody that binds to or is reactive with another antigen on the surface of the target cell. Preferred additional MAbs comprise at least one humanized, chimeric or human MAb selected from the group consisting of a MAb reactive with CD4, CD5, CD8, CD14, CD15, CD16, CD19, IGF-1R, CD20, CD21, CD22, CD23, CD25, CD30, CD32b, CD33, CD37, CD38, CD40, CD40L, CD45, CD46, CD52, CD54, CD70, CD74, CD79a, CD80, CD95, CD126, CD133, CD138, CD154, CEACAM5, CEACAM6, B7, AFP, PSMA, EGP-1, EGP-2, carbonic anhydrase IX, PAM4 antigen, MUC1, MUC2, MUC3, MUC4, MUC5, Ia, MIF, HM1.24, HLA-DR, tenascin, Flt-3, VEGFR, PlGF, ILGF, IL-6, IL-25, tenascin, TRAIL-R1, TRAIL-R2, complement factor C5, oncogene product, or a combination thereof. Various antibodies of use, such as anti-CD19, anti-CD20, and anti-CD22 antibodies, are known to those of skill in the art. See, for example, Ghetie *et al.*, *Cancer Res. 48*:2610 (1988); Hekman *et al.*, *Cancer Immunol. Immunother. 32*:364 (1991); Longo, *Curr. Opin. Oncol. 8*:353 (1996), U.S. Patent Nos. 5,798,554; 6,187,287; 6,306,393; 6,676,924; 7,109,304; 7,151,164; 7,230,084; 7,230,085; 7,238,785; 7,238,786; 7,282,567; 7,300,655; 7,312,318; and U.S. Patent Application Publ. Nos. 20080131363; 20080089838; 20070172920; 20060193865; 20060210475; 20080138333; and 20080146784, the Examples section of each incorporated herein by reference.

[0113] The DNL construct therapy can be further supplemented with the administration, either concurrently or sequentially, of at least one therapeutic agent. For example, "CVB" (1.5 g/m^2 cyclophosphamide, 200-400 mg/m^2 etoposide, and 150-200 mg/m^2 carmustine) is a regimen used to treat non-Hodgkin's lymphoma. Patti *et al.*, *Eur. J. Haematol. 51*: 18 (1993). Other suitable combination chemotherapeutic regimens are well-known to those of skill in the art. See, for example, Freedman *et al.*, "Non-Hodgkin's Lymphomas," in CANCER MEDICINE, VOLUME 2, 3rd Edition, Holland *et al.* (eds.), pages 2028-2068 (Lea & Febiger 1993). As

an illustration, first generation chemotherapeutic regimens for treatment of intermediate-grade non-Hodgkin's lymphoma (NHL) include C-MOPP (cyclophosphamide, vincristine, procarbazine and prednisone) and CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone). A useful second generation chemotherapeutic regimen is m-BACOD (methotrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine, dexamethasone and leucovorin), while a suitable third generation regimen is MACOP-B (methotrexate, doxorubicin, cyclophosphamide, vincristine, prednisone, bleomycin and leucovorin). Additional useful drugs include phenyl butyrate, bendamustine, and bryostatin-1.

[0114] The subject DNL constructs can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby The DNL construct is combined in a mixture with a pharmaceutically suitable excipient. Sterile phosphate-buffered saline is one example of a pharmaceutically suitable excipient. Other suitable excipients are well-known to those in the art. See, for example, Ansel *et al.*, PHARMACEUTICAL DOSAGE FORMS AND DRUG DELIVERY SYSTEMS, 5th Edition (Lea & Febiger 1990), and Gennaro (ed.), REMINGTON'S PHARMACEUTICAL SCIENCES, 18th Edition (Mack Publishing Company 1990), and revised editions thereof.

[0115] The subject DNL constructs can be formulated for intravenous administration via, for example, bolus injection or continuous infusion. Preferably, DNL construct is infused over a period of less than about 4 hours, and more preferably, over a period of less than about 3 hours. For example, the first 25-50 mg could be infused within 30 minutes, preferably even 15 min, and the remainder infused over the next 2-3 hrs. Formulations for injection can be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions can take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and can contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient can be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

[0116] Additional pharmaceutical methods may be employed to control the duration of action of the DNL constructs. Control release preparations can be prepared through the use of polymers to complex or adsorb the DNL constructs. For example, biocompatible polymers include matrices of poly(ethylene-co-vinyl acetate) and matrices of a polyanhydride copolymer of a stearic acid dimer and sebacic acid. Sherwood *et al.*, *Bio/Technology* 10: 1446 (1992). The rate of release from such a matrix depends upon the molecular weight of the DNL construct, the amount of DNL construct within the matrix, and the size of dispersed particles. Saltzman *et al.*, *Biophys. J.* 55: 163 (1989); Sherwood *et al.*, *supra*. Other solid

dosage forms are described in Ansel *et al.*, PHARMACEUTICAL DOSAGE FORMS AND DRUG DELIVERY SYSTEMS, 5th Edition (Lea & Febiger 1990), and Gennaro (ed.), REMINGTON'S PHARMACEUTICAL SCIENCES, 18th Edition (Mack Publishing Company 1990), and revised editions thereof.

[0117] The DNL construct may also be administered to a mammal subcutaneously or even by other parenteral routes. Moreover, the administration may be by continuous infusion or by single or multiple boluses. Preferably, the DNL construct is infused over a period of less than about 4 hours, and more preferably, over a period of less than about 3 hours.

[0118] More generally, the dosage of an administered DNL construct for humans will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition and previous medical history. It may be desirable to provide the recipient with a dosage of DNL construct that is in the range of from about 1 mg/kg to 25 mg/kg as a single intravenous infusion, although a lower or higher dosage also may be administered as circumstances dictate. A dosage of 1-20 mg/kg for a 70 kg patient, for example, is 70-1,400 mg, or 41-824 mg/m^2 for a 1.7-m patient. The dosage may be repeated as needed, for example, once per week for 4-10 weeks, once per week for 8 weeks, or once per week for 4 weeks. It may also be given less frequently, such as every other week for several months, or monthly or quarterly for many months, as needed in a maintenance therapy.

[0119] Alternatively, a DNL construct may be administered as one dosage every 2 or 3 weeks, repeated for a total of at least 3 dosages. Or, the construct may be administered twice per week for 4-6 weeks. If the dosage is lowered to approximately 200-300 mg/m^2 (340 mg per dosage for a 1.7-m patient, or 4.9 mg/kg for a 70 kg patient), it may be administered once or even twice weekly for 4 to 10 weeks. Alternatively, the dosage schedule may be decreased, namely every 2 or 3 weeks for 2-3 months. It has been determined, however, that even higher doses, such as 20 mg/kg once weekly or once every 2-3 weeks can be administered by slow i.v. infusion, for repeated dosing cycles. The dosing schedule can optionally be repeated at other intervals and dosage may be given through various parenteral routes, with appropriate adjustment of the dose and schedule.

[0120] In preferred embodiments, the DNL constructs are of use for therapy of cancer. Examples of cancers include, but are not limited to, carcinoma, lymphoma, glioblastoma, melanoma, sarcoma, and leukemia, myeloma, or lymphoid malignancies. More particular examples of such cancers are noted below and include: squamous cell cancer (e.g., epithelial squamous cell cancer), Ewing sarcoma, Wilms tumor, astrocytomas, lung cancer including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung and squamous

carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioblastoma multiforme, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, hepatocellular carcinoma, neuroendocrine tumors, medullary thyroid cancer, differentiated thyroid carcinoma, breast cancer, ovarian cancer, colon cancer, rectal cancer, endometrial cancer or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulvar cancer, anal carcinoma, penile carcinoma, as well as head-and-neck cancer. The term "cancer" includes primary malignant cells or tumors (e.g., those whose cells have not migrated to sites in the subject's body other than the site of the original malignancy or tumor) and secondary malignant cells or tumors (e.g., those arising from metastasis, the migration of malignant cells or tumor cells to secondary sites that are different from the site of the original tumor). Cancers conducive to treatment methods of the present invention involves cells which express, over-express, or abnormally express IGF-1R.

[0121] Other examples of cancers or malignancies include, but are not limited to: Acute Childhood Lymphoblastic Leukemia, Acute Lymphoblastic Leukemia, Acute Lymphocytic Leukemia, Acute Myeloid Leukemia, Adrenocortical Carcinoma, Adult (Primary) Hepatocellular Cancer, Adult (Primary) Liver Cancer, Adult Acute Lymphocytic Leukemia, Adult Acute Myeloid Leukemia, Adult Hodgkin's Lymphoma, Adult Lymphocytic Leukemia, Adult Non-Hodgkin's Lymphoma, Adult Primary Liver Cancer, Adult Soft Tissue Sarcoma, AIDS-Related Lymphoma, AIDS-Related Malignancies, Anal Cancer, Astrocytoma, Bile Duct Cancer, Bladder Cancer, Bone Cancer, Brain Stem Glioma, Brain Tumors, Breast Cancer, Cancer of the Renal Pelvis and Ureter, Central Nervous System (Primary) Lymphoma, Central Nervous System Lymphoma, Cerebellar Astrocytoma, Cerebral Astrocytoma, Cervical Cancer, Childhood (Primary) Hepatocellular Cancer, Childhood (Primary) Liver Cancer, Childhood Acute Lymphoblastic Leukemia, Childhood Acute Myeloid Leukemia, Childhood Brain Stem Glioma, Childhood Cerebellar Astrocytoma, Childhood Cerebral Astrocytoma, Childhood Extracranial Germ Cell Tumors, Childhood Hodgkin's Disease, Childhood Hodgkin's Lymphoma, Childhood Hypothalamic and Visual Pathway Glioma, Childhood Lymphoblastic Leukemia, Childhood Medulloblastoma, Childhood Non-Hodgkin's Lymphoma, Childhood Pineal and Supratentorial Primitive Neuroectodermal Tumors, Childhood Primary Liver Cancer, Childhood Rhabdomyosarcoma, Childhood Soft Tissue Sarcoma, Childhood Visual Pathway and Hypothalamic Glioma, Chronic Lymphocytic Leukemia, Chronic Myelogenous Leukemia, Colon Cancer, Cutaneous T-Cell Lymphoma, Endocrine Pancreas Islet Cell Carcinoma, Endometrial Cancer,

Ependymoma, Epithelial Cancer, Esophageal Cancer, Ewing's Sarcoma and Related Tumors, Exocrine Pancreatic Cancer, Extracranial Germ Cell Tumor, Extragonadal Germ Cell Tumor, Extrahepatic Bile Duct Cancer, Eye Cancer, Female Breast Cancer, Gaucher's Disease, Gallbladder Cancer, Gastric Cancer, Gastrointestinal Carcinoid Tumor, Gastrointestinal Tumors, Germ Cell Tumors, Gestational Trophoblastic Tumor, Hairy Cell Leukemia, Head and Neck Cancer, Hepatocellular Cancer, Hodgkin's Lymphoma, Hypergammaglobulinemia, Hypopharyngeal Cancer, Intestinal Cancers, Intraocular Melanoma, Islet Cell Carcinoma, Islet Cell Pancreatic Cancer, Kaposi's Sarcoma, Kidney Cancer, Laryngeal Cancer, Lip and Oral Cavity Cancer, Liver Cancer, Lung Cancer, Lymphoproliferative Disorders, Macroglobulinemia, Male Breast Cancer, Malignant Mesothelioma, Malignant Thymoma, Medulloblastoma, Melanoma, Mesothelioma, Metastatic Occult Primary Squamous Neck Cancer, Metastatic Primary Squamous Neck Cancer, Metastatic Squamous Neck Cancer, Multiple Myeloma, Multiple Myeloma/Plasma Cell Neoplasm, Myelodysplastic Syndrome, Myelogenous Leukemia, Myeloid Leukemia, Myeloproliferative Disorders, Nasal Cavity and Paranasal Sinus Cancer, Nasopharyngeal Cancer, Neuroblastoma, Non-Hodgkin's Lymphoma, Nonmelanoma Skin Cancer, Non-Small Cell Lung Cancer, Occult Primary Metastatic Squamous Neck Cancer, Oropharyngeal Cancer, Osteo-/Malignant Fibrous Sarcoma, Osteosarcoma/Malignant Fibrous Histiocytoma, Osteosarcoma/Malignant Fibrous Histiocytoma of Bone, Ovarian Epithelial Cancer, Ovarian Germ Cell Tumor, Ovarian Low Malignant Potential Tumor, Pancreatic Cancer, Paraproteinemias, Polycythemia vera, Parathyroid Cancer, Penile Cancer, Pheochromocytoma, Pituitary Tumor, Primary Central Nervous System Lymphoma, Primary Liver Cancer, Prostate Cancer, Rectal Cancer, Renal Cell Cancer, Renal Pelvis and Ureter Cancer, Retinoblastoma, Rhabdomyosarcoma, Salivary Gland Cancer, Sarcoidosis Sarcomas, Sezary Syndrome, Skin Cancer, Small Cell Lung Cancer, Small Intestine Cancer, Soft Tissue Sarcoma, Squamous Neck Cancer, Stomach Cancer, Supratentorial Primitive Neuroectodermal and Pineal Tumors, T-Cell Lymphoma, Testicular Cancer, Thymoma, Thyroid Cancer, Transitional Cell Cancer of the Renal Pelvis and Ureter, Transitional Renal Pelvis and Ureter Cancer, Trophoblastic Tumors, Ureter and Renal Pelvis Cell Cancer, Urethral Cancer, Uterine Cancer, Uterine Sarcoma, Vaginal Cancer, Visual Pathway and Hypothalamic Glioma, Vulvar Cancer, Waldenstrom's Macroglobulinemia, Wilms' Tumor, and any other hyperproliferative disease, besides neoplasia, located in an organ system listed above.

[0122] The methods and compositions described and claimed herein may be used to treat malignant or premalignant conditions and to prevent progression to a neoplastic or malignant

state, including but not limited to those disorders described above. Such uses are indicated in conditions known or suspected of preceding progression to neoplasia or cancer, in particular, where non-neoplastic cell growth consisting of hyperplasia, metaplasia, or most particularly, dysplasia has occurred (for review of such abnormal growth conditions, see Robbins and Angell, Basic Pathology, 2d Ed., W. B. Saunders Co., Philadelphia, pp. 68-79 (1976)).

[0123] Dysplasia is frequently a forerunner of cancer, and is found mainly in the epithelia. It is the most disorderly form of non-neoplastic cell growth, involving a loss in individual cell uniformity and in the architectural orientation of cells. Dysplasia characteristically occurs where there exists chronic irritation or inflammation. Dysplastic disorders which can be treated include, but are not limited to, anhidrotic ectodermal dysplasia, anterofacial dysplasia, asphyxiating thoracic dysplasia, atriodigital dysplasia, bronchopulmonary dysplasia, cerebral dysplasia, cervical dysplasia, chondroectodermal dysplasia, cleidocranial dysplasia, congenital ectodermal dysplasia, craniodiaphysial dysplasia, craniocarpotarsal dysplasia, craniometaphysial dysplasia, dentin dysplasia, diaphysial dysplasia, ectodermal dysplasia, enamel dysplasia, encephalo-ophthalmic dysplasia, dysplasia epiphysialis hemimelia, dysplasia epiphysialis multiplex, dysplasia epiphysialis punctata, epithelial dysplasia, faciodigitogenital dysplasia, familial fibrous dysplasia of jaws, familial white folded dysplasia, fibromuscular dysplasia, fibrous dysplasia of bone, florid osseous dysplasia, hereditary renal-retinal dysplasia, hidrotic ectodermal dysplasia, hypohidrotic ectodermal dysplasia, lymphopenic thymic dysplasia, mammary dysplasia, mandibulofacial dysplasia, metaphysial dysplasia, Mondini dysplasia, monostotic fibrous dysplasia, mucoepithelial dysplasia, multiple epiphysial dysplasia, oculoauriculovertebral dysplasia, oculodentodigital dysplasia, oculovertebral dysplasia, odontogenic dysplasia, opthalmomandibulomelic dysplasia, periapical cemental dysplasia, polyostotic fibrous dysplasia, pseudoachondroplastic spondyloepiphysial dysplasia, retinal dysplasia, septo-optic dysplasia, spondyloepiphysial dysplasia, and ventriculoradial dysplasia.

[0124] Additional pre-neoplastic disorders which can be treated include, but are not limited to, benign dysproliferative disorders (e.g., benign tumors, fibrocystic conditions, tissue hypertrophy, intestinal polyps or adenomas, and esophageal dysplasia), leukoplakia, keratoses, Bowen's disease, Farmer's Skin, solar cheilitis, and solar keratosis.

[0125] In preferred embodiments, the method of the invention is used to inhibit growth, progression, and/or metastasis of cancers, in particular those listed above.

[0126] Additional hyperproliferative diseases, disorders, and/or conditions include, but are not limited to, progression, and/or metastases of malignancies and related disorders such as

leukemia (including acute leukemias (e.g., acute lymphocytic leukemia, acute myelocytic leukemia (including myeloblastic, promyelocytic, myelomonocytic, monocytic, and erythroleukemia)) and chronic leukemias (e.g., chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia)), polycythemia vera, lymphomas (e.g., Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, and solid tumors including, but not limited to, sarcomas and carcinomas such as fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, emangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, and retinoblastoma.

Expression Vectors

[0127] Still other embodiments may concern DNA sequences comprising a nucleic acid encoding an antibody, antibody fragment, cytokine or constituent fusion protein of a DNL construct. Fusion proteins may comprise an antibody or fragment or cytokine attached to, for example, an AD or DDD moiety.

[0128] Various embodiments relate to expression vectors comprising the coding DNA sequences. The vectors may contain sequences encoding the light and heavy chain constant regions and the hinge region of a human immunoglobulin to which may be attached chimeric, humanized or human variable region sequences. The vectors may additionally contain promoters that express the encoded protein(s) in a selected host cell, enhancers and signal or leader sequences. Vectors that are particularly useful are pdHL2 or GS. More preferably, the light and heavy chain constant regions and hinge region may be from a human EU myeloma immunoglobulin, where optionally at least one of the amino acid in the allotype positions is changed to that found in a different IgG1 allotype, and wherein optionally amino acid 253 of the heavy chain of EU based on the EU number system may be replaced with alanine. See Edelman

et al., *Proc. Natl. Acad. Sci USA* 63: 78-85 (1969). In other embodiments, an IgG1 sequence may be converted to an IgG4 sequence.

[0129] The skilled artisan will realize that methods of genetically engineering expression constructs and insertion into host cells to express engineered proteins are well known in the art and a matter of routine experimentation. Host cells and methods of expression of cloned antibodies or fragments have been described, for example, in U.S. Patent Nos. 7,531,327 and 7,537,930, the Examples section of each incorporated herein by reference.

Kits

[0130] Various embodiments may concern kits containing components suitable for treating or diagnosing diseased tissue in a patient. Exemplary kits may contain one or more DNL constructs as described herein. If the composition containing components for administration is not formulated for delivery via the alimentary canal, such as by oral delivery, a device capable of delivering the kit components through some other route may be included. One type of device, for applications such as parenteral delivery, is a syringe that is used to inject the composition into the body of a subject. Inhalation devices may also be used. In certain embodiments, a therapeutic agent may be provided in the form of a prefilled syringe or autoinjection pen containing a sterile, liquid formulation or lyophilized preparation.

[0131] The kit components may be packaged together or separated into two or more containers. In some embodiments, the containers may be vials that contain sterile, lyophilized formulations of a composition that are suitable for reconstitution. A kit may also contain one or more buffers suitable for reconstitution and/or dilution of other reagents. Other containers that may be used include, but are not limited to, a pouch, tray, box, tube, or the like. Kit components may be packaged and maintained sterilely within the containers. Another component that can be included is instructions to a person using a kit for its use.

EXAMPLES

[0132] The following examples are provided to illustrate, but not to limit, the claims of the present invention.

Example 1. General strategy for production of modular Fab subunits

[0133] Fab modules may be produced as fusion proteins containing either a DDD or AD sequence. Independent transgenic cell lines are developed for each fusion protein. Once produced, the modules can be purified if desired or maintained in the cell culture supernatant

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fluid. Following production, any DDD_2 module can be combined with any AD module to generate a trivalent DNL construct.

[0134] The plasmid vector pdHL2 has been used to produce a number of antibodies and antibody-based constructs. See Gillies et al., J Immunol Methods (1989), 125:191-202; Losman et al., Cancer (Phila) (1997), 80:2660-6. The di-cistronic mammalian expression vector directs the synthesis of the heavy and light chains of IgG. The vector sequences are mostly identical for many different IgG-pdHL2 constructs, with the only differences existing in the variable domain (VH and VL) sequences. Using molecular biology tools known to those skilled in the art, these IgG expression vectors can be converted into Fab-DDD or Fab-AD expression vectors. To generate Fab-DDD expression vectors, the coding sequences for the hinge, CH2 and CH3 domains of the heavy chain are replaced with a sequence encoding the first 4 residues of the hinge, a 14 residue Gly-Ser linker and the first 44 residues of human RIIα (referred to as DDD1). To generate Fab-AD expression vectors, the sequences for the hinge, CH2 and CH3 domains of IgG are replaced with a sequence encoding the first 4 residues of the hinge, a 15 residue Gly-Ser linker and a 17 residue synthetic AD called AKAP-*IS* (referred to as AD1), which was generated using bioinformatics and peptide array technology and shown to bind RIIα dimers with a very high affinity (0.4 nM). See Alto, et al. Proc. Natl. Acad. Sci., U.S.A (2003), 100:4445-50.

[0135] Two shuttle vectors were designed to facilitate the conversion of IgG-pdHL2 vectors to either Fab-DDD1 or Fab-AD1 expression vectors, as described below.

Preparation of CH1

[0136] The CH1 domain was amplified by PCR using the pdHL2 plasmid vector as a template. The left PCR primer consists of the upstream (5') of the CH1 domain and a SacII restriction endonuclease site, which is 5' of the CH1 coding sequence. The right primer consists of the sequence coding for the first 4 residues of the hinge followed by a short linker, with the final two codons comprising a Bam HI restriction site.

5' of CH1 Left Primer

5'GAACCTCGCGGACAGTTAAG-3' (SEQ ID NO:63)

CH1+G_4S-Bam Right ("G_4S" disclosed as SEQ ID NO: 96)

5'GGATCCTCCGCCGCCGCAGCTCTTAGGTTTCTTGTCCACCTTGGTGTTGCTGG -3' (SEQ ID NO:64)

[0137] The 410 bp PCR amplimer was cloned into the pGemT PCR cloning vector (Promega, Inc.) and clones were screened for inserts in the T7 (5') orientation.

Construction of $(G_4S)_2$DDD1 ("$(G_4S)_2$" disclosed as SEQ ID NO: 97)

[0138] A duplex oligonucleotide, designated $(G_4S)_2$DDD1 ("$(G_4S)_2$" disclosed as SEQ ID NO: 97), was synthesized by Sigma Genosys (Haverhill, UK) to code for the amino acid sequence of DDD1 preceded by 11 residues of the linker peptide, with the first two codons comprising a BamHI restriction site. A stop codon and an EagI restriction site are appended to the 3'end. The encoded polypeptide sequence is shown below.

GSGGGGSGGGGSHIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:65)

[0139] The two oligonucleotides, designated RIIA1-44 top and RIIA1-44 bottom, that overlap by 30 base pairs on their 3' ends, were synthesized (Sigma Genosys) and combined to comprise the central 154 base pairs of the 174 bp DDD1 sequence. The oligonucleotides were annealed and subjected to a primer extension reaction with Taq polymerase.

RIIA1-44 top

5'GTGGCGGGTCTGGCGGAGGTGGCAGCCACATCCAGATCCCGCCGGGGCTCA CGGAGCTGCTGCAGGGCTACACGGTGGAGGTGCTGCGACAG-3' (SEQ ID NO:66)

RIIA1-44 bottom

5'GCGCGAGCTTCTCTCAGGCGGGTGAAGTACTCCACTGCGAATTCGACGAGGT CAGGCGGCTGCTGTCGCAGCACCTCCACCGTGTAGCCCTG-3' (SEQ ID NO:67)

[0140] Following primer extension, the duplex was amplified by PCR using the following primers:

G4S Bam-Left ("G4S" disclosed as SEQ ID NO: 96)

5'-GGATCCGGAGGTGGCGGGTCTGGCGGAGGT-3' (SEQ ID NO:68)

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1-44 stop Eag Right

5'-CGGCCGTCAAGCGCGAGCTTCTCTCAGGCG-3' (SEQ ID NO:69)

[0141] This amplimer was cloned into pGemT and screened for inserts in the T7 (5') orientation.

Construction of $(G_4S)_2$-AD1 ("$(G_4S)_2$" disclosed as SEQ ID NO: 97)

[0142] A duplex oligonucleotide, designated $(G_4S)_2$-AD1 ("$(G_4S)_2$" disclosed as SEQ ID NO: 97), was synthesized (Sigma Genosys) to code for the amino acid sequence of AD1 preceded by 11 residues of the linker peptide with the first two codons comprising a BamHI restriction site. A stop codon and an EagI restriction site are appended to the 3'end. The encoded polypeptide sequence is shown below.

GSGGGGSGGGGSQIEYLAKQIVDNAIQQA (SEQ ID NO:70)

[0143] Two complimentary overlapping oligonucleotides, designated AKAP-IS Top and AKAP-IS Bottom, were synthesized.

AKAP-IS Top

5'GGATCCGGAGGTGGCGGGTCTGGCGGAGGTGGCAGCCAGATCGAGTACCTG GCCAAGCAGATCGTGGACAACGCCATCCAGCAGGCCTGACGGCCG-3' (SEQ ID NO:71)

AKAP-IS Bottom

5'CGGCCGTCAGGCCTGCTGGATGGCGTTGTCCACGATCTGCTTGGCCAGGTAC TCGATCTGGCTGCCACCTCCGCCAGACCCGCCACCTCCGGATCC-3' (SEQ ID NO:72)

[0144] The duplex was amplified by PCR using the following primers:

G4S Bam-Left ("G4S" disclosed as SEQ ID NO: 96)

5'-GGATCCGGAGGTGGCGGGTCTGGCGGAGGT-3' (SEQ ID NO:73)

AKAP-IS stop Eag Right

5'-CGGCCGTCAGGCCTGCTGGATG-3' (SEQ ID NO:74)

[0145] This amplimer was cloned into the pGemT vector and screened for inserts in the T7 (5') orientation.

Ligating DDD1 with CH1

[0146] A 190 bp fragment encoding the DDD1 sequence was excised from pGemT with BamHI and NotI restriction enzymes and then ligated into the same sites in CH1-pGemT to generate the shuttle vector CH1-DDD1-pGemT.

Ligating AD1 with CH1

[0147] A 110 bp fragment containing the AD1 sequence was excised from pGemT with BamHI and NotI and then ligated into the same sites in CH1-pGemT to generate the shuttle vector CH1-AD1-pGemT.

Cloning CH1-DDD1 or CH1-AD1 into pdHL2-based vectors

[0148] With this modular design either CH1-DDD1 or CH1-AD1 can be incorporated into any IgG construct in the pdHL2 vector. The entire heavy chain constant domain is replaced with one of the above constructs by removing the SacII/EagI restriction fragment (CH1-CH3) from pdHL2 and replacing it with the SacII/EagI fragment of CH1-DDD1 or CH1-AD1, which is excised from the respective pGemT shuttle vector.

N-terminal DDD domains

[0149] The location of the DDD or AD is not restricted to the carboxyl terminal end of CH1. A construct was engineered in which the DDD1 sequence was attached to the amino terminal end of the VH domain.

Example 2: Expression vectors

Construction of h679-Fd-AD1-pdHL2

[0150] h679-Fd-AD1-pdHL2 is an expression vector for production of h679 Fab with AD1 coupled to the carboxyl terminal end of the CH1 domain of the Fd via a flexible Gly/Ser peptide spacer composed of 14 amino acid residues. A pdHL2-based vector containing the variable domains of h679 was converted to h679-Fd-AD1-pdHL2 by replacement of the

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SacII/EagI fragment with the CH1-AD1 fragment, which was excised from the CH1-AD1-SV3 shuttle vector with SacII and EagI.

Construction of C-DDD1-Fd-hMN-14-pdHL2

[0151] C-DDD1-Fd-hMN-14-pdHL2 is an expression vector for production of fusion protein C-DDD1-Fab-hMN-14, in which DDD1 is linked to hMN-14 Fab at the carboxyl terminus of CH1 via a flexible peptide spacer. The plasmid vector hMN14(I)-pdHL2, which has been used to produce hMN-14 IgG, was converted to C-DDD1-Fd-hMN-14-pdHL2 by digestion with SacII and EagI restriction endonucleases to remove the CH1-CH3 domains and insertion of the CH1-DDD1 fragment, which was excised from the CH1-DDD1-SV3 shuttle vector with SacII and EagI.

Construction of N-DDD1-Fd-hMN-14-pdHL2

[0152] N-DDD1-Fd-hMN-14-pdHL2 is an expression vector for production of a stable dimer that comprises two copies of a fusion protein N-DDD1-Fab-hMN-14, in which DDD1 is linked to hMN-14 Fab at the amino terminus of VH via a flexible peptide spacer. The expression vector was engineered as follows. The DDD1 domain was amplified by PCR using the two primers shown below.

DDD1 Nco Left

5' CCATGGGCAGCCACATCCAGATCCCGCC –3' (SEQ ID NO:75)

DDD1-G_4S Bam Right ("G_4S" disclosed as SEQ ID NO: 96)

5'GGATCCGCCACCTCCAGATCCTCCGCCGCCAGCGCGAGCTTCTCTCAGGC GGGTG-3' (SEQ ID NO:76)

[0153] As a result of the PCR, an NcoI restriction site and the coding sequence for part of the linker containing a BamHI restriction were appended to the 5' and 3' ends, respectively. The 170 bp PCR amplimer was cloned into the pGemT vector and clones were screened for inserts in the T7 (5') orientation. The 194 bp insert was excised from the pGemT vector with NcoI and SalI restriction enzymes and cloned into the SV3 shuttle vector, which was prepared by digestion with those same enzymes, to generate the intermediate vector DDD1-SV3.

[0154] The hMN-14 Fd sequence was amplified by PCR using the oligonucleotide primers shown below.

hMN-14VH left G4S Bam ("G4S" disclosed as SEQ ID NO: 96)

5'-GGATCCGGCGGAGGTGGCTCTGAGGTCCAACTGGTGGAGAGCGG-3' (SEQ ID NO:77)

CH1-C stop Eag

5'- CGGCCGTCAGCAGCTCTTAGGTTTCTTGTC –3' (SEQ ID NO:78)

[0155] As a result of the PCR, a BamHI restriction site and the coding sequence for part of the linker were appended to the 5' end of the amplimer. A stop codon and EagI restriction site was appended to the 3'end. The 1043 bp amplimer was cloned into pGemT. The hMN-14-Fd insert was excised from pGemT with BamHI and EagI restriction enzymes and then ligated with DDD1-SV3 vector, which was prepared by digestion with those same enzymes, to generate the construct N-DDD1-hMN-14Fd-SV3.

[0156] The N-DDD1-hMN-14 Fd sequence was excised with XhoI and EagI restriction enzymes and the 1.28 kb insert fragment was ligated with a vector fragment that was prepared by digestion of C-hMN-14-pdHL2 with those same enzymes. The final expression vector is N-DDD1-Fd-hMN-14-pDHL2.

Example 3. Production and purification of h679-Fab-AD1

[0157] The 679 antibody binds to an HSG target antigen and may be purified by affinity chromatography. The h679-Fd-AD1-pdHL2 vector was linearized by digestion with Sal I restriction endonuclease and transfected into Sp/EEE myeloma cells by electroporation. The di-cistronic expression vector directs the synthesis and secretion of both h679 kappa light chain and h679 Fd-AD1, which combine to form h679 Fab-AD1. Following electroporation, the cells were plated in 96-well tissue culture plates and transfectant clones were selected with 0.05 μM methotrexate (MTX). Clones were screened for protein expression by ELISA using microtitre plates coated with a BSA- IMP-260 (HSG) conjugate and detection with HRP-conjugated goat anti-human Fab. BIAcore analysis using an HSG (IMP-239) sensorchip was used to determine the productivity by measuring the initial slope obtained from injection of diluted media samples. The highest producing clone had an initial

productivity of approximately 30 mg/L. A total of 230 mg of h679-Fab-AD1 was purified from 4.5 liters of roller bottle culture by single-step IMP-291 affinity chromatography. Culture media was concentrated approximately 10-fold by ultrafiltration before loading onto an IMP-291-affigel column. The column was washed to baseline with PBS and h679-Fab-AD1 was eluted with 1 M imidazole, 1 mM EDTA, 0.1 M NaAc, pH 4.5. SE-HPLC analysis of the eluate showed a single sharp peak with a retention time (9.63 min) consistent with a 50 kDa protein (not shown). Only two bands, which represent the polypeptide constituents of h679-AD1, were evident by reducing SDS-PAGE analysis (not shown).

Example 4. Production and purification of N-DDD1-Fab-hMN-14 and C-DDD1-Fab-hMN-14

[0158] The C-DDD1-Fd-hMN-14-pdHL2 and N-DDD1-Fd-hMN-14-pdHL2 vectors were transfected into Sp2/0-derived myeloma cells by electroporation. C-DDD1-Fd-hMN-14-pdHL2 is a di-cistronic expression vector, which directs the synthesis and secretion of both hMN-14 kappa light chain and hMN-14 Fd-DDD1, which combine to form C-DDD1-hMN-14 Fab. N-DDD1-hMN-14-pdHL2 is a di-cistronic expression vector, which directs the synthesis and secretion of both hMN-14 kappa light chain and N-DDD1-Fd-hMN-14, which combine to form N-DDD1-Fab-hMN-14. Each fusion protein forms a stable homodimer via the interaction of the DDD1 domain.

[0159] Following electroporation, the cells were plated in 96-well tissue culture plates and transfectant clones were selected with 0.05 μM methotrexate (MTX). Clones were screened for protein expression by ELISA using microtitre plates coated with WI2 (a rat anti-id monoclonal antibody to hMN-14) and detection with HRP-conjugated goat anti-human Fab. The initial productivity of the highest producing C-DDD1-Fab-hMN14 Fab and N-DDD1-Fab-hMN14 Fab clones was 60 mg/L and 6 mg/L, respectively.

Affinity purification of N-DDD1-hMN-14 and C-DDD1-hMN-14 with AD1-Affigel

[0160] The DDD/AD interaction was utilized to affinity purify DDD1-containing constructs. AD1-C is a peptide that was made synthetically consisting of the AD1 sequence and a carboxyl terminal cysteine residue, which was used to couple the peptide to Affigel following reaction of the sulfhydryl group with chloroacetic anhydride. DDD-containing a_2 structures specifically bind to the AD1-C-Affigel resin at neutral pH and can be eluted at low pH (e.g., pH 2.5).

[0161] A total of 81 mg of C-DDD1-Fab-hMN-14 was purified from 1.2 liters of roller bottle culture by single-step AD1-C affinity chromatography. Culture media was concentrated approximately 10-fold by ultrafiltration before loading onto an AD1-C-affigel column. The column was washed to baseline with PBS and C-DDD1-Fab-hMN-14 was eluted with 0.1 M Glycine, pH 2.5. SE-HPLC analysis of the eluate showed a single protein peak with a retention time (8.7 min) consistent with a 107 kDa protein (not shown). The purity was also confirmed by reducing SDS-PAGE, showing only two bands of molecular size expected for the two polypeptide constituents of C-DDD1-Fab-hMN-14 (not shown).

[0162] A total of 10 mg of N-DDD1-hMN-14 was purified from 1.2 liters of roller bottle culture by single-step AD1-C affinity chromatography as described above. SE-HPLC analysis of the eluate showed a single protein peak with a retention time (8.77 min) similar to C-DDD1-Fab-hMN-14 and consistent with a 107 kDa protein (not shown). Reducing SDS-PAGE showed only two bands attributed to the polypeptide constituents of N-DDD1-Fab-hMN-14 (not shown).

[0163] The binding activity of C-DDD1-Fab-hMN-14 was determined by SE-HPLC analysis of samples in which the test article was mixed with various amounts of WI2. A sample prepared by mixing WI2 Fab and C-DDD1-Fab-hMN-14 at a molar ratio of 0.75:1 showed three peaks, which were attributed to unbound C-DDD1-Fab-hMN14 (8.71 min), C-DDD1-Fab-hMN-14 bound to one WI2 Fab (7.95 min), and C-DDD1-Fab-hMN14 bound to two WI2 Fabs (7.37 min) (not shown). When a sample containing WI2 Fab and C-DDD1-Fab-hMN-14 at a molar ratio of 4 was analyzed, only a single peak at 7.36 minutes was observed (not shown). These results demonstrate that hMN14-Fab-DDD1 is dimeric and has two active binding sites. Very similar results were obtained when this experiment was repeated with N-DDD1-Fab-hMN-14.

[0164] A competitive ELISA demonstrated that both C-DDD1-Fab-hMN-14 and N-DDD1-Fab-hMN-14 bind to CEA with an avidity similar to hMN-14 IgG, and significantly stronger than monovalent hMN-14 Fab (not shown). ELISA plates were coated with a fusion protein containing the epitope (A3B3) of CEA for which hMN-14 is specific.

Example 5. Formation of a_2b complexes

[0165] Evidence for the formation of an $\mathbf{a_2b}$ complex was provided by SE-HPLC analysis of a mixture containing C-DDD1-Fab-hMN-14 (as $\mathbf{a_2}$) and h679-Fab-AD1 (as **b**) in an equal

molar amount. When such a sample was analyzed, a single peak was observed having a retention time of 8.40 minutes, which is consistent with the formation of a new protein that is larger than either h679-Fab-AD1 (9.55 min) or C-DDD1-Fab-hMN-14 (8.73 min) alone (not shown). The upfield shift was not observed when hMN-14 F(ab')$_2$ was mixed with h679-Fab-AD1 or C-DDD1-Fab-hMN-14 was mixed with 679-Fab-NEM, demonstrating that the interaction is mediated specifically via the DDD1 and AD1 domains. Very similar results were obtained using h679-Fab-AD1 and N-DDD1-Fab-hMN-14 (not shown).

[0166] BIAcore was used to further demonstrate and characterize the specific interaction between the DD1 and AD1 fusion proteins. The experiments were performed by first allowing either h679-Fab-AD1 or 679-Fab-NEM to bind to the surface of a high density HSG-coupled (IMP239) sensorchip, followed by a subsequent injection of C- DDD1-Fab-hMN-14 or hMN-14 F(ab')$_2$. As expected, only the combination of h679-Fab-AD1 and C-DDD1-Fab-hMN-14 resulted in a further increase in response units when the latter was injected (not shown). Similar results were obtained using N-DDD1-Fab-hMN-14 and h679-Fab-AD1 (not shown).

[0167] Equilibrium SE-HPLC experiments were carried out to determine the binding affinity of the specific interaction between AD1 and DDD1 present in the respective fusion proteins. The dissociation constants (K_d) for the binding of h679-Fab-AD1 with C-DDD1-Fab-hMN-14, N-DDD1-hMN-14 and a commercial sample of recombinant human RIIα were found to be 15 nM, 8 nM and 30 nM, respectively.

Example 6. Affinity purification of either DDD or AD fusion proteins

[0168] Universal affinity purification systems can be developed by production of DDD or AD proteins, which have lower affinity docking. The DDD formed by RIα dimers binds AKAP-IS (AD1) with a 500-fold weaker affinity (225 nM) compared to RIIα. Thus, RIα dimers formed from the first 44 amino acid resides can be produced and coupled to a resin to make an affinity matrix for purification of any AD1-containing fusion protein.

[0169] Many lower affinity (0.1μM) AKAP anchoring domains exist in nature. If necessary, highly predicable amino acid substitutions can be introduced to further lower the binding affinity. A low affinity AD can be produced either synthetically or biologically and coupled to resin for use in affinity purification of any DDD1 fusion protein.

Example 7. Vectors for producing disulfide stabilized structures

N-DDD2-Fd-hMN-14-pdHL2

[0170] N-DDD2-hMN-14-pdHL2 is an expression vector for production of N-DDD2-Fab-hMN-14, which possesses a dimerization and docking domain sequence of DDD2 appended to the amino terminus of the Fd. The DDD2 is coupled to the V_H domain via a 15 amino acid residue Gly/Ser peptide linker. DDD2 has a cysteine residue preceding the dimerization and docking sequences, which are identical to those of DDD1.

[0171] The expression vector was engineered as follows. Two overlapping, complimentary oligonucleotides (DDD2 Top and DDD2 Bottom), which comprise residues 1 – 13 of DDD2, were made synthetically. The oligonucleotides were annealed and phosphorylated with T4 polynucleotide kinase (PNK), resulting in overhangs on the 5' and 3' ends that are compatible for ligation with DNA digested with the restriction endonucleases NcoI and PstI, respectively.

DDD2 Top

5'CATGTGCGGCCACATCCAGATCCCGCCGGGGCTCACGGAGCTGCTGCA-3' (SEQ ID NO:79)

DDD2 Bottom

5'GCAGCTCCGTGAGCCCCGGCGGGATCTGGATGTGGCCGCA-3' (SEQ ID NO:80)

[0172] The duplex DNA was ligated with a vector fragment, DDD1-hMN14 Fd-SV3 that was prepared by digestion with NcoI and PstI, to generate the intermediate construct DDD2-hMN14 Fd-SV3. A 1.28 kb insert fragment, which contained the coding sequence for DDD2-hMN14 Fd, was excised from the intermediate construct with XhoI and EagI restriction endonucleases and ligated with hMN14-pdHL2 vector DNA that was prepared by digestion with those same enzymes. The final expression vector is N-DDD2-Fd-hMN-14-pdHL2.

C-DDD2-Fd-hMN-14-pdHL2

[0173] C-DDD2-Fd-hMN-14-pdHL2 is an expression vector for production of C-DDD2-Fab-hMN-14, which possesses a dimerization and docking domain sequence of DDD2 appended to the carboxyl terminus of the Fd via a 14 amino acid residue Gly/Ser peptide linker. The

expression vector was engineered as follows. Two overlapping, complimentary oligonucleotides, which comprise the coding sequence for part of the linker peptide (GGGGSGGGCG, SEQ ID NO:81) and residues 1 – 13 of DDD2, were made synthetically. The oligonucleotides were annealed and phosphorylated with T4 PNK, resulting in overhangs on the 5' and 3' ends that are compatible for ligation with DNA digested with the restriction endonucleases BamHI and PstI, respectively.

G4S-DDD2 top ("G4S" disclosed as SEQ ID NO: 96)

5'GATCCGGAGGTGGCGGGTCTGGCGGAGGTTGCGGCCACATCCAGATCCCGC CGGGGCTCACGGAGCTGCTGCA-3' (SEQ ID NO:82)

G4S-DDD2 bottom ("G4S" disclosed as SEQ ID NO: 96)

5'GCAGCTCCGTGAGCCCCGGCGGGATCTGGATGTGGCCGCAACCTCCGCCAGA CCCGCCACCTCCG-3' (SEQ ID NO:83)

[0174] The duplex DNA was ligated with the shuttle vector CH1-DDD1-pGemT, which was prepared by digestion with BamHI and PstI, to generate the shuttle vector CH1-DDD2-pGemT. A 507 bp fragment was excised from CH1-DDD2-pGemT with SacII and EagI and ligated with the IgG expression vector hMN14(I)-pdHL2, which was prepared by digestion with SacII and EagI. The final expression construct is C-DDD2-Fd-hMN-14-pdHL2.

h679-Fd-AD2-pdHL2

[0175] h679-Fd-AD2-pdHL2 is an expression vector for the production of h679-Fab-AD2, which possesses an anchor domain sequence of AD2 appended to the carboxyl terminal end of the CH1 domain via a 14 amino acid residue Gly/Ser peptide linker. AD2 has one cysteine residue preceding and another one following the anchor domain sequence of AD1.

[0176] The expression vector was engineered as follows. Two overlapping, complimentary oligonucleotides (AD2 Top and AD2 Bottom), which comprise the coding sequence for AD2 and part of the linker sequence, were made synthetically. The oligonucleotides were annealed and phosphorylated with T4 PNK, resulting in overhangs on the 5' and 3' ends that are compatible for ligation with DNA digested with the restriction endonucleases BamHI and SpeI, respectively.

AD2 Top

5'GATCCGGAGGTGGCGGGTCTGGCGGATGTGGCCAGATCGAGTACCTGGCCAAG CAGATCGTGGACAACGCCATCCAGCAGGCCGGCTGCTGAA-3' (SEQ ID NO:84)

AD2 Bottom

5'TTCAGCAGCCGGCCTGCTGGATGGCGTTGTCCACGATCTGCTTGGCCAGGTACT CGATCTGGCCACATCCGCCAGACCCGCCACCTCCG-3'(SEQ ID NO:85)

[0177] The duplex DNA was ligated into the shuttle vector CH1-AD1-pGemT, which was prepared by digestion with BamHI and SpeI, to generate the shuttle vector CH1-AD2-pGemT. A 429 base pair fragment containing CH1 and AD2 coding sequences was excised from the shuttle vector with SacII and EagI restriction enzymes and ligated into h679-pdHL2 vector that prepared by digestion with those same enzymes. The final expression vector is h679-Fd-AD2-pdHL2.

Example 8. Generation of TF1

[0178] A large scale preparation of a trivalent DNL construct, referred to as TF1, was carried out as follows. N-DDD2-Fab-hMN-14 (Protein L-purified) and h679-Fab-AD2 (IMP-291-purified) were first mixed in roughly stoichiometric concentrations in 1mM EDTA, PBS, pH 7.4. Before the addition of TCEP, SE-HPLC did not show any evidence of a_2b formation (not shown). Instead there were peaks representing $\mathbf{a_4}$ (7.97 min; 200 kDa), $\mathbf{a_2}$ (8.91 min; 100 kDa) and B (10.01 min; 50 kDa). Addition of 5 mM TCEP rapidly resulted in the formation of the $\mathbf{a_2b}$ complex as demonstrated by a new peak at 8.43 min, consistent with a 150 kDa protein (not shown). Apparently there was excess B in this experiment as a peak attributed to h679-Fab-AD2 (9.72 min) was still evident yet no apparent peak corresponding to either $\mathbf{a_2}$ or $\mathbf{a_4}$ was observed. After reduction for one hour, the TCEP was removed by overnight dialysis against several changes of PBS. The resulting solution was brought to 10% DMSO and held overnight at room temperature.

[0179] When analyzed by SE-HPLC, the peak representing $\mathbf{a_2b}$ appeared to be sharpen with a slight reduction of the retention time by 0.1 min to 8.31 min (not shown), which, based on our previous findings, indicates an increase in binding affinity. The complex was further purified by IMP-291 affinity chromatography to remove the kappa chain contaminants. As expected, the excess h679-AD2 was co-purified and later removed by preparative SE-HPLC (not shown).

[0180] TF1 is a highly stable complex. When TF1 was tested for binding to an HSG (IMP-239) sensorchip, there was no apparent decrease of the observed response at the end of sample injection. In contrast, when a solution containing an equimolar mixture of both C-DDD1-Fab-hMN-14 and h679-Fab-AD1 was tested under similar conditions, the observed increase in response units was accompanied by a detectable drop during and immediately after sample injection, indicating that the initially formed $\mathbf{a_2b}$ structure was unstable. Moreover, whereas subsequent injection of WI2 gave a substantial increase in response units for TF1, no increase was evident for the C-DDD1/AD1 mixture.

[0181] The additional increase of response units resulting from the binding of WI2 to TF1 immobilized on the sensorchip corresponds to two fully functional binding sites, each contributed by one subunit of N-DDD2-Fab-hMN-14. This was confirmed by the ability of TF1 to bind two Fab fragments of WI2 (not shown). When a mixture containing h679-AD2 and N-DDD1-hMN14, which had been reduced and oxidized exactly as TF1, was analyzed by BIAcore, there was little additional binding of WI2 (not shown), indicating that a disulfide-stabilized $\mathbf{a_2b}$ complex such as TF1 could only form through the interaction of DDD2 and AD2.

[0182] Two improvements to the process were implemented to reduce the time and efficiency of the process. First, a slight molar excess of N-DDD2-Fab-hMN-14 present as a mixture of $\mathbf{a_4/a_2}$ structures was used to react with h679-Fab-AD2 so that no free h679-Fab-AD2 remained and any $\mathbf{a_4/a_2}$ structures not tethered to h679-Fab-AD2, as well as light chains, would be removed by IMP-291 affinity chromatography. Second, hydrophobic interaction chromatography (HIC) has replaced dialysis or diafiltration as a means to remove TCEP following reduction, which would not only shorten the process time but also add a potential viral removing step. N-DDD2-Fab-hMN-14 and 679-Fab-AD2 were mixed and reduced with 5 mM TCEP for 1 hour at room temperature. The solution was brought to 0.75 M ammonium sulfate and then loaded onto a Butyl FF HIC column. The column was washed with 0.75 M ammonium sulfate, 5 mM EDTA, PBS to remove TCEP. The reduced proteins were eluted from the HIC column with PBS and brought to 10% DMSO. Following incubation at room temperature overnight, highly purified TF1 was isolated by IMP-291 affinity chromatography (not shown). No additional purification steps, such as gel filtration, were required.

Example 9. Generation of TF2

[0183] Following the successful creation of TF1, an analog designated TF2 was obtained by reacting C-DDD2-Fab-hMN-14 with h679-Fab-AD2. A pilot batch of TF2 was generated with >90% yield as follows. Protein L-purified C-DDD2-Fab-hMN-14 (200 mg) was mixed with h679-Fab-AD2 (60 mg) at a 1.4:1 molar ratio. The total protein concentration was 1.5 mg/ml in PBS containing 1 mM EDTA. Subsequent steps involving TCEP reduction, HIC chromatography, DMSO oxidation, and IMP-291 affinity chromatography were the same as described for TF1. Before the addition of TCEP, SE-HPLC did not show any evidence of $\mathbf{a_2b}$ formation (not shown). Instead there were peaks corresponding to $\mathbf{a_4}$ (8.40 min; 215 kDa), $\mathbf{a_2}$ (9.32 min; 107 kDa) and **b** (10.33 min; 50 kDa). Addition of 5 mM TCEP rapidly resulted in the formation of $\mathbf{a_2b}$ complex as demonstrated by a new peak at 8.77 min (not shown), consistent with a 157 kDa protein expected for the binary structure. TF2 was purified to near homogeneity by IMP-291 affinity chromatography (not shown). SE-HPLC analysis of the IMP-291 unbound fraction demonstrated the removal of $\mathbf{a_4}$, $\mathbf{a_2}$ and free kappa chains from the product (not shown).

[0184] The functionality of TF2 was determined by BIACORE as descibed for TF1. TF2, C-DDD1-hMN-14 + h679-AD1 (used as a control sample of noncovalent $\mathbf{a_2b}$ complex), or C-DDD2-hMN-14 + h679-AD2 (used as a control sample of unreduced $\mathbf{a_2}$ and **b** components) were diluted to 1 μg/ml (total protein) and pass over a sensorchip immoblized with HSG. The response for TF2 was approximately two-fold that of the two control samples, indicating that only the h679-Fab-AD component in the control samples would bind to and remains on the sensorchip. Subsequent WI2 IgG injections demonstrated that only TF2 had a DDD-Fab-hMN-14 component that was tightly associated with h679-Fab-AD as indicated by an additional signal response. The additional increase of response units resulting from the binding of WI2 to TF2 immobilized on the sensorchip also corresponds to two fully functional binding sites, each contributed by one subunit of C-DDD2-Fab-hMN-14. This was confirmed by the ability of TF2 to bind two Fab fragments of WI2 (not shown).

[0185] The relative CEA-binding avidity of TF2 was determined by competitive ELISA. Plates were coated (0.5 μg/well) with a fusion protein containing the A3B3 domain of CEA , which is recognized by hMN-14. Serial dilutions of TF1, TF2 and hMN-14 IgG were made in quadruplicate and incubated in wells containing HRP-conjugated hMN-14 IgG (1 nM). The

data indicate that TF2 binds CEA with an avidity that is at least equivalent to that of IgG and two-fold stronger than TF1 (not shown).

Example 10. Serum stability of TF1 and TF2

[0186] TF1 and TF2 were designed to be stably tethered structures that could be used *in vivo* where extensive dilution in blood and tissues would occur. The stability of TF2 in human sera was assessed using BIACORE. TF2 was diluted to 0.1 mg/ml in fresh human serum, which was pooled from four donors, and incubated at 37°C under 5% CO_2 for seven days. Daily samples were diluted 1:25 and then analyzed by BIACORE using an IMP-239 HSG sensorchip. An injection of WI2 IgG was used to quantify the amount of intact and fully active TF2. Serum samples were compared to control samples that were diluted directly from the stock. TF2 is highly stable in serum, retaining 98% of its bispecific binding activity after 7 days (not shown). Similar results were obtained for TF1 in either human or mouse serum (not shown).

Example 11. Creation of C-H-AD2-IgG-pdHL2 expression vectors.

[0187] The pdHL2 mammalian expression vector has been used to mediate the expression of many recombinant IgGs (Qu et al., Methods 2005, 36:84-95). A plasmid shuttle vector was produced to facilitate the conversion of any IgG-pdHL2 vector into a C-H-AD2-IgG-pdHL2 vector. The gene for the Fc (CH2 and CH3 domains) was amplified using the pdHL2 vector as a template and the oligonucleotides *Fc BglII Left* and *Fc Bam-EcoRI Right* as primers.

Fc BglII Left

5'-AGATCTGGCGCACCTGAACTCCTG-3' (SEQ ID NO:86)

Fc Bam-EcoRI Right

5'-GAATTCGGATCCTTTACCCGGAGACAGGGAGAG-3' (SEQ ID NO:87)

[0188] The amplimer was cloned in the pGemT PCR cloning vector. The Fc insert fragment was excised from pGemT with XbaI and BamHI restriction enzymes and ligated with AD2-pdHL2 vector that was prepared by digestion of h679-Fab-AD2-pdHL2 with XbaI and BamHI, to generate the shuttle vector Fc-AD2-pdHL2.

[0189] To convert any IgG-pdHL2 expression vector to a C-H-AD2-IgG-pdHL2 expression vector, an 861 bp BsrGI / NdeI restriction fragment is excised from the former and replaced with a 952 bp BsrGI / NdeI restriction fragment excised from the Fc-AD2-pdHL2 vector. BsrGI cuts in the CH3 domain and NdeI cuts downstream (3') of the expression cassette.

Example 12. Production of C-H-AD2-hLL2 IgG

[0190] Epratuzumab, or hLL2 IgG, is a humanized anti-human CD22 MAb. An expression vector for C-H-AD2-hLL2 IgG was generated from hLL2 IgG-pdHL2, as described in Example 11, and used to transfect Sp2/0 myeloma cells by electroporation. Following transfection, the cells were plated in 96-well plates and transgenic clones were selected in media containing methotrexate. Clones were screened for C-H-AD2-hLL2 IgG productivity by a sandwich ELISA using 96-well microtitre plates coated with an hLL2-specific anti-idiotype MAb and detection with peroxidase-conjugated anti-human IgG. Clones were expanded to roller bottles for protein production and C-H-AD2-hLL2 IgG was purified from the spent culture media in a single step using Protein-A affinity chromatography. SE-HPLC analysis resolved two protein peaks (not shown). The retention time of the slower eluted peak (8.63 min) was similar to hLL2 IgG. The retention time of the faster eluted peak (7.75 min) was consistent with a ~300 kDa protein. It was later determined that this peak represents disulfide linked dimers of C-H-AD2-hLL2-IgG. This dimer is reduced to the monomeric form during the DNL reaction. SDS-PAGE analysis demonstrated that the purified C-H-AD2-hLL2-IgG consisted of both monomeric and disulfide-linked dimeric forms of the module (not shown). Protein bands representing these two forms were evident by SDS-PAGE under non-reducing conditions, while under reducing conditions all of the forms were reduced to two bands representing the constituent polypeptides (Heavy chain-AD2 and kappa chain) (not shown). No other contaminating bands were detected.

Example 13. Production of C-H-AD2-hA20 IgG

[0191] hA20 IgG is a humanized anti-human CD20 MAb. An expression vector for C-H-AD2-hA20 IgG was generated from hA20 IgG-pDHL2, as described in Example 27, and used to transfect Sp2/0 myeloma cells by electroporation. Following transfection, the cells were plated in 96-well plates and transgenic clones were selected in media containing methotrexate. Clones were screened for C-H-AD2-hA20 IgG productivity by a sandwich ELISA using 96-well microtitre plates coated with an hA20-specific anti-idiotype MAb and

detection with peroxidase-conjugated anti-human IgG. Clones were expanded to roller bottles for protein production and C-H-AD2-hA20 IgG was purified from the spent culture media in a single step using Protein-A affinity chromatography. SE-HPLC and SDS-PAGE analyses gave very similar results to those obtained for C-H-AD2-hLL2 IgG in Example 28.

Example 14. Production of AD- and DDD-linked Fab and IgG Fusion Proteins From Multiple Antibodies

[0192] Using the techniques described in the preceding Examples, the following IgG or Fab fusion proteins were constructed and incorporated into DNL constructs. The fusion proteins retained the antigen-binding characteristics of the parent antibodies and the DNL constructs exhibited the antigen-binding activities of the incorporated antibodies or antibody fragments.

Table 2. Fusion proteins comprising IgG or Fab Moieties

Fusion Protein	Binding Specificity
C-AD1-Fab-h679	HSG
C-AD2-Fab-h679	HSG
C-$(AD2)_2$-Fab-H679	HSG
C-AD2-IgG-h734	Indium-DTPA
C-AD2-IgG-hA20	CD20
C-AD2-IgG-hA20L	CD20
C-AD2-IgG-hL243	HLA-DR
C-AD2-IgG-hLL2	CD22
N-AD2-IgG-hLL2	CD22
C-AD2-IgG-hMN-14	CEA
C-AD2-IgG-hR1	IGF-1R

C-AD2-IgG-hRS7	EGP-1
C-AD2-IgG-hPAM4	MUC1
C-AD2-IgG-hLL1	CD74
C-DDD1-Fab-hMN-14	CEACAM5
C-DDD2-Fab-hMN-14	CEACAM5
C-DDD2-Fab-h679	
C-DDD2-Fab-hA19	CD19
C-DDD2-Fab-hA20	CD20
C-DDD2-Fab-hAFP	AFP
C-DDD2-Fab-hL243	HLA-DR
C-DDD2-Fab-hLL1	CD74
C-DDD2-Fab-hLL2	CD22
C-DDD2-Fab-hMN-3	CEACAM6
C-DDD2-Fab-hMN-15	CEACAM6
C-DDD2-Fab-hPAM4	MUC1
C-DDD2-Fab-hR1	IGF-1R
C-DDD2-Fab-hRS7	IGP-1
N-DDD2-Fab-hMN-14	CEACAM5

Example 15. Generation of DDD-module based on Interferon (IFN)-α2b

Construction of IFN-α2b-DDD2-pdHL2 for expression in mammalian cells

[0193] The cDNA sequence for IFN-α2b was amplified by PCR resulting in sequences comprising IFN-α2b fused at its C-terminus to a polypeptide of the following sequence: KSHHHHHHGSGGGGSGGGCGHIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTR LREARA (SEQ ID NO:88).

[0194] PCR amplification was accomplished using a full length human IFNα2b cDNA clone (Invitrogen Ultimate ORF human clone cat# HORF01Clone ID IOH35221) as a template and the following oligonucleotides as primers:

IFNA2 Xba I Left

TCTAGACACAGGACCTCATCATGGCCTTGACCTTTGCTTTACTGG (SEQ ID NO:89)

IFNA2 BamHI right

GGATCCATGATGGTGATGATGGTGTGACTTTTCCTTACTTCTTAAACTTTCTTGC (SEQ ID NO:90)

[0195] The PCR amplimer was cloned into the pGemT vector. A DDD2-pdHL2 mammalian expression vector was prepared for ligation with IFN-α2b as follows. The C_{H1}-DDD2-Fab-hMN-14-pdHL2 (Rossi et al., Proc Natl Acad Sci USA 2006, 103:6841-6) vector was digested with Xba I and Bam HI, which removes all of the Fab gene sequences but leaves the DDD2 coding sequence. The IFN-α2b amplimer was excised from pGemT with Xba I and Bam HI and ligated into the DDD2-pdHL2 vector to generate the expression vector IFN-α2b-DDD2-pdHL2.

Mammalian cell expression of IFN-α2b-DDD2

[0196] IFN-α2b-DDD2-pdHL2 was linearized by digestion with Sal I and stably transfected by electroporation into Sp/ESF myeloma cells. Two clones were found to have detectable levels of IFN-α2b by ELISA. One of the two clones, designated 95, was adapted to growth in serum-free media without substantial decrease in productivity. The clone was subsequently amplified with increasing MTX concentrations from 0.1 to 0.8 μM over five weeks. At this stage, it was sub-cloned by limiting dilution and the highest producing sub-clone (95-5) was expanded. The productivity of 95-5 grown in shake-flasks was estimated to be 2.5 mg/L using commercial rIFN-α2b (Chemicon IF007, Lot 06008039084) as standards.

Purification of IFN-α2b-DDD2 from batch cultures grown in roller bottles

[0197] Clone 95-5 was expanded to 34 roller bottles containing a total of 20 L of serum-free Hybridoma SFM with 0.8 μM MTX and allowed to reach terminal culture. The culture broth was processed and IFN-α2b-DDD2 was purified by immobilized metal affinity chromatography (IMAC) as follows. The supernatant fluid was clarified by centrifugation, 0.2 μM filtered, diafiltered into 1X Binding buffer (10 mM imidazole, 0.5 M NaCl, 50 mM NaH_2PO_4, pH 7.5), concentrated to 310 mL, added Tween 20 to a final concentration of 0.1%, and loaded onto a 30-mL Ni-NTA column. Following sample loading, the column was washed with 500 mL of 0.02% Tween 20 in 1X binding buffer and then 290 mL of 30 mM imidazole, 0.02% Tween 20, 0.5 M NaCl, 50 mM NaH_2PO_4, pH 7.5. The product was eluted with 110 mL of 250 mM imidazole, 0.02% Tween 20, 150 mM NaCl, 50 mM NaH_2PO_4, pH 7.5. Approximately 6 mg of IFNα2b-DDD2 was purified.

Production of IFN-α2b-DDD2 in E. coli

[0198] IFN-α2b-DDD2 was also expressed by microbial fermentation as a soluble protein in *E. coli*. The coding sequence was amplified by PCR using IFN-α2b-DDD2-pdHL2 DNA as a template. The amplimer was cloned into the pET26b *E. coli* expression vector using Nde I and Xho I restriction sites. Protein was expressed intracellularly in BL21pLysS host cells by induction of LB shake flasks with 100 μM IPTG at 18°C for 12 hours. Soluble IFN-α2b-DDD2 was purified from cell lysates by IMAC as described above.

Example 16. Generation of a DNL conjugate comprising IFN-α2b-DDD2 linked to C_{H3}-AD2-IgG

[0199] A DNL construct designated 20-2b, comprising four copies of IFNα2b-DDD2 attached to one C_{H3}-AD2-IgG, was produced by DNL via the combination of two DNL modules, C_{H3}-AD2-IgG-v-mab and IFNα2b-DDD2, which were each expressed in Sp/ESF. Additional DNL-generated MAb-IFNα constructs, of similar design as 20-2b (humanized IgG1 + 4 IFNα2b) but with different targeting MAbs, were used as controls in several experiments: 22-2b has C_{H3}-AD2-IgG-e-mab (epratuzumab) as its AD2 module, which is directed against CD22 and binds lymphoma; 734-2b has C_{H3}-AD2-IgG-h734 as its AD2 module, which is directed against the hapten, In-DTPA and does not bind to any animal proteins or tissues; and R1-2b uses C_{H3}-AD2-IgG-hR1, which binds human insulin-like growth factor 1 receptor (IGF-1R).

[0200] The 20-2b DNL construct was made as follows. A select C_{H3}-AD2-IgG was

combined with approximately two mole-equivalents of IFN-α2b-DDD2 and the mixture was reduced under mild conditions overnight at room temperature after adding 1 mM EDTA and 2 mM reduced glutathione (GSH). Oxidized glutathione was added to 2 mM and the mixture was held at room temperature for an additional 12-24 hours. The DNL conjugate was purified over a Protein A affinity column. Four such DNL conjugates designed 20-2b, 22-2b, hR1-2b, and 243-2b, each comprising four copies of IFN-α2b anchored on C_{H3}-AD2-IgG-hA20 (with specificity for CD20), C_{H3}-AD2-IgG-hLL2 (with specificity for CD22), C_{H3}-AD2-IgG-hR1 (with specificity for IGF-1R) and C_{H3}-AD2-IgG-hL243 (with specificity for HLA-DR), respectively, were prepared. SE-HPLC analyses of 20-2b generated from mammalian (m) or *E. coli* (e)-produced IFN-α2b-DDD2 each showed a major peak having a retention time consistent with a covalent complex composed of an IgG and 4 IFN-α2b groups (not shown). Similar SE-HPLC profiles were observed for the other three IFN-IgG conjugates.

In vitro activity of the IFN-IgG conjugates

[0201] The *in vitro* IFNα biological activity of 20-2b was compared to that of commercial PEGylated IFNα2 agents, PEGASYS and PEG-Intron, using cell-based reporter, viral protection, and lymphoma proliferation assays. Specific activities were determined using a cell-based kit, which utilizes a transgenic human pro-monocyte cell line carrying a reporter gene fused to an interferon-stimulated response element (**FIG. 1A-1D**). The specific activity of 20-2b (5300 IU/pmol) was greater than both PEGASYS (170 IU/pmol) and PEG-Intron (3400 IU/pmol) (**FIG. 1A**). 734-2b, 1R-2b and five additional MAb-IFNα constructs (data not shown), which were produced similarly to 20-2b, each exhibited similar specific activities (4000 – 8000 IU/pmol), demonstrating the consistency of the DNL method for generating such structures (**FIG. 1A**). Having four IFNα2b groups contributed to the enhanced potency of MAb-IFNα. When normalized to IFNα equivalents, the specific activity/IFNα was about 10-fold greater than PEGASYS and only about 2-fold less than PEG-Intron.

[0202] Comparison of MAb-IFNα, PEGASYS and PEG-Intron in an *in vitro* viral protection assay demonstrated that MAb-IFNα retains IFNα2b antiviral activity with specific activities similar to PEG-Intron and 10-fold greater than PEGASYS (**FIG. 1B**).

[0203] IFNα2b can have a direct antiproliferative or cytotoxic effect on some tumor lines. The activity of 20-2b was measured in an *in vitro* proliferation assay with a Burkitt lymphoma cell line (Daudi) that is highly sensitive to IFNα (**FIG. 1C**). Each of the IFNα2 agents efficiently inhibited (>90%) Daudi *in vitro* with high potency (EC_{50}=4 -10 pM).

However, 20-2b (EC_{50}=0.25 pM) was about 30-fold more potent than the non-targeting MAb-IFNα constructs.

The parent anti-CD20 MAb of 20-2b has anti-proliferative activity *in vitro* on many lymphoma cell lines, including Daudi (Rossi et al., 2008, Cancer Res 68:8384-92), at considerably greater concentrations (EC_{50}>10nM).

[0204] The *in vitro* activity of 20-2b was also assessed using Jeko-1, which is a mantle cell lymphoma line that has lower sensitivity to both IFNα and anti-CD20 (**FIG. 1D**). Jeko-1 is only modestly sensitive to the parent anti-CD20 MAb, having 10% maximal inhibition (I_{max}) with an EC_{50} near 1 nM. As shown with 734-2b, Jeko-1 (I_{max} =43%; EC_{50} = 23 pM) is less responsive to IFNα2b than Daudi (I_{max} =90%; EC_{50} = 7.5 pM). Compared to 734-2b, 20-2b inhibited Jeko-1 to a greater extent (I_{max} =65%) and exhibited a biphasic dose-response curve (**FIG. 1D**). At <10 pM, a low-concentration response attributed to IFNα2b activity was observed, which plateaus at I_{max} =43%, similar to 734-2b. A high-concentration response was evident above 100 pM, where I_{max} reached 65%. The low-concentration IFNα2b response of 20-2b (EC_{50}=0.97 pM) was 25-fold more potent than 734-2b, similar to the results with Daudi.

[0205] A combination of the parent anti-CD20 antibody and 734-2b (v-mab+734-2b) was assayed to elucidate whether the increased potency of 20-2b is due to an additive/synergistic effect of CD20 and IFNα signaling. The dose response curve for v-mab+734-2b was largely similar to 734-2b alone, except at >1 nM, where inhibition increased for the former but not the latter. These results suggest that MAb targeting is responsible for the lower EC_{50} of 20-2b, but its greater I_{max} is apparently due to the additive activity of IFNα2b and CD-20 signaling. The effect of CD20 signaling was only evident in the high-concentration response for 20-2b (EC_{50}=0.85 nM), which parallels the response to v-mab (EC_{50}=1.5 nM). A biphasic dose-response curve was not obvious for v-mab+734-2b, because the two responses overlap. However, an additive effect was evident at >1 nM concentrations. The I_{max} of 20-2b (65%) was greater than the added responses of IFNα2b (I_{max} =43%) and v-mab (I_{max} =10%), suggesting possible synergism between the actions of IFNα2b and v-mab.

ADCC activity

[0206] IFNα can potentiate ADCC activity, which is a fundamental mechanism of action (MOA) for anti-CD20 immunotherapy, by activating NK cells and macrophages. We compared ADCC of 20-2b and v-mab with two NHL cell lines using peripheral blood mononuclear cells (PBMCs) as effector cells. Replicate assays using PBMCs from multiple

donors consistently demonstrated that 20-2b had enhanced ADCC compared to v-mab, as shown for both Daudi and Raji cells (**FIG. 3A**). This effect was also shown with 22-2b, a MAb-IFNα comprising the anti-CD22 MAb, epratuzumab, which shows modest ADCC (Carnahan et al., 2007, Mol Immunol 44:1331-41.

CDC activity

[0207] CDC is thought to be an important MOA for Type-I anti-CD20 MAbs (including v-mab and rituximab). However, this function is lacking in the Type-II MAbs, represented by tositumomab (Cardarelli et al., 2002, Cancer Immunol Immunother 51:15-24), which nonetheless has anti-lymphoma activity. Unlike v-mab, 20-2b does not show CDC activity *in vitro* (**FIG. 3B**). These results are consistent with those for other DNL structures based on the C_{H3}-AD2-IgG-v-mab module, in which complement fixation is apparently impaired, perhaps by steric interference (Rossi et al., 2008).

Example 17. Pharmacokinetic (PK) analysis of 20-2b.

[0208] The pharmacokinetic (PK) properties of 20-2b were evaluated in male Swiss-Webster mice and compared to those of PEGASYS, PEG-INTRON and α2b-413 (Pegylated IFN made by DNL, see U.S. Patent Application Serial No. 11/925,408). Concentrations of IFN-α in the serum samples at various times were determined by ELISA. IFNα2b specific activities were determined using the iLite Human Interferon Alpha Cell-Based Assay Kit following the manufacturer's suggested protocol (PBL Interferon Source). **FIG. 2** presents the results of the PK analysis, which showed significantly slower elimination and longer serum residence of 20-2b compared to the other agents. At an injected dose of 210 pmol, the calculated pharmacokinetic serum half-life in hours was 8.0 hr (20-2b), 5.7 hr (α2b-413), 4.7 hr (PEGASYS) and 2.6 hr (PEG-INTRON). The elimination rate (1/h) was 0.087 (20-2b), 0.121 (α2b-413), 0.149 (PEGASYS) and 0.265 (PEG-INTRON). The calculated $\mathbf{MRT}_{0.08\rightarrow\infty}$ (hr) was 22.2 (20-2b), 12.5 (α2b-413), 10.7 (PEGASYS) and 6.0 (PEG-INTRON). Because the pharmacokinetic parameters are determined more by the nature of the complex than the individual antibody or cytokine, it is expected that the PK characteristics of the cytokine-DNL complex are generalizable to other cytokine moieties and antibody moieties and are not limited to the specific 20-2b construct discussed above.

Example 18. *In vivo* activity of 20-2b

Serum stability.

[0209] 20-2b was stable in human sera (≥10 days) or whole blood (≥6 days) at 37°C (not shown). Concentration of 20-2b complex was determined using a bispecific ELISA assay. There was essentially no detectable change in serum 20-2b levels in either whole blood or serum over the time period of the assay.

Ex vivo efficacy of 20-2b against lymphoma cells from whole human blood

[0210] We compared the abilities of 20-2b, v-mab, 734-2b, or v-mab+734-2b to eliminate lymphoma or normal B-cells from whole blood in an *ex vivo* setting (**FIG. 4**). The therapeutic efficacy of naked anti-CD20 MAbs is believed to be achieved via three mechanisms of action (MOA) - signaling-induced apoptosis or growth arrest, ADCC, and CDC (Glennie et al., 2007, Mol Immunol 44:3823-37). In this assay, v-mab can employ all three MOA, while, based on the *in vitro* findings, 20-2b can potentially take advantage of signaling and enhanced ADCC, but not CDC. In this short-term model, the IFNα2b groups of 20-2b and 734-2b can act directly on tumor cells, augment the ADCC activity of v-mab, and possibly have some immunostimulatory effects. However, the full spectrum of IFNα-mediated activation of the innate and adaptive immune systems that might occur *in vivo* is not realized in this two-day *ex vivo* assay.

[0211] At 0.01 nM, 20-2b depleted Daudi cells (60.5%) significantly more than v-mab (22.8%), 734-2b (38.6%) or v-mab+734-2b (41.7%) (**FIG. 4**). At 0.1 nM, 20-2b and v-mab+734-2b depleted Daudi to a similar extent (88.9%), which was more than for v-mab (82.4%) or 734-2b (40.7%) (**FIG. 4**). At 1 nM, each agent depleted Daudi >95%, except for 734-2b (55.7%) (**FIG. 4**). Each of the differences indicated were statistically significant ($P<0.01$).

[0212] Ramos is less sensitive than Daudi to both IFNα2b and v-mab. The effect of 734-2b was only moderate, resulting in <20% depletion of Ramos at each concentration (**FIG. 4**). At both 0.01 and 0.1 nM, 20-2b depleted Ramos more than v-mab+734-2b, which in turn eliminated more cells than v-mab (**FIG. 4**). At 1 nM, all treatments besides 734-2b resulted in similar Ramos depletion (75%) (**FIG. 4**). Each of the differences indicated were statistically significant ($P<0.02$).

[0213] As demonstrated with 734-2b, IFNα2b alone does not deplete normal B-cells in this assay. At these low concentrations, 20-2b, v-mab, and v-mab+734-2b each show similar dose-responsive depletion of B-cells, which is markedly less than the depletion of either Daudi or Ramos. None of the treatments resulted in significant depletion of T-cells (data not shown).

In vivo efficacy of 20-2b in SCID mice

[0214] A limitation of the mouse model is the very low sensitivity of murine cells to human IFNα2b. The overall therapeutic advantage of 20-2b that might be achieved in humans can involve the enhancement of both innate and adaptive immunity. With these limitations in mind, we studied the anti-lymphoma *in vivo* efficacy of 20-2b against disseminated Burkitt lymphoma models in SCID mice. We initially tested a highly sensitive early Daudi model (**FIG. 5A**). One day after inoculation, groups were administered a single low dose of 20-2b, v-mab, or 734-2b. A single dose of v-mab or 734-2b at 0.7 pmol (170 ng) resulted in significant improvement in survival when compared to saline for v-mab ($P<0.0001$), but not for the irrelevant MAb-IFNα control, 734-2b (**FIG. 5A**). This improvement was modest, with the median survival time (MST) increasing from 27 days for saline to 34 days for v-mab. However, a single dose of 0.7 pmol (170 ng) of 20-2b improved the MST by more than 100 days over both saline control and v-mab groups ($P<0.0001$) (**FIG. 5A**). The study was terminated after 19 weeks, at which time the 7 long-term survivors (LTS) in the 0.7 pmol 20-2b treatment group were necropsied with no visible evidence of disease found (cured) (**FIG. 5A**). Remarkably, even the lowest dose of 0.07 pmol (17 ng) of 20-2b more than doubled the MST (**FIG. 5A**).

[0215] Next, we assessed the efficacy of 20-2b in a more challenging advanced Daudi model, in which mice were allowed to develop a substantially greater tumor burden prior to treatment (**FIG. 5B**). Seven days after tumor inoculation, groups were administered a single low dose (0.7, 7.0 or 70 pmol) of 20-2b, v-mab, 734-2b, or PEGASYS. The MST for the saline control mice was 21 days (**FIG. 5B**). The highest dose (70 pmol) of PEGASYS or 734-2b, each of which have enhanced Pk (compared to recombinant IFNα2b) but do not target tumor, doubled the MST (42 days; $P<0.0001$) (**FIG. 5B**). Treatment with 20-2b at a 100-fold lower dose (0.7 pmol) produced similar results (38.5 days) as the highest dose (70 pmol) of either PEGASYS or 734-2b (**FIG. 5B**). Treatment with 20-2b at a 10-fold lower dose (7 pmol) resulted in significantly improved survival (80.5 days, 20% LTS) over treatment with 70 pmol of PEGASYS or 734-2b ($P<0.0012$) (**FIG. 5B**). At the highest dose tested (70 pmol), 20-2b improved the MST to >105 days with 100% LTS (**FIG. 5B**). We have demonstrated previously with the early tumor model that v-mab can increase survival of Daudi-bearing mice at relatively low doses (3.5 pmol) while higher doses result in LTS. However, in this advanced tumor model, a single dose of 70 pmol of v-mab had only a modest, though significant, effect on survival (MST=24 days, $P=0.0001$) (**FIG. 5B**).

[0216] We subsequently assayed 20-2b in more challenging models, which are less sensitive than Daudi to direct inhibition by IFNα and less responsive to immunotherapy with v-mab. Raji is ~1000-fold less sensitive to the direct action of IFNα2b compared to Daudi. However, Raji has a similar CD20 antigen density to Daudi (Stein et al., 2006, Blood 108:2736-44) and is responsive to v-mab, albeit considerably less so than Daudi (Goldenberg et al., 2009, Blood 113, 1062-70). The efficacy of 20-2b was studied in an advanced Raji model with therapy beginning five days after tumor inoculation (**FIG. 6A**). Groups were administered a total of 6 injections (250 pmol each) over two weeks. 734-2b did not improve survival over saline (MST =16 days), consistent with the insensitivity of Raji to IFNα (**FIG. 6A**). V-mab significantly improved survival over saline (MST=26 days, $P<0.0001$) (**FIG. 6A**). 20-2b was superior to all other treatments (MST=33 days, $P<0.0001$) (**FIG. 6A**).

[0217] Finally, we investigated the efficacy of 20-2b with NAMALWA (**FIG. 6B**), a human lymphoma that has low sensitivity to the direct action of IFNα, ~25-fold lower CD20 antigen density compared to Daudi or Raji, and is considered to be resistant to anti-CD20 immunotherapy (Stein et al., 2006). Groups were administered a total of 6 doses (250 pmol each) of either 20-2b or 734-2b. Another group was administered a total of 7 doses (3.5 nmol each) of v-mab. The group treated with saline had an MST of 17 days (**FIG. 6B**). Treatment with 734-2b very modestly, though significantly, improved survival (MST=20 days, $P=0.0012$) (**FIG. 6B**). 20-2b (MST=34 days) was superior to 734-2b ($P=0.0004$) as well as v-mab (MST=24 days, $P=0.0026$), which was given at a 14-fold higher dose (**FIG. 6B**).

Conclusions

[0218] The results demonstrate unequivocally that targeting of IFNα with an anti-CD20 MAb makes the immunocytokine more potent and effective than either agent alone or in combination. MAb targeting of IFNα to tumors may allow a less frequent dosing schedule of a single agent, reduce or eliminate side effects associated with IFN therapy, and result in profoundly enhanced efficacy. Additionally, targeted IFNα can induce an acute tumor-directed immune response and possibly evoke immune memory via pleiotropic stimulation of innate and adaptive immunity (Belardelli et al, 2002, Cytokine Growth Factor Rev 12:119-34). Other groups have produced MAb-IFNα made by chemical conjugation that revealed some of the potential clinical benefits of such constructs (Pelham et al., 1983, Cancer Immunol Immunother 15:210-16; Ozzello et al., 1998, Breast Cancer Res Treat 48:135-47). A recombinant MAb-IFNα comprising murine IFNα and an anti-HER2/neu MAb exhibited potent inhibition of a transgenic (HER2/neu) murine B-cell lymphoma in immunocompetent

mice and was also capable of inducing a protective adaptive immune response with immunologic memory (Huang et al., 2007, J Immunol 179:6881-88).

[0219] We expect that therapy with 20-2b will stimulate localized recruitment and activation of a number of immune cells, including NK, T4, T8, and dendritic cells, resulting in enhanced cytotoxicity and ADCC, and may potentially induce tumor-directed immunologic memory. However, murine cells are exceedingly less sensitive (~4 logs) than human cells to human IFNα2b (Kramer et al., 1983, J Interferon Res 3:425-35; Weck et al., 1981, J Gen Virol 57:233-37). Therefore, very little, if any, of the anti-lymphoma activity of 20-2b in the mouse model *in vivo* studies described above can be attributed to IFNα2b activation of the mouse immune response. Rather, killing is due primarily to the direct action of IFNα2b on the lymphoma cells.

[0220] We have shown that 20-2b has augmented ADCC, which may be the most important MOA of anti-CD20 immunotherapy. However, since human IFNα2b is only a very weak stimulator of the murine host's immune effector cells, an IFNα-enhanced ADCC is probably not realized as it might be in humans. Even with these limitations, the *in vivo* results demonstrate that 20-2b can be a highly effective anti-lymphoma agent, exhibiting more than 100-times the potency of v-mab or a non-targeting MAb-IFNα in the IFNα-sensitive Daudi model. Even with lymphoma models that are relatively insensitive to the direct action of IFNα (Raji/NAMALWA) or are resistant to anti-CD20 immunotherapy (NAMALWA), 20-2b showed superior efficacy to either v-mab or non-targeted MAb-IFNα.

[0221] Fusion of IFNα2b to v-mab increases its *in vivo* potency by extending circulation times and enabling tumor targeting. The therapeutic significance of Pk was demonstrated in the Daudi model, where the slower clearing PEGASYS was superior to the faster clearing PEG-Intron, which has a higher specific activity (data not shown). 20-2b was considerably more potent than either PEGASYS or 734-2b, suggesting that lymphoma targeting via the anti-CD20 MAb is critical to its superior potency and efficacy. Surprisingly, the impact of targeting was evident even in the *in vitro* assays. In the *in vitro* proliferation experiments, which only allow for lymphoma inhibition via signaling, 20-2b showed activity at a 25-fold lower concentration compared to non-targeting MAb-IFNα, either alone or when combined with v-mab. The *ex vivo* setting allows the involvement of all three of the anti-CD20 MOA. Even without CDC activity, 20-2b was more effective at depleting lymphoma from blood than IFNα or v-mab, either alone or in combination, demonstrating the significance of targeting. The influence of MAb targeting in the *in vitro/ex vivo* studies is somewhat

surprising, because the MAbs, effector, and target cells are all confined throughout the experiments. We expect that 20-2b will have a substantially greater impact *in vivo* in human patients.

[0222] The IFNα2b and v-mab components of 20-2b can apparently act additively or synergistically, to contribute to its enhanced potency. The *in vitro* proliferation assays suggest at least an additive effect, which was substantiated with the results of the *ex vivo* studies where the combination of v-mab and 734-2b was superior to either agent alone. This may be accomplished *ex vivo* via increased ADCC activity of v-mab as part of 20-2b or when combined with 734-2b, yet ADCC is not functional in the *in vitro* proliferation assays, suggesting additional mechanisms. The signal transduced by v-mab-bound CD20 may potentiate the IFNα signal, resulting in enhanced potency. Alternatively, the binding of v-mab, which is a slowly internalizing MAb, may prevent the internalization/down-regulation of the Type-I IFN receptors, resulting in a more prolonged and effective IFNα-induced signal.

Example 19. Generation of DDD module based on Erythropoeitin (EPO)

Construction of EPO-DDD2-pdHL2 for expression in mammalian cells

[0223] The cDNA sequence for EPO was amplified by PCR resulting in EPO fused at its C-terminus to a polypeptide consisting of:
KSHHHHHHGSGGGGSGGGCGHIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTR LREARA (SEQ ID NO:88)

[0224] PCR amplification was accomplished using a full-length human EPO cDNA clone as a template and the following oligonucleotides as primers:

EPO Xba I left

TCTAGACACAGGACCTCATCATGGGGGTGCACGAATGTCC (SEQ ID NO:91)

EPO BamHI Right

GGATCCATGATGGTGATGATGGTGTGACTTTCTGTCCCCTGTCCTGCAG (SEQ ID NO:92)

[0225] The PCR amplimer was cloned into the pGemT vector. A DDD2-pdHL2 mammalian expression vector was prepared for ligation with EPO by digestion with XbaI and Bam HI restriction endonucleases. The EPO amplimer was excised from pGemT with XbaI and Bam HI and ligated into the DDD2-pdHL2 vector to generate the expression vector EPO-DDD2-pdHL2.

Mammalian cell expression of EPO-DDD2

[0226] EPO-pdHL2 was linearized by digestion with SalI enzyme and stably transfected into Sp/ESF myeloma cells by electroporation. Clones were selected with media containing 0.15 μM MTX. Clones # 41, 49 and 37 each were shown to produce ~0.5 mg/L of EPO by an ELISA using Nunc Immobilizer Nickel-Chelate plates to capture the His-tagged fusion protein and detection with anti-EPO antibody. Approximately 2.5 mg of EPO-DDD2 was purified by IMAC from 9.6 liters of serum-free roller bottle culture.

Example 20. Generation of 734-EPO, a DNL conjugate comprising four EPO-DDD2 moieties linked to C_{H3}-AD2-IgG-h734

[0227] 734-EPO was produced as described above for 20-2b. SE-HPLC analysis of the protein A-purified 734-EPO showed a major peak and a shoulder of a higher molecular size (not shown). The retention time of the major peak was consistent with a covalent complex composed of an IgG and 4 EPO groups. The shoulder was likely due to a non-covalent dimer of the IgG-EPO conjugate. SDS-PAGE analysis with Coomassie blue staining and anti-EPO immunoblot analysis showed that under non-reducing conditions the product had a Mr of >260 kDa (not shown), consistent with the deduced MW of ~310 kDa. Under reducing conditions the bands representing the three constituent polypeptides of 734-EPO (EPO-DDD2, Heavy chain-AD2, and light chain) were evident and appeared to be similar in quantity (not shown). Non-product contaminants were not detected.

[0228] EPO-DDD2 and 734-EPO were assayed for their ability to stimulate the growth of EPO-responsive TF1 cells (ATCC) using recombinant human EPO (Calbiochem) as a positive control. TF1 cells were grown in RPMI 1640 media supplemented with 20% FBS without GM-CSF supplementation in 96-well plates containing 1 x 10^4 cells/well. The concentrations (units/ml) of the EPO constructs were determined using a commercial kit (Human erythropoietin ELISA kit, Stem Cell Research, Cat# 01630). Cells were cultured in the presence of rhEPO, EPO-DDD2 or 734-EPO at concentrations ranging from 900 u/ml to 0.001 U/ml for 72 hours. The viable cell densities were compared by MTS assay using 20 μl of MTS reagent/well incubated for 6 hours before measuring the OD490 in a 96-well plate reader. Dose response curves and EC_{50} values were generated using Graph Pad Prism software (**FIG. 7**). Both EPO-DDD2 and 734-EPO showed in vitro biological activity that was approximately 10% of rhEPO.

Example 21. Generation of DDD module based on Granulocyte-Colony Stimulating Factor (G-CSF)

Construction of G-CSF-DDD2-pdHL2 for expression in mammalian cells

[0229] The cDNA sequence for G-CSF was amplified by PCR, resulting in G-CSF fused at its C-terminus to a polypeptide consisting of SEQ ID NO:88. PCR amplification was accomplished using a full-length human G-CSF cDNA clone (Invitrogen IMAGE human cat# 97002RG Clone ID 5759022) as a template and the following oligonucleotides as primers:

G-CSF XbaI Left

TCTAGACACAGGACCTCATCATGGCTGGACCTGCCACCCAG (SEQ ID NO:93)

G-CSF BamHI-Right

GGATCCATGATGGTGATGATGGTGTGACTTGGGCTGGGCAAGGTGGCGTAG (SEQ ID NO:94).

[0230] The PCR amplimer was cloned into the pGemT vector. A DDD2-pdHL2 mammalian expression vector was prepared for ligation with G-CSF by digestion with XbaI and Bam HI restriction endonucleases. The G-CSF amplimer was excised from pGemT with XbaI and Bam HI and ligated into the DDD2-pdHL2 vector to generate the expression vector G-CSF-DDD2-pdHL2.

Mammalian cell expression of G-CSF-DDD2

[0231] G-CSF-pdHL2 was linearized by digestion with Sal I enzyme and stably transfected into Sp/ESF myeloma cells by electroporation. Clones were selected with media containing 0.15 μM MTX. Clone # 4 was shown to produce 0.15 mg/L of G-CSF-DDD2 by sandwich ELISA.

Purification of G-CSF-DDD2 from batch cultures grown in roller bottles

[0232] Clone #4 was expanded to 34 roller bottles containing a total of 20 L of Hybridoma SFM with 0.4 μM MTX and allowed to reach terminal culture. The supernatant fluid was clarified by centrifugation, filtered (0.2 μM), diafiltered into 1X Binding buffer (10 mM Imidazole, 0.5 M NaCl, 50 mM NaH_2PO_4, pH 7.5 and concentrated. The concentrate was purified by IMAC.

Construction and expression of G-CSF-DDD2 in E. coli.

[0233] G-CSF-DDD2 was also expressed by microbial fermentation as a soluble protein in *E. coli*. The coding sequence was amplified by PCR using G-CSF-DDD2-pdHL2 DNA as a template. The amplimer was cloned into the pET26b *E. coli* expression vector using Nde I and Xho I restriction sites. Protein was expressed intracellularly in BL21pLysS host cells by

induction of LB shake flasks with 100 μM IPTG at 18°C for 12 hours. Soluble G-CSF-DDD2 was purified from cell lysates by IMAC.

Construction and expression of N-DDD2-G-CSF(C17S) in E. coli.

[0234] An alternative G-CSF-DDD2 module was made by fusing the DDD2 sequence and a peptide spacer at the N-terminus of G-CSF(C17S), which differs from the wild-type by substituting the unpaired cysteine residue at the 17th position with a serine. N-DDD2-G-CSF(C17S) was expressed in E. coli and purified by IMAC.

Example 22. Generation of hR1-17S, a DNL conjugate comprising four N-DDD2-G-CSF(C17S) moieties linked to C_{H3}-AD2-IgG-hR1.

[0235] hR1-17S was produced by combining C_{H3}-AD2-IgG-hR1 with excess N-DDD2-G-CSF(C17S) under redox conditions following purification by Protein A affinity chromatography. SE-HPLC analysis of the protein A-purified hR1-17S showed a major peak and a shoulder of a higher molecular size (not shown). The retention time of the major peak was consistent with a covalent complex composed of an IgG and 4 G-CSF groups. The shoulder was likely due to a non-covalent dimer of the IgG-G-CSF conjugate. SDS-PAGE analysis with Coomassie blue staining and anti-G-CSF immunoblot analysis showed that under non-reducing conditions the product had an Mr consistent with the deduced MW of ~260 kDa. Under reducing conditions, bands representing the three constituent polypeptides of hR1-17S (N-DDD2-G-CSF(C17S), Heavy chain-AD2, and light chain) were detected (not shown).

Example 23. Production and Use of a DNL Construct Comprising Two Different Antibody Moieties and a Cytokine

[0236] As discussed above, 20-2b, a monospecific immunocytokine generated by the dock-and-lock (DNL) method to comprise tetrameric IFN-α2b covalently linked to veltuzumab, a humanized anti-CD20 mAb, exhibited very potent anti-tumor activity *in vitro* and in human lymphoma xenografts. However, lymphomas and leukemias that express little or no CD20 are expected to be resistant to therapy with 20-2b. HLA-DR is expressed on many hematopoietic tumors and some solid cancers. A bispecific immunocytokine that could target IFN-α to both CD20 and HLA-DR might be a more effective therapeutic against a wide variety of hematopoietic malignancies, including those that express CD20, HLA-DR, or both. Since each component of the multifunctional complex (veltuzumab, anti-HLA-DR $F(ab)_2$, and IFN-α2b) has anti-tumor activity independently, we evaluated if the bispecific immunocytokine can potentially be even more potent than the monospecific immunocytokine, 20-2b.

[0237] We report here the generation and characterization of the first bispecific MAb-IFNα, designated 20-C2-2b, which comprises two copies of IFN-α2b and a stabilized $F(ab)_2$ of hL243 (humanized anti-HLA-DR; IMMU-114) site-specifically linked to veltuzumab (humanized anti-CD20). *In vitro*, 20-C2-2b inhibited each of four lymphoma and eight myeloma cell lines, and was more effective than monospecific CD20-targeted MAb-IFNα or a mixture comprising the parental antibodies and IFNα in all but one ($HLA\text{-}DR^-/CD20^-$) myeloma line, suggesting that 20-C2-2b should be useful in the treatment of various hematopoietic malignancies. The 20-C2-2b displayed greater cytotoxicity against KMS12-BM ($CD20^+/HLA\text{-}DR^+$ myeloma) than monospecific MAb-IFNα that targets only HLA-DR or CD20, indicating that all three components in 20-C2-2b can contribute to toxicity. Our findings indicate that a given cell's responsiveness to MAb-IFNα depends on its sensitivity to IFNα and the specific antibodies, as well as the expression and density of the targeted antigens.

[0238] Because 20-C2-2b has antibody-dependent cellular cytotoxicity (ADCC), but not CDC, and can target both CD20 and HLA-DR, it is useful for therapy of a broad range of hematopoietic cancers that express either or both antigens. The bispecific immunocytokine appears to be particularly effective in the elimination of the putative cancer stem cells associated with myeloma, which are resistant to current therapy regimens and reportedly express CD20.

Materials and Methods

[0239] Antibodies and cell culture The abbreviations used in the following discussion are: 20 (C_H3-AD2-IgG-v-mab, anti-CD20 IgG DNL module); C2 (C_H1-DDD2-Fab-hL243, anti-HLA-DR Fab_2 DNL module); 2b (dimeric IFNα2B-DDD2 DNL module); 734 (anti-in-DTPA IgG DNL module used as non-targeting control). The following MAbs were provided by Immunomedics, Inc.: veltuzumab or v-mab (anti-CD20 IgG_1), hL243γ4p (Immu-114, anti-HLA-DR IgG_4), a murine anti-IFNα MAb, and rat anti-idiotype MAbs to v-mab (WR2) and hL243 (WT). Heat-inactivated FBS was obtained from Hyclone (Logan, UT). All other cell culture media and supplements were purchased from Invitrogen Life Technologies (Carlsbad, CA).

[0240] Sp/ESF cells, a cell line derived from Sp2/0 with superior growth properties were maintained in Hybridoma Serum-Free Media. The NHL and MM cells were grown in RPMI 1640 medium with 10% FBS, 1 mM sodium pyruvate, 10 mM L-glutamine, and 25 mM HEPES. Daudi, Ramos, Raji, Jeko-1, NCI-H929, and U266 human lymphoma cell lines were

purchased from ATCC (Manassas, VA). The sources of MM cell lines are as follows: KMS11, KMS12-PE, and KMS12-BM from Dr. Takemi Otsuki (Kawasaki Medical School, Okayama, Japan); CAG, OPM-6 and MM.1R from Dr. Joshua Epstein (University of Arkansas, Little Rock, AK), Dr. Kenji Oritani (Osaka University, Osaka, Japan) and Dr. Steven Rosen (Northwestern University, Chicago, IL), respectively. All cell lines were authenticated by the supplier, obtained within 6 months of their use and passaged less than 50 times. We did not re-authenticate the cell lines.

[0241] DNL constructs Monospecific MAb-IFNα (20-2b-2b, 734-2b-2b and C2-2b-2b) and the bispecific HexAb (20-C2-C2) were generated by combination of an IgG-AD2-module with DDD2-modules using the DNL method, as described in the preceding Examples. The 734-2b-2b, which comprises tetrameric IFNα2b and MAb h734 [anti-Indium-DTPA IgG_1], was used as a non-targeting control MAb-IFNα.

[0242] 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-v-mab are disclosed in the preceding Examples. The expressed recombinant fusion protein has the AD2 peptide linked to the carboxyl terminus of the C_H3 domain of v-mab 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-vmab productivity via a sandwich ELISA. The module was produced in roller bottle culture with serum-free media.

[0243] The DDD-module, IFNα2b-DDD2, was generated as discussed in Example 16 by recombinant fusion of the DDD2 peptide to the carboxyl terminus of human IFNα2b via an 18 amino acid long flexible linker peptide. As is the case for all DDD-modules, the expressed fusion protein spontaneously forms a stable homodimer.

[0244] The production, characterization and use of a variety of C_H1-DDD2-Fab modules was performed as discussed in Examples 1-7. The C_H1-DDD2-Fab-hL243 expression vector was generated from hL243-IgG-pdHL2 vector by excising the sequence for the C_H1-Hinge-C_H2-C_H3 domains with SacII and EagI restriction enzymes and replacing it with a 507 bp sequence encoding C_H1-DDD2, which was excised from the C-DDD2-hMN-14-pdHL2

expression vector with the same enzymes. Following transfection of C_H1-DDD2-Fab-hL243-pdHL2 into Sp/ESF cells by electroporation, stable, MTX-resistant clones were screened for productivity via a sandwich ELISA using 96-well microtiter plates coated with mouse anti-human kappa chain to capture the fusion protein, which was detected with horseradish peroxidase-conjugated goat anti-human Fab. The module was produced in roller bottle culture.

[0245] Roller bottle cultures in serum-free H-SFM media and fed-batch bioreactor production resulted in yields comparable to other IgG-AD2 modules and cytokine-DDD2 modules generated to date. C_H3-AD2-IgG-v-mab 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 (Rossi et al., Blood 2009, 114:3864-71). The culture broth containing the C_H1-DDD2-Fab-hL243 module was applied directly to KappaSelect affinity gel (GE-Healthcare), which was washed to baseline with PBS and eluted with 0.1 M Glycine, pH 2.5.

[0246] The purity of the DNL modules was assessed by SDS-PAGE and SE-HPLC (not shown). Analysis under non-reducing conditions shows that, prior to the DNL reaction, IFNα2b-DDD2 and C_H1-DDD2-Fab-hL243 exist as disulfide-linked dimers (not shown). 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-v-mab (not shown) 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 (not shown). For C_H3-AD2-IgG-v-mab, heavy chain-AD2 and kappa light chains were identified. hL243-Fd-DDD2 and kappa light chain polypeptides were resolved for C_H1-DDD2-Fab-hL243 (not shown). One major and one minor band were resolved for IFNα2b-DDD2 (not shown), which were determined to be non-glycosylated and O-glycosylated species, respectively.

[0247] Generation of 20-C2-2b by DNL Three DNL modules (C_H3-AD2-IgG-v-mab, C_H1-DDD2-Fab-hL243, and IFN-α2b-DDD2) were combined in equimolar quantities to generate the bsMAb-IFNα, 20-C2-2b. Following an overnight docking step under mild reducing

conditions (1mM reduced glutathione) at room temperature, oxidized glutathione was added (2mM) to facilitate disulfide bond formation (locking). The 20-C2-2b was purified to near homogeneity using three sequential affinity chromatography steps. Initially, the DNL mixture was purified with Protein A (MAbSelect), which binds the C_H3-AD2-IgG-v-mab group and eliminates un-reacted IFNα2b-DDD2 or C_H1-DDD2-Fab-hL243. The Protein A-bound material was further purified by IMAC using His-Select HF Nickel Affinity Gel, which binds specifically to the IFNα2b-DDD2 moiety and eliminates any constructs lacking this group. The final process step, using an hL243-anti-idiotype affinity gel removed any molecules lacking C_H1-DDD2-Fab-hL243.

[0248] The skilled artisan will realize that affinity chromatography may be used to purify DNL complexes comprising any combination of effector moieties, so long as ligands for each of the three effector moieties can be obtained and attached to the column material. The selected DNL construct is the one that binds to each of three columns containing the ligand for each of the three effector moieties and can be eluted after washing to remove unbound complexes.

[0249] The following example is representative of several similar preparations of 20-C2-2b. Equimolar amounts of C_H3-AD2-IgG-v-mab (15 mg), C_H1-DDD2-Fab-hL243 (12 mg), and IFN-α2b-DDD2 (5 mg) were combined in 30-mL reaction volume and 1 mM reduced glutathione was added to the solution. Following 16 h at room temperature, 2 mM oxidized glutathione was added to the mixture, which was held at room temperature for an additional 6 h. The reaction mixture was applied to a 5-mL Protein A affinity column, which was washed to baseline with PBS and eluted with 0.1 M Glycine, pH 2.5. The eluate, which contained ~20 mg protein, was neutralized with 3 M Tris-HCl, pH 8.6 and dialyzed into HisSelect binding buffer (10 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0) prior to application to a 5-mL HisSelect IMAC column. The column was washed to baseline with HisSelect binding buffer and eluted with 250 mM imidazole, 150 mM NaCl, 50 mM NaH_2PO_4, pH 8.0.

[0250] The IMAC eluate, which contained ~11.5 mg of protein, was applied directly to a WP (anti-hL243) affinity column, which was washed to baseline with PBS and eluted with 0.1 M Glycine, pH 2.5. The process resulted in 7 mg of highly purified 20-C2-2b. This was approximately 44% of the theoretical yield of 20-C2-2b, which is 50% of the total starting material (16 mg in this example) with 25% each of 20-2b-2b and 20-C2-C2 produced as side products.

[0251] Analytical methods Size-exclusion HPLC (SE-HPLC) was performed using an

Alliance HPLC System with a BioSuite 250, 4 μm UHR SEC column (Waters Corp., Milford MA). Immunoreactivity was assessed by mixing excess WT, anti-IFNα, or WR2 with 20-C2-2b prior to analysis of the resulting immune complex by SE-HPLC. SDS-PAGE was performed under reducing and non-reducing conditions using 12% and 4 - 20% gradient Tris-glycine gels (Invitrogen, Gaithersburg, MD), respectively.

[0252] Electrospray ionization time of flight (ESI-TOF) liquid chromatography/mass spectrometry (LC/MS) was performed with a 1200-series HPLC coupled with a 6210 TOF MS (Agilent Technologies, Santa Clara, CA). The 20-C2-2b was reduced with 10 mM tris(2-carboxyethyl)phosphine at 60°C for 30 min and resolved by reversed phase HPLC (RP-HPLC), using a 10-min gradient of 20 – 90% acetonitrile in 0.1% aqueous formic acid with a Poroshell 300 SB, 5 μm C8 column (Agilent). For the TOF MS, the capillary and fragmentor voltages were set to 5500 and 200 V, respectively.

[0253] IFNα2b specific activities were determined using the iLite Human Interferon Alpha Cell-Based Assay Kit (PBL Interferon Source, Piscataway, NJ). Peginterferon alfa-2b (Schering Corp) was used as a positive control.

[0254] Cell binding and apoptosis Cell binding and apoptosis were assessed by flow cytometry using a Guava PCA and the reagents, software and suggested protocols for Guava Express and Guava Nexin, respectively (Millipore, Billerica, MA). For binding assays, live cells were incubated for 1 h at 4°C with MAbs or MAb-IFNα diluted in 1% BSA-PBS. Cells were pelleted and washed twice with 1% BSA-PBS before incubation for 1 h at 4°C with 2 μg/mL PE-conjugated mouse-anti human IgG-Fc (Southern Biotech, Birmingham, AL). After three washes, binding was measured by flow cytometry. For apoptosis assays, cells (5 x 10^5/mL) were incubated with the indicated MAb or MAb-IFNα in 24-well plates for 48 h before quantification of the % annexin-V-positive cells.

[0255] *In-vitro* cytotoxicity Cells were seeded in 48-well plates (300 μL/well) at pre-determined optimal initial densities (1 – 2.5 x 10^5 cells/mL) in the presence of increasing concentrations of the indicated agents and incubated at 37°C until the density of untreated cells increased ≥10-fold (4 - 7 days). Relative viable cell densities at the end of the assay were determined using a CellTiter 96 Cell Proliferation Assay (Promega, Madison, WI).

[0256] *Ex-vivo* depletion of Daudi from whole blood Blood specimens were collected under a protocol approved by the New England Institutional Review Board (Wellesley, MA). Daudi (5 x 10^4) cells were mixed with heparinized whole blood (150 μL) from healthy volunteers and incubated with MAbs or MAb-IFNα at 1 nM for 2 days at 37°C and 5% CO_2. Cells were

stained with FITC-anti-CD19, FITC-anti-CD14, APC-anti-CD3 or APC-mouse IgG_1 isotype control MAb (BD Biosciences, San Jose, CA) and analyzed by flow cytometry using a FACSCalibur (BD Biosciences). Daudi cells are CD19+ and in the monocyte gate. Normal B and T cells are CD19+ and CD3+ cells, respectively, in the lymphocyte gate. Monocytes are CD14+ cells in the monocyte gate.

Results

[0257] Generation and characterization of 20-C2-2b The bispecific MAb-IFNα was generated by combining the IgG-AD2 module, C_H3-AD2-IgG-v-mab, with two different dimeric DDD-modules, C_H1-DDD2-Fab-hL243 and IFNα2b-DDD2. Due to the random association of either DDD-module with the two AD2 groups, two side-products, 20-C2-C2 and 20-2b-2b are expected to form, in addition to 20-C2-2b.

[0258] Non-reducing SDS-PAGE (not shown) resolved 20-C2-2b (~305 kDa) as a cluster of bands positioned between those of 20-C2-C2 (~365 kDa) and 20-2b-2b (255 kDa). Reducing SDS-PAGE resolved the five polypeptides (v-mab HC-AD2, hL243 Fd-DDD2, IFNα2b-DDD2 and co-migrating v-mab and hL243 kappa light chains) comprising 20-C2-2b (not shown). IFNα2b-DDD2 and hL243 Fd-DDD2 are absent in 20-C2-C2 and 20-2b-2b. MAbSelect binds to all three of the major species produced in the DNL reaction, but removes any excess IFNα2b-DDD2 and C_H1-DDD2-Fab-hL243. The His-Select unbound fraction contained mostly 20-C2-C2 (not shown). The unbound fraction from WT affinity chromatography comprised 20-2b-2b (not shown). Each of the samples was subjected to SE-HPLC and immunoreactivity analyses, which corroborated the results and conclusions of the SDS-PAGE analysis.

[0259] Following reduction of 20-C2-2b, its five component polypeptides were resolved by RP-HPLC and individual ESI-TOF deconvoluted mass spectra were generated for each peak (not shown). Native, but not bacterially-expressed recombinant IFNα2, is O-glycosylated at Thr-106 (Adolf et al., Biochem J 1991;276 (Pt 2):511-8). We 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 (not shown). LC/MS analysis of 20-C2-2b identified both the O-glycosylated and non-glycosylated species of IFNα2b-DDD2 with mass accuracies of 15 ppm and 2 ppm, respectively (not shown). The observed mass of the O-glycosylated form indicates an O-linked glycan having the structure NeuGc-NeuGc-Gal-GalNAc, which was also predicted (<1 ppm) for 20-2b-2b (not shown). LC/MS identified both v-mab and hL243 kappa chains as well as hL243-Fd-DDD2 (not

shown) as single, unmodified species, with observed masses matching the calculated ones (<35 ppm). Two major glycoforms of v-mab HC-AD2 were identified as having masses of 53,714.73 (70%) and 53,877.33 (30%), indicating G0F and G1F N-glycans, respectively, which are typically associated with IgG (not shown). 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.

[0260] SE-HPLC analysis of 20-C2-2b resolved a predominant protein peak with a retention time (6.7 min) consistent with its calculated mass and between those of the larger 20-C2-C2 (6.6 min) and smaller 20-2b-2b (6.85 min), as well as some higher molecular weight peaks that likely represent non-covalent dimers formed via self-association of IFNα2b (not shown).

[0261] Immunoreactivity assays demonstrated the homogeneity of 20-C2-2b with each molecule containing the three functional groups (not shonw). Incubation of 20-C2-2b with an excess of antibodies to any of the three constituent modules resulted in quantitative formation of high molecular weight immune complexes and the disappearance of the 20-C2-2b peak. The His-Select and WT affinity unbound fractions were not immunoreactive with WT and anti-IFNα, respectively (not shown).

[0262] Cell binding The MAb-IFNα showed similar binding avidity to their parental MAbs (**FIG. 8A**). At sub-saturating concentrations, similar binding levels were observed for 20-C2-2b and hL243γ4p. The antigen density of HLA-DR is ~6-fold greater than CD20 in these cells, allowing more binding of 20-C2-2b compared to 20-2b-2b. Binding curves, which were analyzed using a one-site binding non-linear regression model, demonstrated that 20-C2-2b can achieve a 4.7-fold higher B_{max} compared to 20-2b-2b, with no significant difference observed between their binding affinities (K_d ~4 nM) (**FIG. 8B**).

[0263] IFNα biological activity The specific activities for various MAb-IFNα were measured using a cell-based reporter gene assay and compared to peginterferon alfa-2b (**FIG. 8C**). Expectedly, the specific activity of 20-C2-2b (2454 IU/pmol), which has two IFNα2b groups, was significantly lower than those of 20-2b-2b (4447 IU/pmol) or 734-2b-2b (3764 IU/pmol), yet greater than peginterferon alfa-2b (P<0.001). The difference between 20-2b-2b and 734-2b-2b was not significant. The specific activity among all agents varies minimally when normalized to IU/pmol of total IFNα. Based on these data, the specific activity of each IFNα2b group of the MAb-IFNα is approximately 30% of recombinant IFNα2b (~4000 IU/pmol).

[0264] *In-vitro* cytotoxicity: NHL The results of *in-vitro* cytotoxicity assays with B-cell

NHL are summarized in **Table 3.** The relative antigen densities of HLA-DR and CD20 for each cell line has been reported (Stein et al., Blood 2010, in Press). The targeting index (TI) represents the fold-increase in potency of a targeted MAb-IFNα compared to non-targeted MAb-IFNα (734-2b-2b), with the EC_{50} values converted to total IFNα concentration (I-EC_{50}). Daudi is very sensitive to cell killing by IFNα2, as demonstrated with the non-targeting MAb-IFNα, 734-2b-2b (I-EC_{50}=14 pM). Targeting CD20 on Daudi with the monospecific 20-2b-2b (I-EC_{50}=0.4 pM) further enhanced the potency 25-fold (TI=25), consistent with previous results (Rossi et al., Blood 2009;114:3864-71). The potency enhancement for the bispecific 20-C2-2b (I-EC_{50}=0.08 pM; TI=125) was 5-fold greater than 20-2b-2b, which can be attributed to the added antigen density of HLA-DR and possibly its high-avidity tetravalent tumor binding. It is less likely that hL243-induced signaling contributes additional cytotoxicity at these low concentrations. The mixture of v-mab, hL243γ4p and 734-2b-2b (v-mab+hL243+734-2b) was equal to 734-2b-2b alone, supporting the conclusion that hL243-induced signaling does not contribute to the high TI of 20-C2-2b.

[0265] Apoptosis was induced in Daudi with only 1 pM of any MAb-IFNα but not with 10 pM of v-mab or hL243γ4p (**FIG. 9*A***). Treatment with 20-2b-2b or 20-C2-2b resulted in significantly more apoptotic cells than 734-2b-2b or v-mab+hL243+734-2b (P<0.0005). There was no significant difference observed between 734-2b-2b and the mixture.

[0266] The 734-2b-2b had less effect on Raji (I-EC_{50}=32 nM) and Ramos (I-EC_{50}>80 nM), resulting in maximal inhibition (I_{max}) of only 62% and 35%, respectively. Under these conditions, hL243γ4p, but not v-mab (not shown), inhibited these Burkitt lymphoma lines. The observed enhancement in potency of 20-C2-2b (TI=118) was >50-fold greater than 20-2b-2b (TI=2) for Raji, which has much greater HLA-DR antigen density than CD20. Unlike Daudi and Raji, the densities of HLA-DR and CD-20 are similar on Ramos, yet the TI for 20-C2-2b was 15-fold greater than 20-2b-2b, indicating additive activities of hL243 and IFNα2b.

[0267] The v-mab+hL234+734-2b mixture was more potent than any of the single agents alone for Raji and Ramos. Targeting the IFNα2b was critical for achieving maximal potency. In each of the three Burkitt lymphoma lines, 20-C2-2b was more effective than v-mab+hL234+734-2b, which comprises the same number of anti-CD20 and anti-HLA-DR Fabs and twice the amount (and activity) of IFNα2b.

[0268] The mantle cell lymphoma, Jeko-1, was considerably more responsive to hL243γ4p (EC_{50}=0.4 nM) and less sensitive to IFNα2b (minimal effect with 734-2b-2b). Any treatment

comprising hL243 was superior to 20-2b-2b (EC_{50}=1 nM). The 20-C2-2b exhibited two-fold enhanced potency compared to hL243γ4p or v-mab+hL243+734-2b. At 0.5 nM, hL243γ4p and 734-2b-2b induced similar levels of apoptosis and their effects are apparently additive, since treatment with v-mab+hL243+734-2b resulted in approximately twice the number of annexin-V-positive cells compared to either agent alone (**FIG. 9*A***). Presumably, v-mab has little contribution in the mixture, since alone it had only a modest effect. Both 20-C2-2b and the mixture were superior to 20-2b-2b (P<0.002), due to the action of hL243.

[0269] *In-vitro* cytotoxicity: Myeloma Whereas the eight MM cell lines vary in HLA-DR levels (and only KMS12-BM expresses CD20) and sensitivity to IFNα2b (**FIG. 10**), all responded to 20-C2-2b. Dose-response curves for each of the eight MM cell lines tested are shown in **FIG. 11,** and the results are summarized in **Table 3**. For example, five were highly responsive to IFNα2 (I-EC_{50} <1 nM for 734-2b-2b), but varied in HLA-DR antigen density. Of these, only CAG, which has high HLA-DR density, was inhibited by hL243γ4p (>1 nM), and showed an increased (additive) response to a mixture of hL243γ4p and 734-2b-2b (hL243+734-2b) at higher concentrations. The 20-C2-2b (I-EC_{50}=10 pM) exhibited considerably enhanced potency (TI=55) for CAG.

[0270] Apoptosis of CAG was evident following treatment with hL243γ4p, 20-2b-2b, 734-2b-2b, or hL243+734-2b at 1 nM, but not at 0.1 or 0.01 nM (**FIG. 9*B***). The 20-C2-2b induced apoptosis even at 0.01 nM, and the level observed for 0.1 nM 20-C2-2b was equal or higher than that resulting from any other treatment at 10-fold higher (1 nM) concentration.

[0271] Enhanced potency of 20-C2-2b was evident, but lower, for OPM6 (TI=2), U266 (TI=7) and MM.1R (TI=10), which were each highly-IFNα-responsive but have lower HLA-DR density and were not inhibited by hL243γ4p. No increased potency was observed for 20-C2-2b on NCI-H929, which was highly IFNα-responsive but is HLA-DR^-.

[0272] KMS12-BM has high HLA-DR and CD20 antigen densities, and surprisingly, was inhibited by 20-2b-2b (I-EC_{50}=31 nM) but not 734-2b-2b (I-EC_{50} >100 nM) or v-mab. KMS12-BM was more responsive to v-mab+hL243+734-2b (EC_{50}=3 nM) compared to hL243+734-2b (EC_{50}=0.7 nM), which in turn was superior to hL243γ4p alone (EC_{50}=3.5 nM). Each of these treatments resulted in strong induction of apoptosis, with the relative levels consistent with the *in-vitro* cytotoxicity results (**FIG. 9*C***). Additionally, v-mab+hL243 induced more apoptosis than hL243γ4p alone, but less than v-mab+hL243+734-2b. These results suggest that for the HLA-DR^+/$CD20^+$ MM cells, the activity of all three components of 20-C2-2b (EC_{50}=0.1 nM) can contribute to cytotoxicity when combined, even though two

of them have virtually no effect when used alone.

[0273] Evaluation of two additional DNL constructs in KMS12-BM helped elucidate the enhanced potency of 20-C2-2b. A MAb-IFNα designated C2-2b-2b, which comprises hL243 IgG_1 and tetrameric IFNα2b (twice that of 20-C2-2b) exhibited less potent cytotoxicity (EC_{50}=0.4 nM) and weaker apoptosis-induction compared to 20-C2-2b, supporting a contribution of v-mab. More revealing was the finding that 20-C2-C2, a bispecific MAb comprising v-mab and four HLA-DR Fabs, showed high-level induction of apoptosis and >50-fold enhanced potency (EC_{50}= 0.06 nM) compared to hL243γ4p, indicating that crosslinking of HLA-DR and CD20, which occurs with 20-C2-2b, effectively induces cytotoxicity, perhaps via a unique signaling cascade. Although each construct was potent (EC_{50}<0.5 nM), 20-C2-C2 (I_{max}=67%) and C2-2b-2b (I_{max}=70%) did not kill KMS12-BM as effectively as 20-C2-2b (I_{max}=99%), supporting the requirement of all three components for achieving the maximal effect. That the v-mab+hL243+734-2b (I_{max}=87%) mixture was the only other treatment resulting in >70% killing substantiates this hypothesis.

[0274] Together, these data demonstrate that antigen density and sensitivity to the actions of IFNα2b, as well as those of the targeting MAbs, are all important determinants of the *in-vitro* responsiveness of a particular cell line to the various MAb-IFNα. However, *in-vivo* tumor killing may be augmented by ADCC and the actions of immune effector cells, which can be stimulated by the high local concentration of IFNα2b.

[0275] Effector functions and stability in human serum We previously reported that 20-2b-2b exhibited enhanced ADCC compared to its parent v-mab (Rossi et al., Blood 2009;114:3864-71). By design, hL243γ4p has diminished ADCC (Stein et al., Blood 2006;108:2736-44). However, 20-C2-C2 induced significantly (P=0.0091) greater ADCC compared to v-mab (not shown). Notably, 20-C2-2b induced significantly greater ADCC than either 20-2b-2b (P=0.0040) or 20-C2-C2 (P=0.0115), indicating an enhancement of the effector function by the presence of IFNα2b. As was demonstrated previously for 20-2b-2b (Rossi et al., Blood 2009;114:3864-71), 20-C2-2b does not induce CDC *in vitro* (not shown).

[0276] Two different assays for stability of 20-C2-2b in human serum gave very similar results, indicating a loss of ~3.5%/day with roughly 65% remaining after 11 days at 37°C (not shown).

[0277] *Ex-vivo* depletion of NHL from whole human blood As shown in **FIG. 12**, Daudi cells were depleted from whole blood (*ex vivo*) more effectively by 20-C2-2b (91%) compared to 20-2b-2b (69%), v-mab (49% depletion), hL243γ4p (46%) or 734-2b-2b (10%).

Both targeted MAb-IFNα were less toxic to normal B cells compared to Daudi. Under these conditions, B cells were significantly depleted by 20-C2-2b (57%) and hL243γ4p (41%), but not by v-mab, 734-2b, or 20-2b-2b. Monocytes were depleted by hL243γ4p (48%), 734-2b-2b (30%), and 20-2b-2b (21%), but not by v-mab. The 20-C2-2b (98%) was highly toxic to monocytes. None of the agents had a significant effect on T cells. Statistical significance with $P<0.001$ was determined by Student's *t*-test for each of the differences in % depletion indicated above.

Discussion

[0278] We and others have reported that fusion proteins comprising CD20-targeting MAbs and IFNα are more effective against NHL compared to combinations of MAb and IFNα in xenograft and syngeneic mouse models, indicating that MAb-IFNα can overcome the toxicity and Pk limitations associated with IFNα (Rossi et al., Blood 2009;114:3864-71; Xuan et al., Blood 2010;115:2864-71). Although CD20 is an attractive candidate for targeted MAb-IFNα therapy of B-cell lymphoma, its expression is largely limited to malignancies of this lineage, with some individuals exhibiting low antigen density. Here we report the first bispecific immunocytokine, 20-C2-2b, which specifically targets IFNα2b to both CD20 and HLA-DR, thus potentially expanding the hematopoietic tumor types amenable to this immunocytokine therapy.

[0279] Anti-HLA-DR MAbs efficiently induce apoptosis, which is mediated by direct signaling without the requirement of additional crosslinking, and are also potent inducers of ADCC and CDC (Stein et al., Blood 2006;108:2736-44; Rech et al., Leuk Lymphoma 2006;47:2147-54) Where ADCC may enhance therapeutic potential, CDC is largely responsible for the pathogenesis of the side effects associated with the MAb infusion (van der Kolk et al., Br J Haematol 2001;115:807-11). The humanized anti-HLA-DR MAb, hL243γ4p, used as a control in this study was engineered for improved clinical safety by using the constant region of the human IgG_4 isotype, resulting in diminished ADCC and CDC. The 20-C2-2b is unique among anti-HLA-DR MAbs in that it lacks CDC, similar to hL243γ4p, but has potent (enhanced) ADCC, making this agent an attractive candidate for immunotherapy.

[0280] In the *ex-vivo* setting, v-mab can deplete cells via signaling-induced apoptosis, ADCC, and CDC. MAb-IFNα can employ enhanced ADCC as well as both MAb- and IFNα2b-induced signaling, but not CDC; and hL243-γ4p is limited to only direct signaling

(Stein et al., Blood 2006;108:2736-44). Although the full spectrum of IFNα-mediated activation of innate and adaptive immunity that might occur *in vivo* is not realized in this setting, it provides pharmacodynamic data. The 20-C2-2b depleted lymphoma cells more effectively than normal B cells and had no effect on T cells. However, it did efficiently eliminate monocytes. Where v-mab had no effect on monocytes, depletion was observed following treatment with hL243α4p and MAb-IFNα, with 20-2b-2b and 734-2b-2b exhibiting similar toxicity. Therefore, the predictably higher potency of 20-C2-2b is attributed to the combined actions of anti-HLA-DR and IFNα, which may be augmented by HLA-DR targeting. These data suggest that monocyte depletion may be a pharmacodynamic effect associated anti-HLA-DR as well as IFNα therapy; however, this side affect would likely be transient because the monocyte population should be repopulated from hematopoeitic stem cells.

[0281] The four NHL and eight MM cell lines we studied encompass the naturally-occurring heterogeneity in expression and antigen density of HLA-DR and CD20, as well as responsiveness to the actions of IFNα, hL243 and v-mab, which all impact MAb-IFNα immunotherapy. Six and eight (of twelve lines) were inhibited (I_{max} >30%) to varying degrees by hL243γ4p and 734-2b-2b, respectively. The 20-C2-2b potently inhibited (EC_{50} <1 nM) 11 of the 12 cell lines, with an EC_{50} ≤0.01 nM for five. Even the least affected MM line (KMS11), which was not inhibited by 734-2b-2b, was responsive to 20-C2-2b (EC_{50} = 17 nM).

[0282] An enhancement in potency of 20-C2-2b over 734-2b-2b was observed in all of the lines besides NCI-H929, which is HLA-DR$^-$/CD20$^-$. Higher levels of HLA-DR antigen density as well as responsiveness to hL243 correlated with a greater TI for 20-C2-2b, demonstrating additive activities of IFNα and hL243, as well as the significance of targeting, even *in vitro*. The 20-C2-2b was superior to the mixture of v-mab+hL243+734-2b in 10 of the lines, further highlighting the impact of tumor targeting, which will be considerably greater *in vivo*, as demonstrated previously for 20-2b-2b (Rossi et al., Blood 2009;114:3864-71). Further, *in-vivo*-targeted MAb-IFNα might elicit a potent anti-tumor immune response.

[0283] Whereas the vast majority of cells comprising primary MM specimens are non-clonogenic and have a plasma cell phenotype (CD138$^+$/CD19$^-$/CD20$^-$), putative MM cancer stem cells are CD138$^-$ and express B-cell surface antigens, including CD45, CD19, CD20, and CD22, reminiscent of memory B cells (Matsui et al., Blood 2004;103:2332-6). Although a variety of clinical approaches have produced responses, MM remains largely incurable due

to relapses thought to be mediated by cancer stem cells, which are resistant to the various therapies. The B-cell phenotype of the putative stem cells prompted clinical investigation with rituximab in MM. However, limited effects on outcome were realized (Treon et al., J Immunother 2002;25:72-81).

[0284] The *in vitro* results with KMS12-BM are compelling, because it is $CD20^+$, similar to the proposed MM stem cells. The 20-C2-2b exhibited potent cytotoxicity and robustly induced apoptosis of KMS12-BM. Even though non-targeted MAb-IFNα and v-mab were ineffective as single agents, they both apparently contribute to cytotoxicity when used in combination with hL243. The results also indicate that bispecific binding of CD20 and HLA-DR may induce an additional (potent) signal that further enhances toxicity to these cells and may sensitize them to IFNα.

[0285] MAb-IFNα produced by DNL exhibits comparable activity to recombinant IFNα. Recently, Xuan et al. reported that anti-CD20-IFNα fusion proteins made by traditional recombinant engineering showed a 300-fold reduction in IFNα activity (Xuan et al., Blood 2010;115:2864-71). This is noteworthy in comparisons of similar Daudi xenograft studies, where a single 17 ng dose of 20-2b-2b significantly improved survival (Rossi et al., Blood 2009;114:3864-71), compared to three 30 μg doses (>5000-fold more) used for recombinant anti-CD20-hIFNα (Xuan et al., Blood 2010;115:2864-71). Studies using IFNα-secreting tumors demonstrated enhanced immune responses elicited by a localized concentration of IFNα (Ferrantini et al., Biochimie 2007;89:884-93). Where this might also be achieved with highly active MAb-IFNα, the reduced activity of traditional recombinant MAb-IFNα may not efficiently recruit and stimulate an anti-tumor immune response, as was reported by Xuan et al. (Blood 2010;115:2864-71)

[0286] The bispecific MAb-IFNα 20-C2-2b is attractive for the treatment of NHL, because each of the three components is active against this disease. This study shows that 20-C2-2b may also be useful for the therapy of MM and other hematopoietic malignancies.

[0287] The skilled artisan will realize that the approach described here to produce and use bispecific immunocytokine, or other DNL constructs comprising three different effector moieties, may be utilized with any combinations of antibodies, antibody fragments, cytokines or other effectors that may be incorporated into a DNL construct.

* * *

[0288] All of the COMPOSITIONS and METHODS disclosed and claimed herein can be made and used without undue experimentation in light of the present disclosure. While the

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compositions and methods have been described in terms of preferred embodiments, it is apparent to those of skill in the art that variations maybe applied to the COMPOSITIONS and METHODS and in the steps or in the sequence of steps of the METHODS described herein without departing from the concept, spirit and scope of the invention. More specifically, certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

[0289] Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

[0290] The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should be taken as an acknowledgement or admission or any form of suggestion that the prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

What is Claimed is:

1. A DNL (dock and lock) construct comprising three different effector moieties, wherein the effector moieties are attached to two DDD (dimerization and docking domain) moieties from protein kinase A (PKA) and one AD (anchoring domain) moiety from an AKAP protein, and wherein the two DDD moieties form a dimer and bind to the AD moiety to form the DNL construct.

2. The DNL construct of claim 1, wherein the DDD moiety has an amino acid sequence from human RIα, RIβ, RIIα or RIIβ PKA.

3. The DNL construct of claim 1, wherein the effector moieties comprise a first antibody or antibody fragment, a second antibody or antibody fragment, and one or more copies of a cytokine.

4. The DNL construct of claim 3, wherein the first antibody or antibody fragment and the second antibody or antibody fragment bind to two different antigens.

5. The DNL construct of claim 4, wherein the first and second antibodies or antibody fragments bind to antigens selected from the group consisting of carbonic anhydrase IX, CCCL19, CCCL21, CSAp, CD1, CD1a, CD2, CD3, CD4, CD5, CD8, CD11A, CD14, CD15, CD16, CD18, CD19, IGF-1R, CD20, CD21, CD22, CD23, CD25, CD29, CD30, CD32b, CD33, CD37, CD38, CD40, CD40L, CD45, CD46, CD52, CD54, CD55, CD59, CD64, CD66a-e, CD67, CD70, CD74, CD79a, CD80, CD83, CD95, CD126, CD133, CD138, CD147, CD154, AFP, PSMA, CEACAM5, CEACAM-6, B7, ED-B of fibronectin, Factor H, FHL-1, Flt-3, folate receptor, GROB, HMGB-1, hypoxia inducible factor (HIF), HM1.24, insulin-like growth factor-1 (ILGF-1), IFN-γ, IFN-α, IFN-β, IL-2, IL-4R, IL-6R, IL-13R, IL-15R, IL-17R, IL-18R, IL-6, IL-8, IL-12, IL-15, IL-17, IL-18, IL-25, IP-10, MAGE, mCRP, MCP-1, MIP-1A, MIP-1B, MIF, MUC1, MUC2, MUC3, MUC4, MUC5, PAM4 antigen, NCA-95, NCA-90, Ia, HM1.24, EGP-1, EGP-2, HLA-DR, tenascin, Le(y), RANTES, T101, TAC, Tn antigen, Thomson-Friedenreich antigens, tumor necrosis antigens, TNF-α, TRAIL receptor (R1 and R2), VEGFR, EGFR, PlGF, complement factors C3, C3a, C3b, C5a, C5, and an oncogene product.

6. The DNL construct of claim 5, wherein the first and second antibodies or antibody fragments are selected from the group consisting of hR1 (anti-IGF-1R), hPAM4 (anti-

mucin), hA20 (anti-CD20), hA19 (anti-CD19), hIMMU31 (anti-AFP), hLL1 (anti-CD74), hLL2 (anti-CD22), hMu-9 (anti-CSAp), hL243 (anti-HLA-DR), hMN-14 (anti-CEACAM5), hMN-15 (anti-CEACAM6), hRS7 (anti-EGP-1) and hMN-3 (anti-CEACAM6).

7. The DNL construct of claim 4, wherein the cytokine is selected from the group consisting of human MIF (macrophage migration inhibitory factor), HMGB-1 (high mobility group box protein 1), TNF-α, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, IL-17, IL-18, IL-19, IL-23, IL-24, CCL19, CCL21, IL-8, MCP-1, RANTES, MIP-1A, MIP-1B, ENA-78, MCP-1, IP-10, Gro-ß, Eotaxin, interferon-α, -β, -λ, G-CSF, GM-CSF, SCF, PDGF, MSF, Flt-3 ligand, erythropoietin, thrombopoietin, CNTF, leptin, oncostatin M, VEGF, EGF, FGF, PlGF, insulin, hGH, calcitonin, Factor VIII, IGF, somatostatin, tissue plasminogen activator and LIF.

8. The DNL construct of claim 4, wherein the first antibody or antibody fragment is veltuzumab, the second antibody or antibody fragment is hL243, and the cytokine is human interferon-α2b.

9. The DNL construct of claim 8, wherein the hL243 antibody or antibody fragment comprises the heavy chain CDR sequences CDR1 (NYGMN, SEQ ID NO:1), CDR2 (WINTYTREPTYADDFKG, SEQ ID NO:2) and CDR3 (DITAVVPTGFDY, SEQ ID NO:3) and the light chain CDR sequences CDR1 (RASENIYSNLA, SEQ ID NO:4), CDR2 (AASNLAD, SEQ ID NO:5), and CDR3 (QHFWTTPWA, SEQ ID NO:6).

10. The DNL construct of claim 1, wherein the three effector moieties are fusion proteins, each fusion protein comprising an AD or DDD moiety.

11. The DNL construct of claim 1, wherein the effector moieties are selected from the group consisting of a protein, a peptide, an antibody, an antigen-binding antibody, an immunomodulator, a cytokine, a hormone, an enzyme, an antisense oligonucleotide, an siRNA, a toxin, a ribonuclease, a xenoantigen, polyethylene glycol (PEG), an anti-angiogenic agent, a cytotoxic agent and a pro-apoptosis agent.

12. The DNL construct of claim 1, wherein the DDD moiety has the amino acid sequence of SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:17, SEQ ID NO:18, the first 44 amino acids of SEQ ID NO:20, the first 44 amino acids of SEQ ID NO:21, SEQ ID NO:22 or SEQ ID

NO:59.

13. The DNL construct of claim 1, wherein the AD moiety has the amino acid sequence of SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:19, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, M SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57 or SEQ ID NO:58.

14. A method of administering a cytokine to a subject, comprising administering a DNL complex according to claim 4 to a subject.

15. The method of claim 14, wherein the first and second antibodies or antibody fragments bind to antigens selected from the group consisting of carbonic anhydrase IX, CCCL19, CCCL21, CSAp, CD1, CD1a, CD2, CD3, CD4, CD5, CD8, CD11A, CD14, CD15, CD16, CD18, CD19, IGF-1R, CD20, CD21, CD22, CD23, CD25, CD29, CD30, CD32b, CD33, CD37, CD38, CD40, CD40L, CD45, CD46, CD52, CD54, CD55, CD59, CD64, CD66a-e, CD67, CD70, CD74, CD79a, CD80, CD83, CD95, CD126, CD133, CD138, CD147, CD154, AFP, PSMA, CEACAM5, CEACAM-6, B7, ED-B of fibronectin, Factor H, FHL-1, Flt-3, folate receptor, GROB, HMGB-1, hypoxia inducible factor (HIF), HM1.24, insulin-like growth factor-1 (ILGF-1), IFN-γ, IFN-α, IFN-β, IL-2, IL-4R, IL-6R, IL-13R, IL-15R, IL-17R, IL-18R, IL-6, IL-8, IL-12, IL-15, IL-17, IL-18, IL-25, IP-10, MAGE, mCRP, MCP-1, MIP-1A, MIP-1B, MIF, MUC1, MUC2, MUC3, MUC4, MUC5, PAM4 antigen, NCA-95, NCA-90, Ia, HM1.24, EGP-1, EGP-2, HLA-DR, tenascin, Le(y), RANTES, T101, TAC, Tn antigen, Thomson-Friedenreich antigens, tumor necrosis antigens, TNF-α, TRAIL receptor (R1 and R2), VEGFR, EGFR, PlGF, complement factors C3, C3a, C3b, C5a, C5, and an oncogene product.

16. The method of claim 15, wherein the first and second antibodies or antibody fragments are selected from the group consisting of hR1 (anti-IGF-1R) hPAM4 (anti-mucin), hA20 (anti-CD20), hA19 (anti-CD19), hIMMU31 (anti-AFP), hLL1 (anti-CD74), hLL2 (anti-CD22), hMu-9 (anti-CSAp), hL243 (anti-HLA-DR), hMN-14 (anti-CEA), hMN-15 (anti-

CEA), hRS7 (anti-EGP-1) and hMN-3 (anti-CEA).

17. The method of claim 14, wherein the cytokine is selected from the group consisting of human MIF (macrophage migration inhibitory factor), HMGB-1 (high mobility group box protein 1), TNF-α, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, IL-17, IL-18, IL-19, IL-23, IL-24, CCL19, CCL21, IL-8, MCP-1, RANTES, MIP-1A, MIP-1B, ENA-78, MCP-1, IP-10, Gro-ß, Eotaxin, interferon-α, -β, -λ, G-CSF, GM-CSF, SCF, PDGF, MSF, Flt-3 ligand, erythropoietin, thrombopoietin, CNTF, leptin, oncostatin M, VEGF, EGF, FGF, PlGF, insulin, hGH, calcitonin, Factor VIII, IGF, somatostatin, tissue plasminogen activator and LIF.

18 The method of claim 14, wherein the first antibody or antibody fragment is veltuzumab, the second antibody or antibody fragment is hL243, and the cytokine is human interferon-α2b.

19. A method of treating a disease selected from the group consisting of cancer, immune dysfunction and autoimmune disease, comprising administering a DNL construct according to claim 1 to a subject with the disease.

20. The method of claim 19, wherein the cancer is selected from the group consisting of non-Hodgkin's lymphoma, B cell lymphoma, B cell leukemia, T cell lymphoma, T cell leukemia, acute lymphoid leukemia, chronic lymphoid leukemia, Burkitt lymphoma, Hodgkin's lymphoma, hairy cell leukemia, acute myeloid leukemia, chronic myeloid leukemia, multiple myeloma, glioma, Waldenstrom's macroglobulinemia, carcinoma, melanoma, sarcoma, glioma, skin cancer, oral cavity cancer, gastrointestinal tract cancer, pulmonary tract cancer, lung cancer, breast cancer, ovarian cancer, prostate cancer, uterine cancer, endometrial cancer, cervical cancer, urinary bladder cancer, pancreatic cancer, bone cancer, liver cancer, gall bladder cancer, kidney cancer, and testicular cancer.

21. The method of claim 19, wherein the autoimmune disease is selected from the group consisting of acute idiopathic thrombocytopenic purpura, chronic idiopathic thrombocytopenic purpura, dermatomyositis, Sydenham's chorea, myasthenia gravis, systemic lupus erythematosus, lupus nephritis, rheumatic fever, polyglandular syndromes, bullous pemphigoid, diabetes mellitus, Henoch-Schonlein purpura, post-streptococcal nephritis, erythema nodosum, Takayasu's arteritis, Addison's disease, rheumatoid arthritis,

multiple sclerosis, sarcoidosis, ulcerative colitis, erythema multiforme, IgA nephropathy, polyarteritis nodosa, ankylosing spondylitis, Goodpasture's syndrome, thromboangitis obliterans, Sjogren's syndrome, primary biliary cirrhosis, Hashimoto's thyroiditis, thyrotoxicosis, scleroderma, chronic active hepatitis, polymyositis/dermatomyositis, polychondritis, pemphigus vulgaris, Wegener's granulomatosis, membranous nephropathy, amyotrophic lateral sclerosis, tabes dorsalis, giant cell arteritis/polymyalgia, pernicious anemia, rapidly progressive glomerulonephritis, psoriasis, or fibrosing alveolitis.

22. The method of claim 19, wherein the first and second antibodies or antibody fragments bind to antigens selected from the group consisting of carbonic anhydrase IX, CCCL19, CCCL21, CSAp, CD1, CD1a, CD2, CD3, CD4, CD5, CD8, CD11A, CD14, CD15, CD16, CD18, CD19, IGF-1R, CD20, CD21, CD22, CD23, CD25, CD29, CD30, CD32b, CD33, CD37, CD38, CD40, CD40L, CD45, CD46, CD52, CD54, CD55, CD59, CD64, CD66a-e, CD67, CD70, CD74, CD79a, CD80, CD83, CD95, CD126, CD133, CD138, CD147, CD154, AFP, PSMA, CEACAM5, CEACAM-6, B7, ED-B of fibronectin, Factor H, FHL-1, Flt-3, folate receptor, GROB, HMGB-1, hypoxia inducible factor (HIF), HM1.24, insulin-like growth factor-1 (ILGF-1), IFN-γ, IFN-α, IFN-β, IL-2, IL-4R, IL-6R, IL-13R, IL-15R, IL-17R, IL-18R, IL-6, IL-8, IL-12, IL-15, IL-17, IL-18, IL-25, IP-10, MAGE, mCRP, MCP-1, MIP-1A, MIP-1B, MIF, MUC1, MUC2, MUC3, MUC4, MUC5, PAM4 antigen, NCA-95, NCA-90, Ia, HM1.24, EGP-1, EGP-2, HLA-DR, tenascin, Le(y), RANTES, T101, TAC, Tn antigen, Thomson-Friedenreich antigens, tumor necrosis antigens, TNF-α, TRAIL receptor (R1 and R2), VEGFR, EGFR, PlGF, complement factors C3, C3a, C3b, C5a, C5, and an oncogene product.

23. The method of claim 19, wherein the first and second antibodies or antibody fragments are selected from the group consisting of hR1 (anti-IGF-1R) hPAM4 (anti-mucin), hA20 (anti-CD20), hA19 (anti-CD19), hIMMU31 (anti-AFP), hLL1 (anti-CD74), hLL2 (anti-CD22), hMu-9 (anti-CSAp), hL243 (anti-HLA-DR), hMN-14 (anti-CEA), hMN-15 (anti-CEA), hRS7 (anti-EGP-1) and hMN-3 (anti-CEA).

24. The method of claim 19, wherein the cytokine is selected from the group consisting of human MIF (macrophage migration inhibitory factor), HMGB-1 (high mobility group box protein 1), TNF-α, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, IL-17, IL-18, IL-19, IL-23, IL-24, CCL19, CCL21, IL-8,

MCP-1, RANTES, MIP-1A, MIP-1B, ENA-78, MCP-1, IP-10, Gro-ß, Eotaxin, interferon-α, -β, -λ, G-CSF, GM-CSF, SCF, PDGF, MSF, Flt-3 ligand, erythropoietin, thrombopoietin, CNTF, leptin, oncostatin M, VEGF, EGF, FGF, PlGF, insulin, hGH, calcitonin, Factor VIII, IGF, somatostatin, tissue plasminogen activator and LIF.

25. The method of claim 19, wherein the first antibody or antibody fragment is veltuzumab, the second antibody or antibody fragment is hL243, and the cytokine is human interferon-α2b.

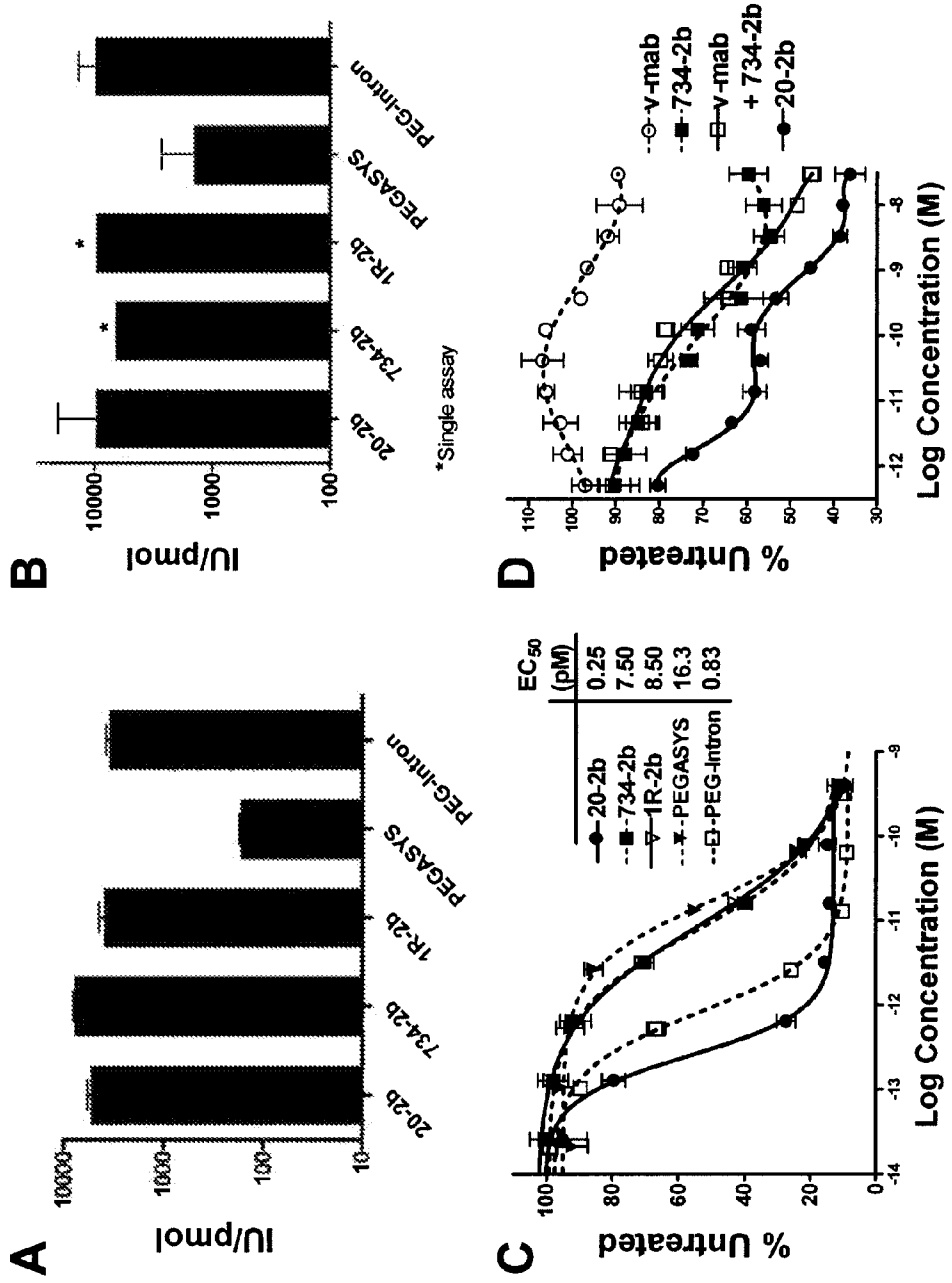
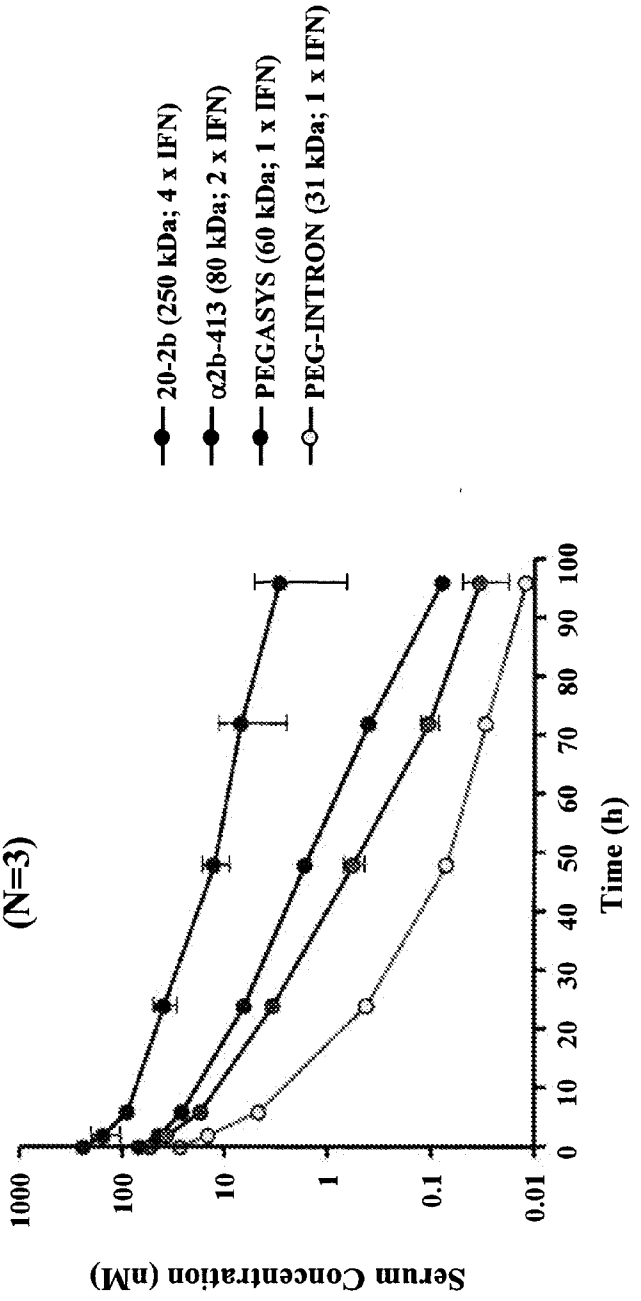

A
IU/pmol
10000
1000
100
10
20-2b
734-2b
1R-2b
PEGASYS
PEG-Intron
B
IU/pmol
10000
1000
100
20-2b
734-2b
1R-2b
PEGASYS
PEG-Intron
*
*
*Single assay
C
% Untreated
100
80
60
40
20
0
-14
-13
-12
-11
-10
-9
Log Concentration (M)
EC50 (pM)
20-2b 0.25
734-2b 7.50
1R-2b 8.50
PEGASYS 16.3
PEG-Intron 0.83
D
% Untreated
110
100
90
80
70
60
50
40
30
-12
-11
-10
-9
-8
Log Concentration (M)
v-mab
734-2b
v-mab + 734-2b
20-2b

FIG. 1

FIG. 2

Pharmacokinetics of Various Interferon-α2b Conjugates in Male Swiss-Webster Mice



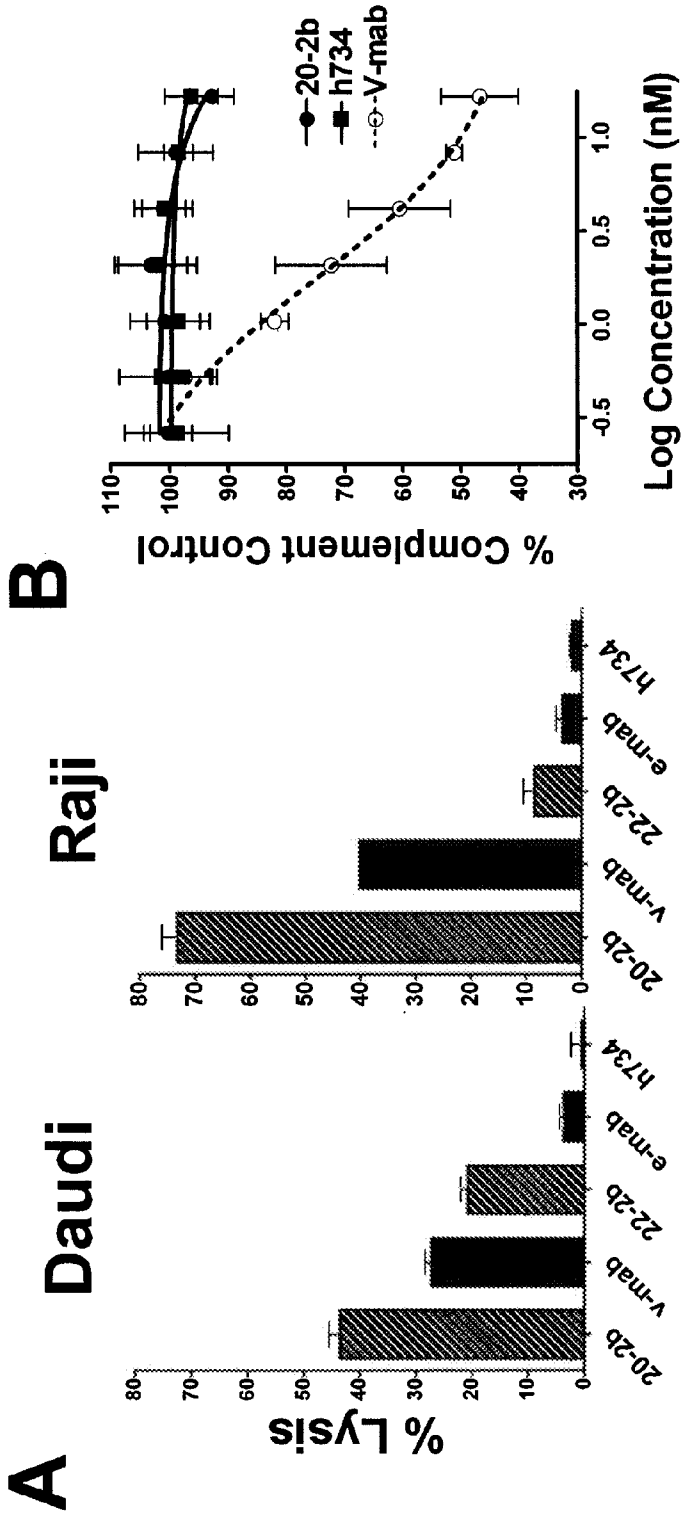

A
Daudi
Raji
% Lysis
20-2b
v-mab
22-2b
e-mab
h734
B
% Complement Control
Log Concentration (nM)
20-2b
h734
V-mab

FIG. 3

FIG. 4

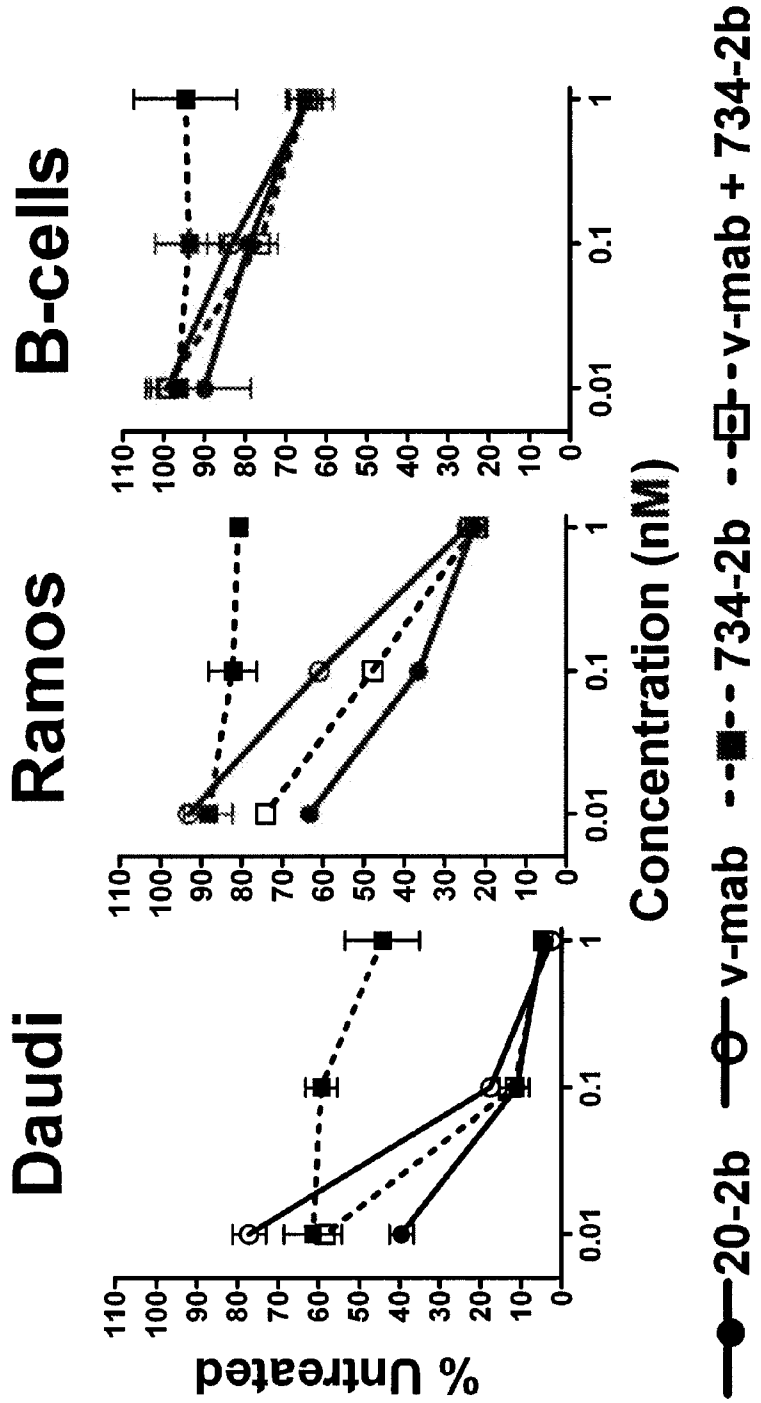

Daudi
Ramos
B-cells
% Untreated
Concentration (nM)
20-2b
v-mab
734-2b
v-mab + 734-2b

FIG. 5

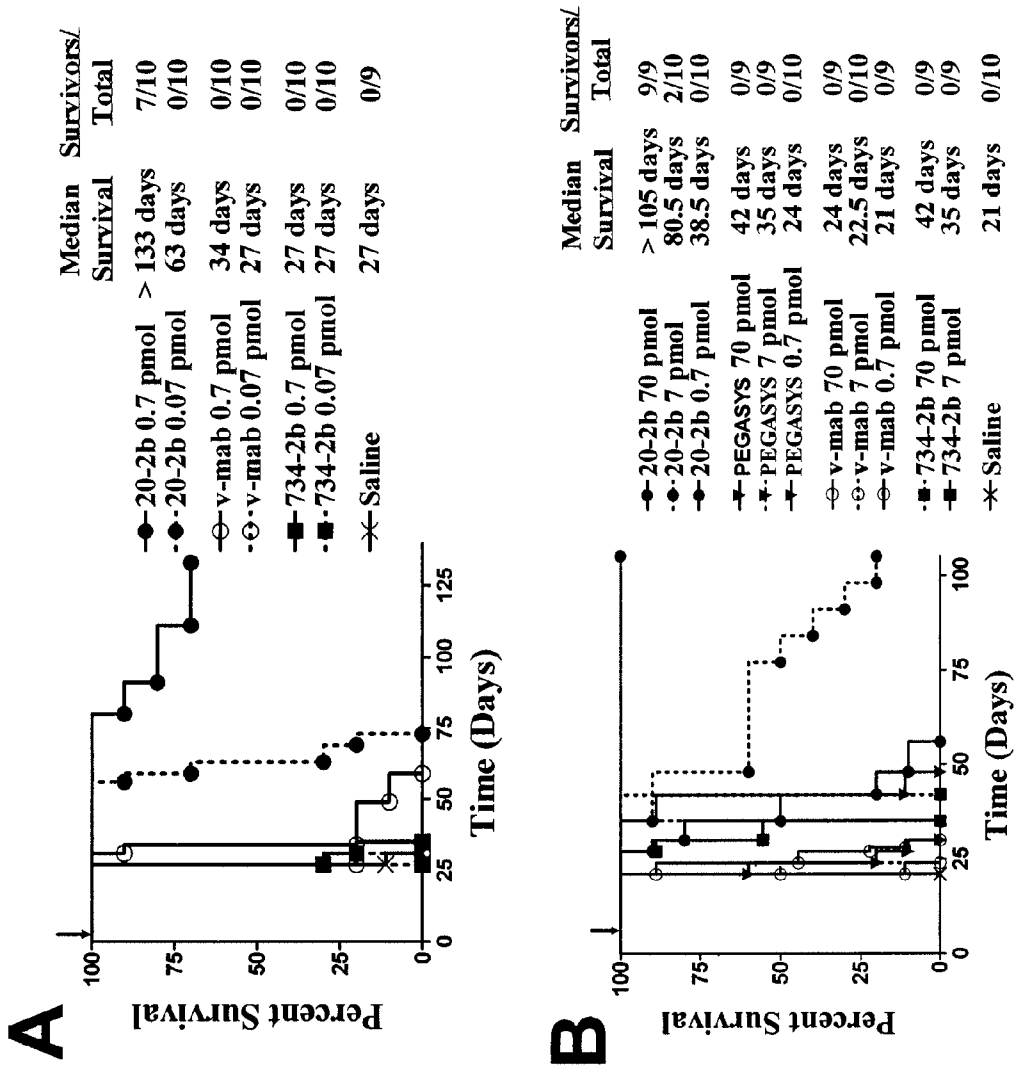

A
Median Survival
Survivors/ Total
20-2b 0.7 pmol > 133 days 7/10
20-2b 0.07 pmol 63 days 0/10
v-mab 0.7 pmol 34 days 0/10
v-mab 0.07 pmol 27 days 0/10
734-2b 0.7 pmol 27 days 0/10
734-2b 0.07 pmol 27 days 0/10
Saline 27 days 0/9
Percent Survival
Time (Days)
B
Median Survival
Survivors/ Total
20-2b 70 pmol > 105 days 9/9
20-2b 7 pmol 80.5 days 2/10
20-2b 0.7 pmol 38.5 days 0/10
PEGASYS 70 pmol 42 days 0/9
PEGASYS 7 pmol 35 days 0/9
PEGASYS 0.7 pmol 24 days 0/10
v-mab 70 pmol 24 days 0/9
v-mab 7 pmol 22.5 days 0/10
v-mab 0.7 pmol 21 days 0/9
734-2b 70 pmol 42 days 0/9
734-2b 7 pmol 35 days 0/9
Saline 21 days 0/10
Percent Survival
Time (Days)

FIG. 6

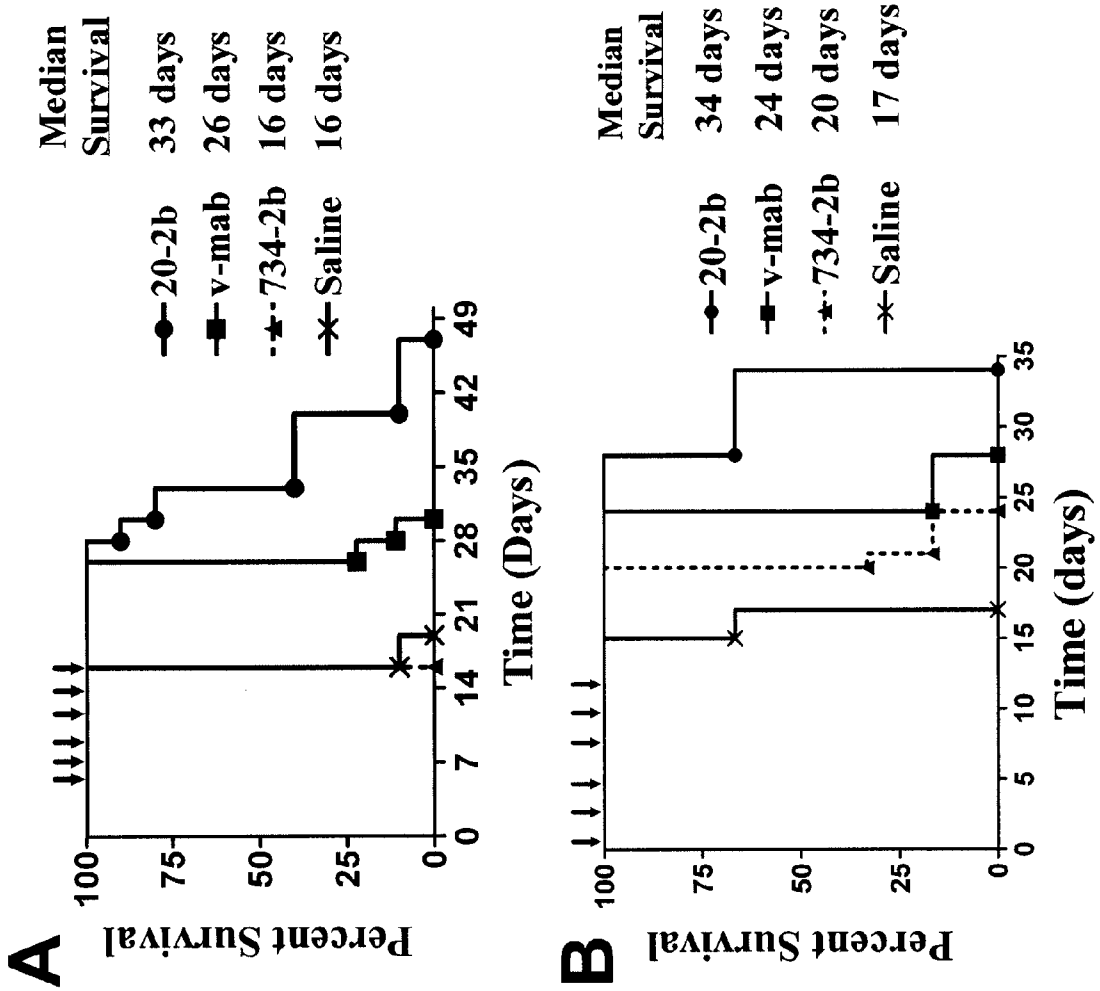

A
Percent Survival
100
75
50
25
0
0 7 14 21 28 35 42 49
Time (Days)
Median Survival
20-2b 33 days
v-mab 26 days
734-2b 16 days
Saline 16 days
B
Percent Survival
100
75
50
25
0
0 5 10 15 20 25 30 35
Time (days)
Median Survival
20-2b 34 days
v-mab 24 days
734-2b 20 days
Saline 17 days

FIG. 7

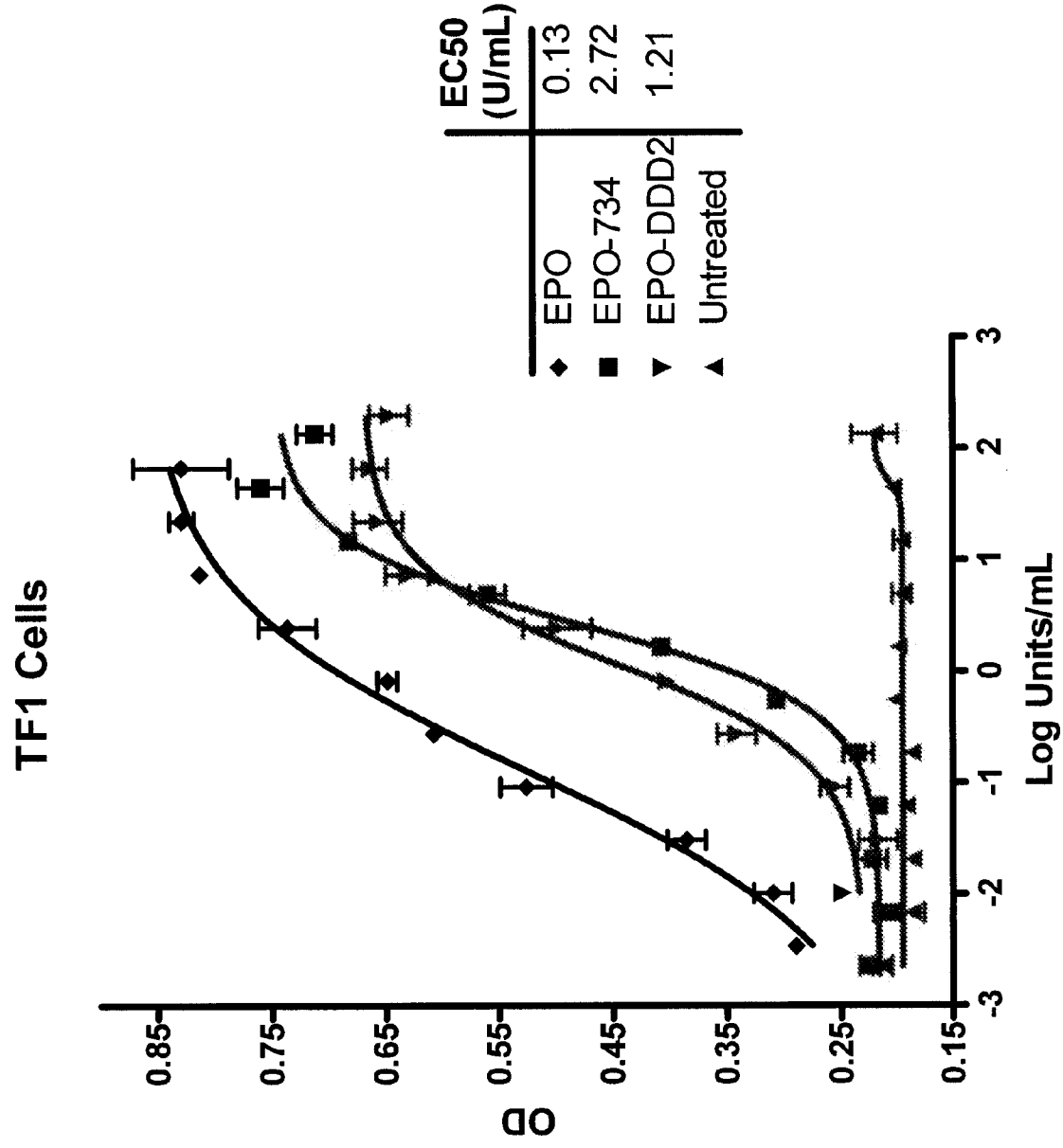

TF1 Cells
OD
0.85
0.75
0.65
0.55
0.45
0.35
0.25
0.15
-3
-2
-1
0
1
2
3
Log Units/mL
EC50 (U/mL)
EPO 0.13
EPO-734 2.72
EPO-DDD2 1.21
Untreated

FIG. 8

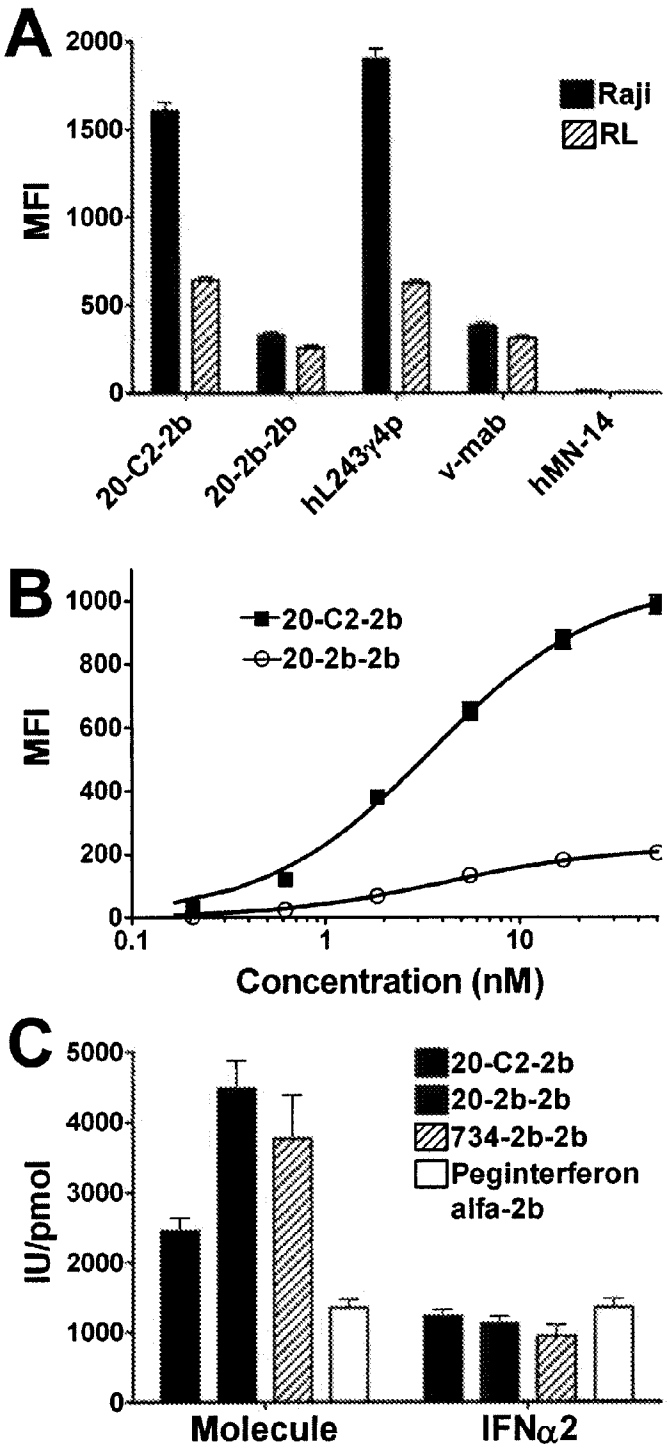

A
2000
1500
1000
500
0
MFI
Raji
RL
20-C2-2b
20-2b-2b
hL243γ4p
v-mab
hMN-14
B
1000
800
600
400
200
0
MFI
20-C2-2b
20-2b-2b
0.1
1
10
Concentration (nM)
C
5000
4000
3000
2000
1000
0
IU/pmol
20-C2-2b
20-2b-2b
734-2b-2b
Peginterferon alfa-2b
Molecule
IFNα2

FIG. 9

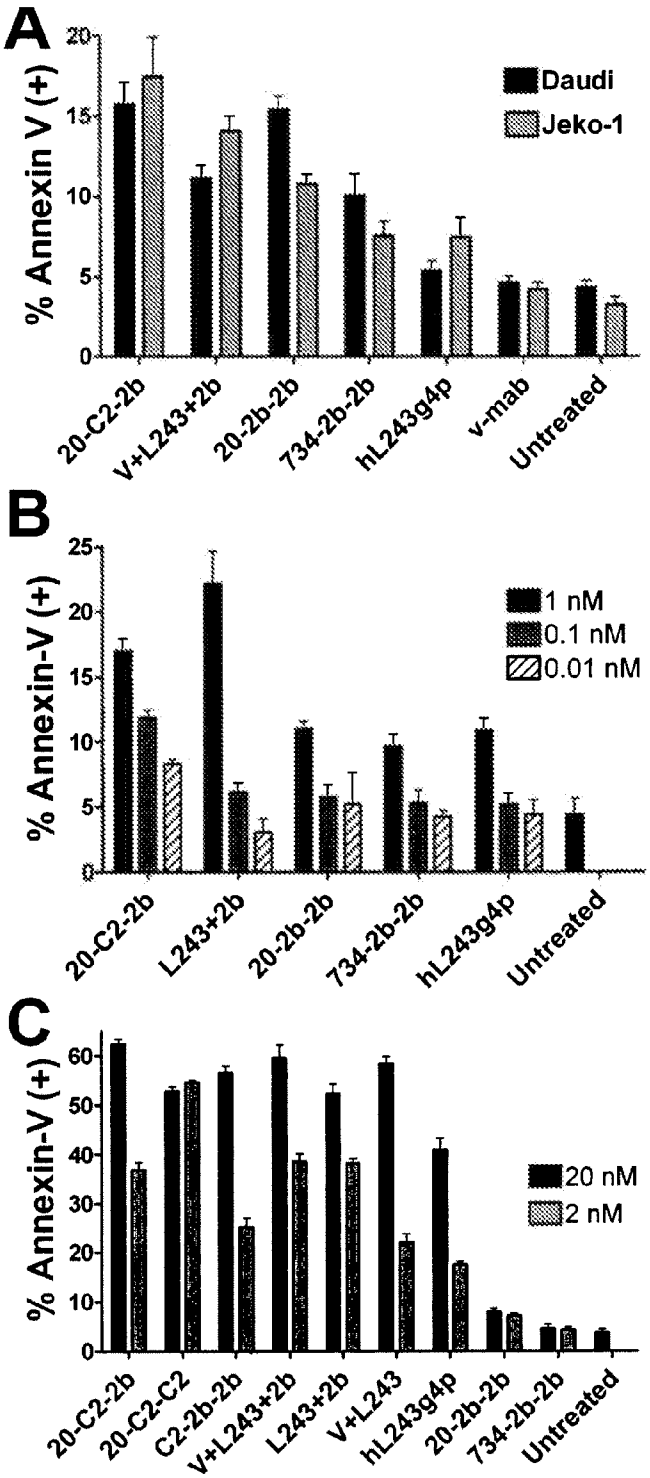

A
% Annexin V (+)
20
15
10
5
0
Daudi
Jeko-1
20-C2-2b
V+L243+2b
20-2b-2b
734-2b-2b
hL243g4p
v-mab
Untreated
B
% Annexin-V (+)
25
20
15
10
5
0
1 nM
0.1 nM
0.01 nM
20-C2-2b
L243+2b
20-2b-2b
734-2b-2b
hL243g4p
Untreated
C
% Annexin-V (+)
60
50
40
30
20
10
0
20 nM
2 nM
20-C2-2b
20-C2-C2
C2-2b-2b
V+L243+2b
L243+2b
V+L243
hL243g4p
20-2b-2b
734-2b-2b
Untreated

FIG. 10

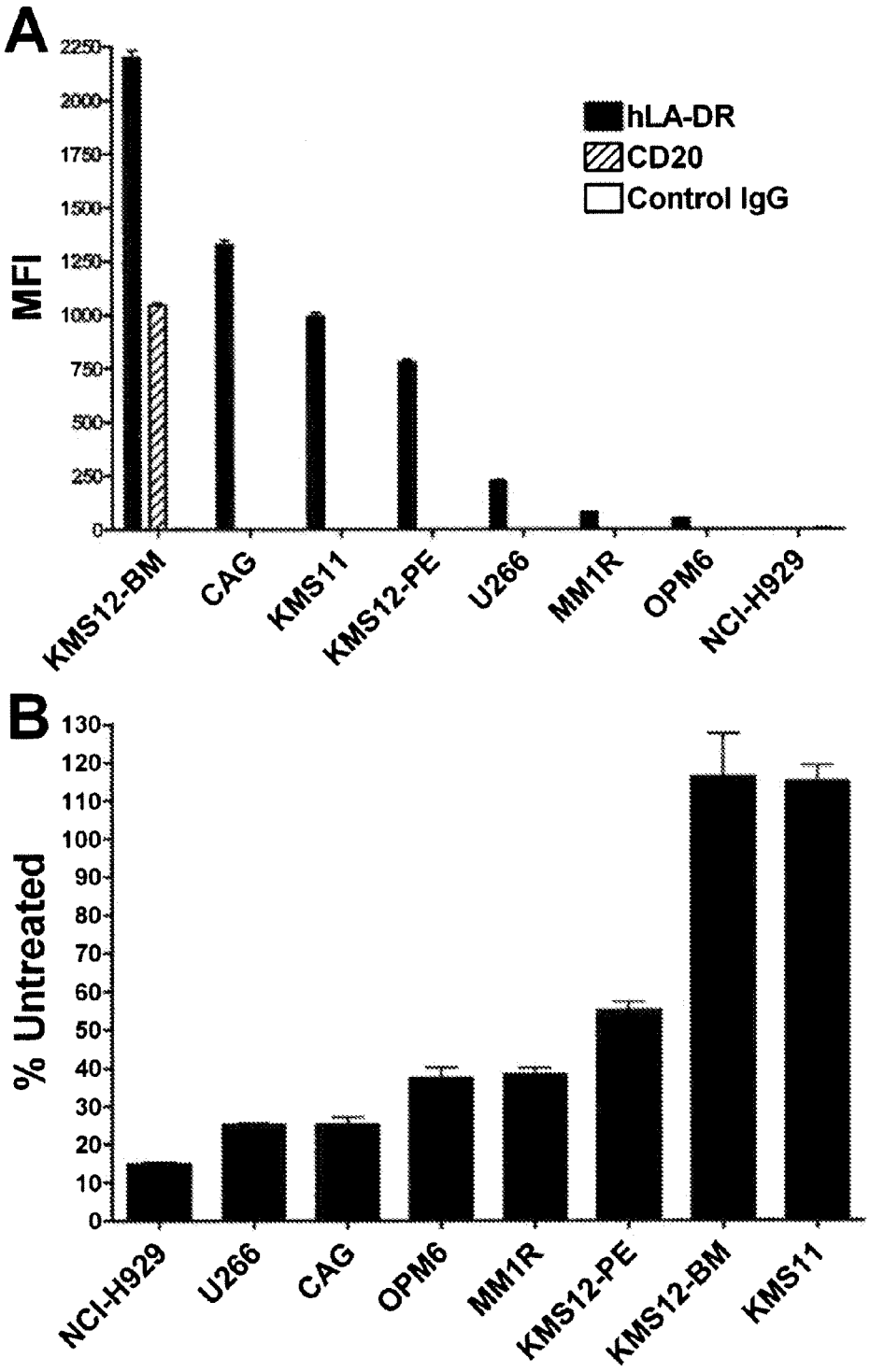

A
MFI
2250
2000
1750
1500
1250
1000
750
500
250
0
hLA-DR
CD20
Control IgG
KMS12-BM
CAG
KMS11
KMS12-PE
U266
MM1R
OPM6
NCI-H929
B
% Untreated
130
120
110
100
90
80
70
60
50
40
30
20
10
0
NCI-H929
U266
CAG
OPM6
MM1R
KMS12-PE
KMS12-BM
KMS11

FIG. 11

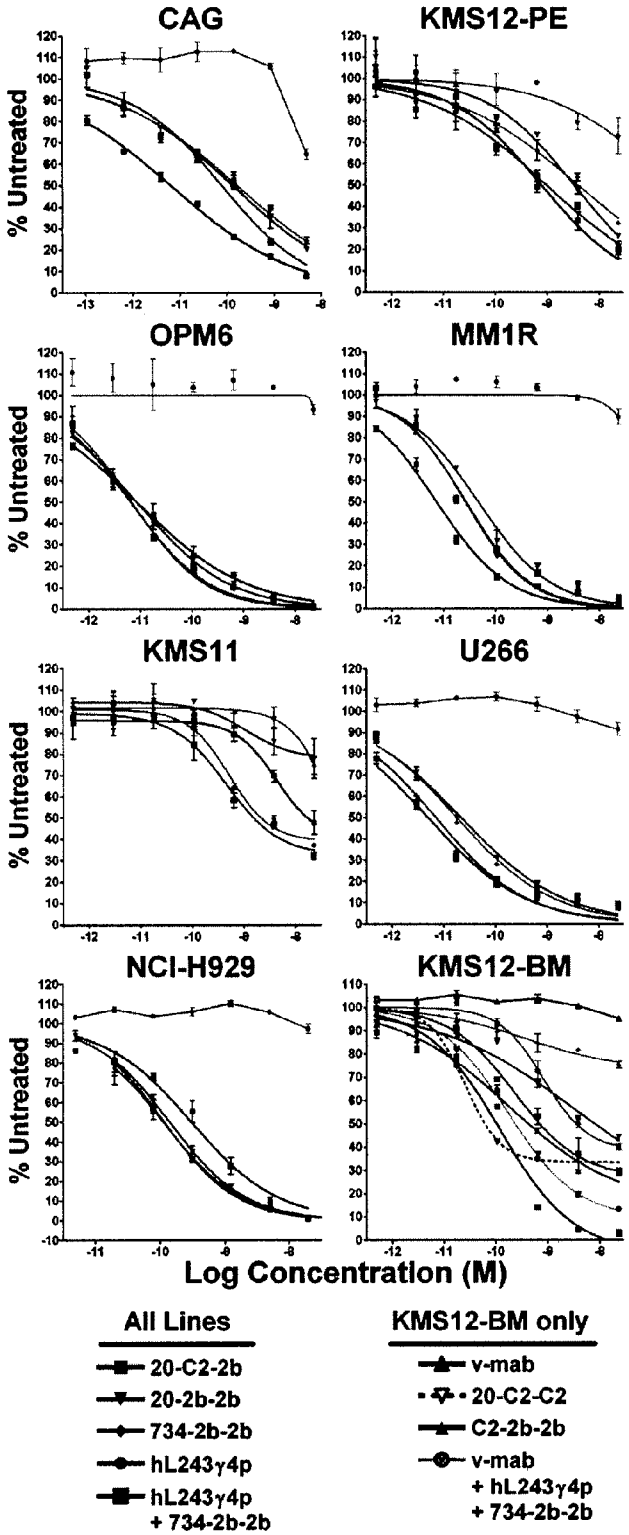

CAG
KMS12-PE
OPM6
MM1R
KMS11
U266
NCI-H929
KMS12-BM
% Untreated
Log Concentration (M)
All Lines
20-C2-2b
20-2b-2b
734-2b-2b
hL243γ4p
hL243γ4p + 734-2b-2b
KMS12-BM only
v-mab
20-C2-C2
C2-2b-2b
v-mab + hL243γ4p + 734-2b-2b

FIG. 12

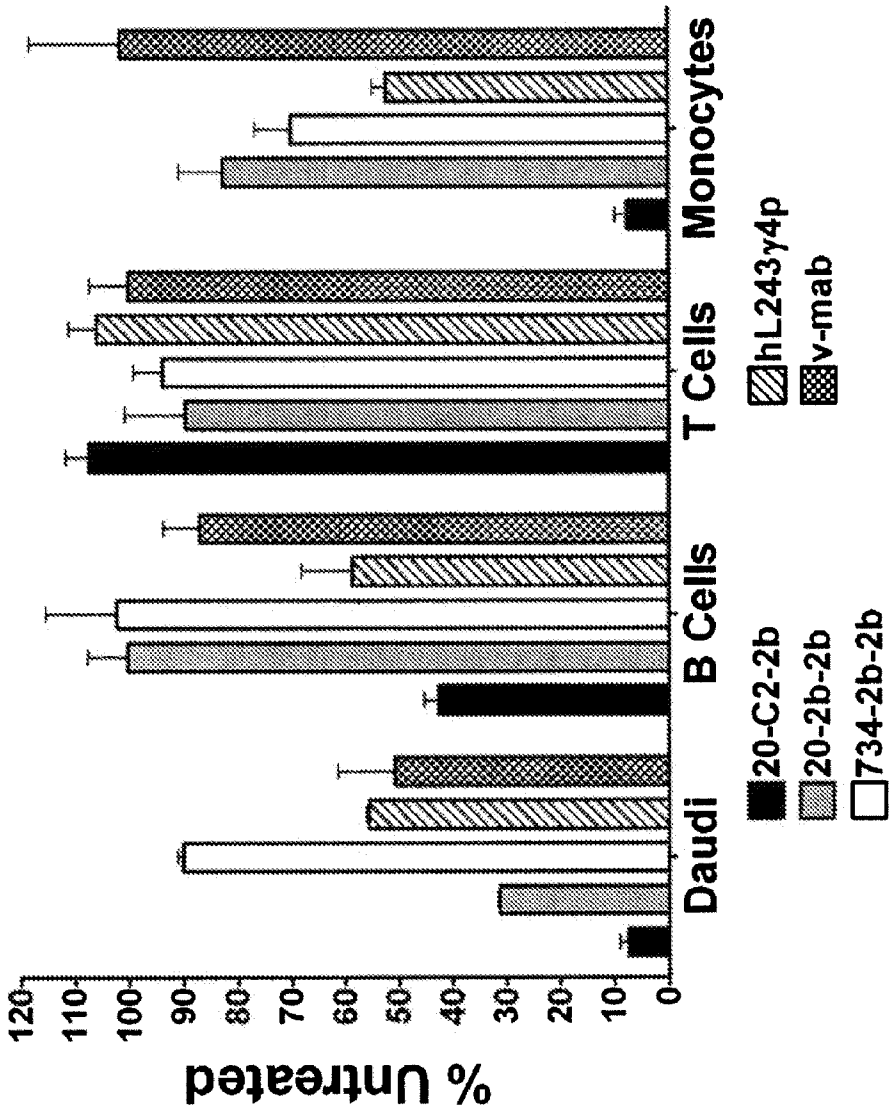

% Untreated
120
110
100
90
80
70
60
50
40
30
20
10
0
Daudi
B Cells
T Cells
Monocytes
20-C2-2b
20-2b-2b
734-2b-2b
hL243γ4p
v-mab

FIG. 13

Table 3. *In vitro* cytotoxicity of MAb-IFNα on NHL and MM cells.

	Cell line	20-C2-2b			20-2b-2b			734-2b-2b		hL243γ4p		v-mab + hL243γ4p + 734-2b-2b	
		I-EC_{50}, nM	TI, fold	I_{max}, %	I-EC_{50}, nM	TI, fold	I_{max}, %	I-EC_{50}, nM	I_{max}, %	EC_{50},* nM	I_{max}, %	I-EC_{50}, nM	I_{max}, %
NHL	Daudi	$8x10^{-5}$	125	95	$4x10^{-4}$	25	95	0.01	95	5.13	67	0.01	95
NHL	Raji	0.30	118	70	15.56	2	60	32.52	62	ND	45	2.25	70
NHL	Ramos	2.04	>40	82	31.04	>2.5	70	ND	35	ND	25	ND	43
NHL	Jeko-1	0.34	>200	90	4.40	>20	59	ND	21	0.40*	98	0.72	90
Myeloma	CAG	0.01	55	95	0.53	1	85	0.66	85	20*	52	0.31†	98
Myeloma	NCI-H929	0.61	1	98	0.56	1	98	0.44	98	ND	0	0.46†	98
Myeloma	KMS11	34.12	>2	52	ND	1	21	ND	24	2.19*	62	6.88†	66
Myeloma	KMS12-PE	1.56	10	83	13.12	1	72	15.60	68	ND	27	3.88†	76
Myeloma	KMS12-BM	0.20	>500	99	31.28	>3	55	ND	22	3.47*	59	2.44	73
Myeloma	MM1R	0.01	10	95	0.19	0.5	95	0.10	97	ND	10	0.11†	98
Myeloma	OPM6	0.02	2	99	0.03	1	99	0.03	99	ND	6	0.03†	99
Myeloma	U266	0.01	7	92	0.09	1	93	0.07	92	ND	10	0.03†	91

I-EC_{50} = Total IFNα concentration (nM) resulting in 50% growth inhibition compared to untreated cells. ND, treatment failed to reach 50% inhibition; *EC_{50} for hL243γ4p IgG; †v-mab excluded. TI, targeting index = fold reduction in EC_{50} compared to non targeted IFNα (734-2b-2b). I_{max} = maximal % decrease in viable cells compared to untreated cells.