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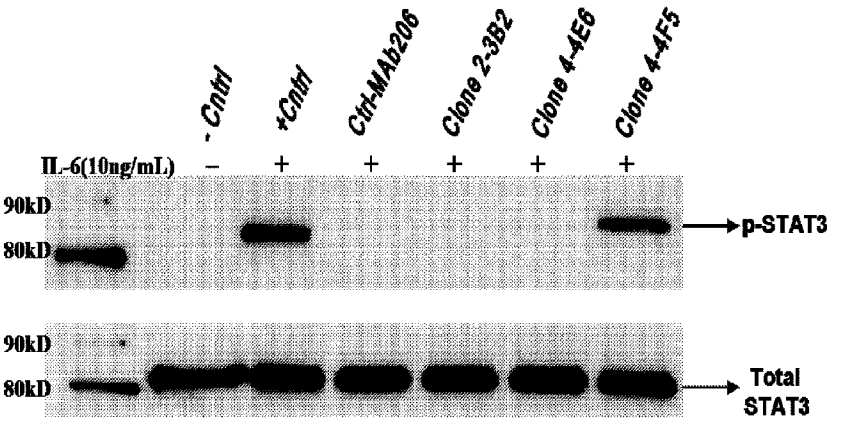
(72) Inventeurs/Inventors:
GOLDENBERG, DAVID M., US;
LI, RONGXIU, US;
CHANG, CHIEN-HSING, US

(73) Propriétaire/Owner:
IBC PHARMACEUTICALS, INC., US

(74) Agent: SMART & BIGGAR LP

(54) Titre : ANTICORPS BISPECIFIQUES QUI NEUTRALISENT A LA FOIS TNF-ALPHA ET IL-6 : NOUVEL AGENT THERAPEUTIQUE POUR TRAITER UNE MALADIE AUTO-IMMUNE

(54) Title: BISPECIFIC ANTIBODIES THAT NEUTRALIZE BOTH TNF-ALPHA AND IL-6: NOVEL THERAPEUTIC AGENT FOR AUTOIMMUNE DISEASE



(57) Abrégé/Abstract:
The present invention concerns compositions and methods of use of bispecific antibodies comprising at least one anti-TNF-α antibody or antigen-binding fragment thereof and at least one anti-IL-6 antibody or antigen-binding fragment thereof. Preferably, the bispecific antibody is in the form of a DNL®? complex. The anti-TNF-α or anti-IL-6 antibodies may comprise specific CDR sequences disclosed herein. The compositions and methods are of use to treat autoimmune disease, immune system dysfunction or inflammatory disease, as disclosed herein.

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

(72) **Inventors**: **GOLDENBERG, David M.**; 300 American Road, Morris Plains, New Jersey 07950 (US). **LI, Rongxiu**; 300 American Road, Morris Plains, New Jersey 07950 (US). **CHANG, Chien-Hsing**; 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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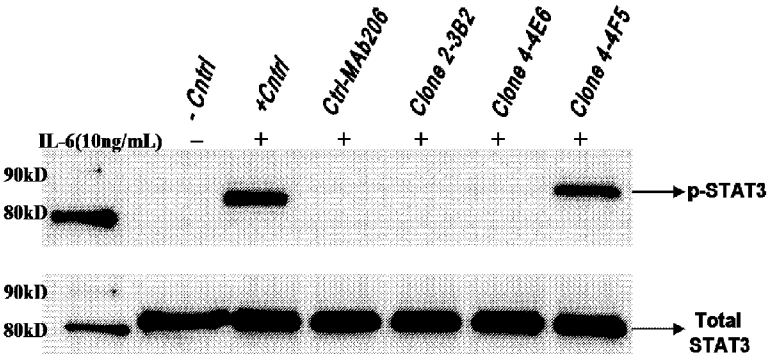
(54) **Title**: BISPECIFIC ANTIBODIES THAT NEUTRALIZE BOTH TNF-ALPHA AND IL-6: NOVEL THERAPEUTIC AGENT FOR AUTOIMMUNE DISEASE

FIG. 1



(57) **Abstract**: The present invention concerns compositions and methods of use of bispecific antibodies comprising at least one anti-TNF-α antibody or antigen-binding fragment thereof and at least one anti-IL-6 antibody or antigen-binding fragment thereof. Preferably, the bispecific antibody is in the form of a DNL®? complex. The anti-TNF-α or anti-IL-6 antibodies may comprise specific CDR sequences disclosed herein. The compositions and methods are of use to treat autoimmune disease, immune system dysfunction or inflammatory disease, as disclosed herein.

BISPECIFIC ANTIBODIES THAT NEUTRALIZE BOTH TNF-ALPHA AND IL-6: NOVEL THERAPEUTIC AGENT FOR AUTOIMMUNE DISEASE

Inventors: David M. Goldenberg, Rongxiu Li, and Chien-Hsing Chang

RELATED APPLICATIONS

[01] This application claims priority from provisional U.S. Patent Application Serial Nos. 61/898,798, filed November 1, 2013.

[02]

FIELD OF THE INVENTION

[03] The present invention relates to compositions and methods of use of complexes comprising at least one anti-TNF-α antibody or antigen-binding fragment thereof and at least one anti-IL-6 antibody or antigen-binding fragment thereof. The complex may be a bispecific or multispecific antibody or fragment thereof. Preferably, the complex is a DOCK-AND-LOCK® (DNL®) complex, in which the components are joined using the binding affinity between a DDD (dimerization and docking domain) moiety of human protein kinase A (PKA) regulatory subunit RIα, RIβ, RIIα or RIIβ, and an AD (anchoring domain) moiety of an A-kinase anchoring protein (AKAP), wherein a pair of DDD moieties forms a dimer that binds to a complementary sequence on the AD moiety. Although the basic DNL® complex is trimeric, complexes with other stoichiometries are possible, such as tetrameric, pentameric or hexameric. The subject complexes are of use to treat autoimmune disease, inflammatory disease or other conditions in which TNF-α and IL-6 play a pathogenic role. In particularly preferred embodiments, the disease or condition is selected from the group consisting of systemic lupus erythematosus (SLE), rheumatoid arthritis, inflammatory bowel disease, type II diabetes, obesity, atherosclerosis and cachexia related to cancer.

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BACKGROUND OF THE INVENTION

[04] TNF-α and IL-6 are proinflammatory cytokines involved in the pathogenesis of various autoimmune diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), inflammatory bowel disease, and type 2 diabetes. Blocking the biological activities of TNF-α has demonstrated clinical benefits in patients with RA and Crohn's disease, as exemplified by five antibody- or receptor-based therapeutics currently on the market. The promise of IL-6 blockade was also reinforced by the regulatory approval of one anti-IL-6R antibody for treating RA and juvenile idiopathic arthritis, with additional antibodies targeting either IL-6R or IL-6 in advanced clinical trials. As reported by Mori et al. (Int Immunol 2011; 23: 701-12), IL-6 directly activates STAT3, whereas TNF-α indirectly activates STAT3 via stimulating the expression of IL-6, which then activates STAT3 and triggers a cytokine amplification loop of IL-6, resulting in sustained STAT3 activation and chronic inflammation.

[05] Numerous antibodies against TNF-α are commercially available and/or publicly known, including infliximab (Jansenn Biotech, Inc.), adalimumab (Abbvie, Inc.), certolizumab pegol (UCB, Inc.) and golimumab (Centocor). Although these therapeutic agents have significantly improved the treatment of certain autoimmune diseases, such as rheumatoid arthritis (RA), it has been reported that about 30% of RA patients treated with TNF inhibitors (including anti-TNFα antibodies) show little to no effect of the therapy, with about two thirds demonstrating moderate to high disease activity at 1 year after treatment (Hirabara et al., 2014, Clin Rheumatol 33:1247-54). Further, loss of therapeutic efficacy is frequently observed with anti-TNF monoclonal antibodies (adalimumab, infliximab) in patients receiving concomitant low-dose methotrexate, due to immunogenicity-related issues (Hirabara et al, 2014). A need exists for more effective compositions and methods for use of anti-TNF antibodies in treating diseases and conditions related to TNF-α.

[06] Dysregulated IL-6 production has been demonstrated to play a pathological role in various autoimmune and chronic inflammatory diseases. Therapies against IL-6 pathways have commonly targeted the IL-6 receptor (IL-6R), including the anti-IL-6R antibodies tocilizumab, and sarilumab. Antibodies targeted directly against IL-6 have also been developed, such as olokizumab (UCB), siltuximab (Janssen), BMS-943429 (Bristol-Myers Squibb) and sirukumab (Centocor). The latter have been used against various autoimmune diseases and cancers. Following regulatory approval of tocilizumab for rheumatoid arthritis, Castleman's disease and systemic juvenile idiopathic arthritis, favorable results of off-label

use have been reported in systemic lupus erythematosus, systemic sclerosis, polymyositis, vasculitis syndrome including giant cell arteritis, Takayasu arteritis, cryoglobulinemia, glomerulonephritis and rheumatoid vasculitis (see, e.g., Tanaka & Kishimoto, 2012, Int J Biol Sci 8:1227-36). While these results are promising, no antibodies against IL-6 (as opposed to IL-6R) have yet been approved for human use in any indication.

[07] A need exists in the field for more effective, well-tolerated therapeutic agents targeted against TNF and IL-6.

SUMMARY OF THE INVENTION

[08] The present invention concerns compositions and methods of use of bispecific or multispecific antibodies comprising at least one anti-TNF-α antibody or antigen-binding fragment thereof and at least one anti- IL-6 antibody or antigen-binding fragment thereof. Preferably, the bispecific or multispecific antibody is in the form of a DNL® complex, comprising AD and DDD moiety binding pairs as described below.

[09] The antibodies may be chimeric, humanized or human antibodies. In certain preferred embodiments, the antibodies are humanized, comprising the CDR sequences of, e.g., a murine anti-IL-6 or anti-TNF-α antibody and the framework (FR) and constant region sequences from one or more human antibodies. Methods of antibody humanization are well known in the art, as discussed in detail below. The antibody can be of various isotypes, preferably human IgG1, IgG2, IgG3 or IgG4, more preferably comprising human IgG1 hinge and constant region sequences. More preferably, the antibody or fragment thereof may be designed or selected to comprise human constant region sequences that belong to specific allotypes, which may result in reduced immunogenicity. Preferred allotypes for administration include a non-G1m1 allotype (nG1m1), such as G1m3, G1m3,1, G1m3,2 or G1m3,1,2. More preferably, the allotype is selected from the group consisting of the nG1m1, G1m3, nG1m1,2 and Km3 allotypes.

[010] Numerous anti-TNF-α antibodies are commercially available and/or publicly known, including but not limited to CDP571 (Ofei et al., 2011, Diabetes 45:881-85); MTNFAI, M2TNFAI, M3TNFAI, M3TNFABI, M302B and M303 (Thermo Scientific); 3H15L1, D13H3, TN3, 17H1L4, MP9-20A4, and 68B6A3 L1 (Life Technologies); NBP1-19532, NB600-587, NBP2-27223, and NBP2-27224, (NOVUS BIOLOGICALS®); ab9635, (ABCAM®); certolizumab pegol (UCB, Brussels, Belgium); adalimumab (Abbvie); infliximab and golimumab (Centocor). These and many other known anti-TNF-α antibodies may be used in the claimed methods and compositions.

[011] Numerous anti-IL-6 antibodies are commercially available and/or publicly known, including but not limited to 5IL6, 4HCLC, 4H16L21, 677B6A2, and 20F3 (Thermo Scientific); NBP1-47810, NBP2025275, NBP1047355, and NBP2021624 (NOVUS BIOLOGICALS®); olokizumab (UCB); siltuximab (Janssen); BMS-943429 (Bristol-Myers Squibb); and sirukumab (Centocor). These and many other known anti-IL-6 antibodies may be used in the claimed methods and compositions.

[012] The subject antibodies may be co-administered with one or more other therapeutic agents. The therapeutic agents may be conjugated to the antibodies or administered separately, either before, concomitantly with or after the antibody. Therapeutic agents of use for treating immune or inflammatory diseases are preferably selected from drugs, anti-angiogenic agents, pro-apoptotic agents, antibiotics, hormones, hormone antagonists, chemokines, prodrugs, enzymes, immunomodulators, cytokines or other known agents of use for immune or inflammatory diseases.

[013] Drugs of use may possess a pharmaceutical property selected from the group consisting of antimitotic, antikinase (e.g., anti-tyrosine kinase), alkylating, antimetabolite, antibiotic, alkaloid, anti-angiogenic, pro-apoptotic agents, immune modulators, and combinations thereof.

[014] Exemplary drugs of use may include 5-fluorouracil, aplidin, azaribine, anastrozole, anthracyclines, bendamustine, bleomycin, bortezomib, bryostatin-1, busulfan, calicheamycin, camptothecin, carboplatin, 10-hydroxycamptothecin, carmustine, celecoxib, 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, nitrosourea, plicomycin, procarbazine, paclitaxel, pentostatin, PSI-341, raloxifene, semustine, streptozocin, tamoxifen, temazolomide (an aqueous form of DTIC), transplatinum, thalidomide, thioguanine, thiotepa, teniposide, topotecan, uracil mustard, vinorelbine, vinblastine, vincristine and vinca alkaloids.

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

[016] 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.

[017] 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. Lenolidamide is yet another

immunomodulator that has shown activity in controlling certain cancers, such as multiple myeloma and hematopoietic tumors.

[018] The antibodies or complexes may be used to treat a variety of diseases or conditions in which TNF-α and IL-6 play a pathogenic role, such as autoimmune, immune dysfunction or inflammatory diseases. Exemplary diseases or conditions may be selected from the group consisting of rheumatoid arthritis (RA), systemic lupus erythematosus, type 2 diabetes, Crohn's disease, Castleman's disease, juvenile idiopathic arthritis, systemic sclerosis, polymyositis, vasculitis syndrome, Takayasu arteritis, cryoglobulinemia, glomerulonephritis, rheumatoid vasculitis, arthritis, sepsis, septic shock, inflammation, non-septic hyperinflammatory disorder, nephritis, inflammatory bowel disease, inflammatory liver injury, acute pancreatitis, acute respiratory distress syndrome, ischemia-reperfusion injury, ischemic stroke, graft-vs.-host disease and cachexia related to cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

[019] **FIG. 1.** Assay for neutralizing anti-IL-6 antibodies. Supernatants from clones were incubated with human IL-6 at 37°C for 1 hour, prior to incubation with HT-29 cells. The cells were incubated with rhIL-6 alone or in combination with serum for 15 min at 37°C and phosphorylation of STAT3 was detected by Western blotting.

[020] **FIG. 2A.** Titration of neutralizing anti-IL-6 antibodies. The ability to block IL-6 induced phosphorylation of STAT3 was determined by Western blot analysis using the indicated concentrations of the 2-3B2 anti-IL-6 antibody. A substantial inhibition of IL-6 dependent phosphorylation was seen as low as 0.067 nM antibody.

[021] **FIG. 2B.** Titration of neutralizing anti-IL-6 antibodies. The ability to block IL-6 induced phosphorylation of STAT3 was determined by Western blot analysis using the indicated concentrations of the 4-4E6 anti-IL-6 antibody. Approximately equivalent effects on phosphorylation were observed at 0.67 nM 4-4E6 vs. 0.0067 nM 2-34B2 antibody (FIG. 2A).

[022] **FIG. 3.** Neutralization activity of TNF-α mediated cytotoxicity by immunized mouse sera on WEHI 164 cells. Serum from mouse #3 was the most effective at inhibiting TNF-α mediated cytotoxicity.

[023] **FIG. 4.** Neutralization activity of TNF-α mediated cytotoxicity by antibodies from clones 4C9D11 and 4D3B11 in WEHI 164 cells.

[024] **FIG. 5.** Neutralization activity of TNF-α mediated cytotoxicity by antibodies from clones 4C9D11G11 and 4D3B11C4 in L929 cells.

[025] FIG. 6. Antibody-based neutralization of rhTNF-α-induced cell surface expression of ICAM-1 in ECV-304 cells (a derivative of T24 bladder cancer cell line).

[026] FIG. 7. Amino acid sequence of the anti-IL-6 antibody (2-3B2) heavy chain (VH) sequence (SEQ ID NO:94). The sequence of a homologous heavy chain of the B34781 antibody (SEQ ID NO:95), obtained from the NCBI protein sequence database, is shown for comparison. Putative CDR sequences (underlined) were identified by comparison with the known sequence of the homologous B34781 antibody.

[027] FIG. 8. Amino acid sequence of the anti-IL-6 antibody (2-3B2) light chain (VK) sequence (SEQ ID NO:96). The sequence of a homologous light chain of AAB53778.1 (SEQ ID NO:97), obtained from the NCBI protein sequence database, is shown for comparison. Putative CDR sequences (underlined) were identified by comparison with the known sequence of the homologous AAB53778.1.

[028] FIG. 9. Activity of cIL6/TNFα DVD construct for neutralizing IL-6 induced phosphorylation of STAT3 in HT-29 cells, compared to parent 2-3B2 anti-IL-6 antibody.

[029] FIG. 10. Amino acid sequence of the anti-TNF-α antibody (4C9) heavy chain (VH) sequence (SEQ ID NO:98). The sequence of a homologous heavy chain of the AAS66033.1 antibody (SEQ ID NO:99), obtained from the NCBI protein sequence database, is shown for comparison. Putative CDR sequences (underlined) were identified by comparison with the known sequence of the homologous AAS66033.1 antibody.

[030] FIG. 11. Amino acid sequence of the anti-IL-6 antibody (4C9) light chain (VK) sequence (SEQ ID NO:100). The sequence of a homologous heavy chain of AAS66032.1 (SEQ ID NO:101), obtained from the NCBI protein sequence database, is shown for comparison. Putative CDR sequences (underlined) were identified by comparison with the known sequence of the homologous AAS66032.1.

[031] FIG. 12. Schematic illustration of the synthesis of C_K-AD2-cIgG-anti-TNF-α-pdHL2.

[032] FIG. 13. Inhibition of IL-6 induced phosphorylation of STAT3 by cT*-(c6)-(c6) complex compared to Fab-DDD2-cIL-6 protein.

[033] FIG. 14. Inhibition of natural IL-6 induced phosphorylation of STAT3 by cT*-(c6)-(c6) complex compared to Fab-DDD2-cIL-6 protein.

[034] FIG. 15. Inhibition of rhTNF-α induced cell death in L929 cells by anti-TNF-α antibody constructs.

[035] FIG. 16. Inhibition of cell death induced by natural TNF-α in L929 cells by anti-TNF-α antibody constructs.

[036] **FIG. 17.** Relative affinities of cT*-(c6)-(c6), c-anti-TNF-α and c-anti-IL-6 for IL-6 and TNF-α from different species.

[037] **FIG. 18A.** Role of STAT3 in IL-6 and TNF-α mediated pathways.

[038] **FIG. 18B.** Role of STAT3 in IL-6 and TNF-α mediated disease processes.

Definitions

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

[040] 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.

[041] 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.

[042] 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).

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

[044] 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.)

[045] An "antibody fragment" is a portion of an intact antibody such as $F(ab')_2$, $F(ab)_2$, Fab', Fab, Fv, sFv, scFv, dAb 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).

[046] An antibody or antibody complex 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®)

[047] In preferred embodiments, a bivalent or multivalent antibody is formed as a DOCK-AND-LOCK® (DNL®) complex (see, e.g., U.S. Patent Nos. 7,521,056; 7,527,787; 7,534,866; 7,550,143 and 7,666,400.

Generally, the technique takes advantage of the specific and high-affinity binding interactions that occur between a dimerization and docking domain (DDD) sequence of the regulatory (R) subunits of cAMP-dependent protein kinase (PKA) and an anchor domain (AD) sequence derived from any of a variety of AKAP proteins (Baillie *et al.*, FEBS Letters. 2005; 579: 3264. Wong and Scott, Nat. Rev. Mol. Cell Biol. 2004; 5: 959). The DDD and AD peptides may be attached to any protein, peptide or other molecule. Because the DDD sequences spontaneously dimerize and bind to the AD sequence, the technique allows the formation of complexes between any selected molecules that may be attached to DDD or AD sequences.

[048] Although the standard DNL® complex comprises a trimer with two DDD-linked molecules attached to one AD-linked molecule, variations in complex structure allow the formation of dimers, trimers, tetramers, pentamers, hexamers and other multimers. In some embodiments, the DNL® complex may comprise two or more antibodies, antibody fragments

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or fusion proteins which bind to the same antigenic determinant or to two or more different antigens. The DNL® complex may also comprise one or more other effectors, such as proteins, peptides, immunomodulators, cytokines, interleukins, interferons, binding proteins, peptide ligands, carrier proteins, toxins, ribonucleases such as onconase, inhibitory oligonucleotides such as siRNA, antigens or xenoantigens, polymers such as PEG, enzymes, therapeutic agents, hormones, cytotoxic agents, anti-angiogenic agents, pro-apoptotic agents or any other molecule or aggregate.

[049] 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). Thus, the four isoforms of PKA regulatory subunits are RIα, RIβ, RIIα and RIIβ. 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 of RIIα (Newlon *et al.*, Nat. Struct. Biol. 1999; 6:222). As discussed below, similar portions of the amino acid sequences of other regulatory subunits are involved in dimerization and docking, each located near the N-terminal end of the regulatory subunit. 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)

[050] 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). 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.

[051] We have developed a platform technology to utilize the DDD of human PKA regulatory subunit RIα, RIβ, RIIα or RIIβ and the AD of AKAP as an excellent pair of linker modules for docking any two entities, referred to hereafter as **A** and **B**, into a noncovalent complex, which could be further locked into a DNL® complex 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 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 binary, trimeric complex composed of $\mathbf{a}_2\mathbf{b}$. 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. Using various combinations of linkers, adaptor modules and precursors, a wide variety of DNL® constructs of different stoichiometry may be produced and used (see, e.g., U.S. Nos. 7,550,143; 7,521,056; 7,534,866; 7,527,787 and 7,666,400.)

[052] By attaching the DDD and AD away from the functional groups of the two precursors, such site-specific ligations are also expected to preserve the original activities of the two precursors. This approach is modular in nature and potentially can be applied to link, site-specifically and covalently, a wide range of substances, including peptides, proteins, antibodies, antibody fragments, and other effector moieties with a wide range of activities. Utilizing the fusion protein method of constructing AD and DDD conjugated effectors described in the Examples below, virtually any protein or peptide may be incorporated into a DNL® construct. However, the technique is not limiting and other methods of conjugation may be utilized.

[053] 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. 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.

Structure-Function Relationships in AD and DDD Moieties

[054] For different types of DNL® constructs, different AD or DDD sequences may be utilized. Exemplary DDD and AD sequences are provided below.

DDD1

SHIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:1)

DDD2

CGHIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:2)

AD1

QIEYLAKQIVDNAIQQA (SEQ ID NO:3)

AD2

CGQIEYLAKQIVDNAIQQAGC (SEQ ID NO:4)

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

DDD3

SLRECELYVQKHNIQALLKDSIVQLCTARPERPMAFLREYFERLEKEEAK (SEQ ID NO:5)

DDD3C

MSCGGSLRECELYVQKHNIQALLKDSIVQLCTARPERPMAFLREYFERLEKEEAK (SEQ ID NO:6)

AD3

CGFEELAWKIAKMIWSDVFQQGC (SEQ ID NO:7)

[056] In other alternative embodiments, other sequence variants of AD and/or DDD moieties may be utilized in construction of the DNL® complexes. For example, there are only four variants of human PKA DDD sequences, corresponding to the DDD moieties of PKA RIα, RIIα, RIβ and RIIβ. The RIIα DDD sequence is the basis of DDD1 and DDD2 disclosed above. The four human PKA DDD sequences are shown below. The DDD sequence represents residues 1-44 of RIIα, 1-44 of RIIβ, 12-61 of RIα and 13-66 of RIβ. (Note that the sequence of DDD1 is modified slightly from the human PKA RIIα DDD moiety.)

PKA RIα

SLRECELYVQKHNIQALLKDVSIVQLCTARPERPMAFLREYFEKLEKEEAK (SEQ ID NO:8)

PKA RIβ

SLKGCELYVQLHGIQQVLKDCIVHLCISKPERPMKFLREHFEKLEKEENRQILA (SEQ ID NO:9)

PKA RIIα

SHIQIPPGLTELLQGYTVEVGQQPPDLVDFAVEYFTRLREARRQ (SEQ ID NO:10)

PKA RIIβ

SIEIPAGLTELLQGFTVEVLRHQPADLLEFALQHFTRLQQENER (SEQ ID NO:11)

[057] 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.

[058] For example, Kinderman et al. (2006, Mol Cell 24:397-408) 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 SEQ ID NO:1 below. (See Figure 1 of Kinderman et al., 2006. 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.

SHIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:1)

[059] As discussed in more detail below, conservative amino acid substitutions have been characterized for each of the twenty common L-amino acids. Thus, based on the data of Kinderman (2006) and conservative amino acid substitutions, potential alternative DDD sequences based on SEQ ID NO:1 are shown in **Table 1**. In devising **Table 1**, only highly conservative amino acid substitutions were considered. For example, charged residues were only substituted for residues of the same charge, residues with small side chains were substituted with residues of similar size, hydroxyl side chains were only substituted with other hydroxyls, etc. Because of the unique effect of proline on amino acid secondary structure, no other residues were substituted for proline. A limited number of such potential alternative DDD moiety sequences are shown in SEQ ID NO:12 to SEQ ID NO:31 below. The skilled artisan will realize that an almost unlimited number of alternative species within the genus of DDD moieties can be constructed by standard techniques, for example using a commercial peptide synthesizer or well known site-directed mutagenesis techniques. The effect of the amino acid substitutions on AD moiety binding may also be readily determined by standard binding assays, for example as disclosed in Alto et al. (2003, Proc Natl Acad Sci USA 100:4445-50).

Table 1. Conservative Amino Acid Substitutions in DDD1 (SEQ ID NO:1). Consensus sequence disclosed as SEQ ID NO:87.

S	H	I	Q	I	P	P	G	L	T	E	L	L	Q	G	Y	T	V	E	V	L	R
T	K R		N				A		S	D			N	A		S		D			K

Q	Q	P	P	D	L	V	E	F	A	V	E	Y	F	T	R	L	R	E	A	R	A

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N	N			E			D		L I V		D			S	K		K	D	L I V	K	L I V

THIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:12)

SKIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:13)

SRIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:14)

SHINIPPGLTELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:15)

SHIQIPPALTELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:16)

SHIQIPPGLSELLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:17)

SHIQIPPGLTDLLQGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:18)

SHIQIPPGLTELLNGYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:19)

SHIQIPPGLTELLQAYTVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:20)

SHIQIPPGLTELLQGYSVEVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:21)

SHIQIPPGLTELLQGYTVDVLRQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:22)

SHIQIPPGLTELLQGYTVEVLKQQPPDLVEFAVEYFTRLREARA (SEQ ID NO:23)

SHIQIPPGLTELLQGYTVEVLRNQPPDLVEFAVEYFTRLREARA (SEQ ID NO:24)

SHIQIPPGLTELLQGYTVEVLRQNPPDLVEFAVEYFTRLREARA (SEQ ID NO:25)

SHIQIPPGLTELLQGYTVEVLRQQPPELVEFAVEYFTRLREARA (SEQ ID NO:26)

SHIQIPPGLTELLQGYTVEVLRQQPPDLVDFAVEYFTRLREARA (SEQ ID NO:27)

SHIQIPPGLTELLQGYTVEVLRQQPPDLVEFLVEYFTRLREARA (SEQ ID NO:28)

SHIQIPPGLTELLQGYTVEVLRQQPPDLVEFIVEYFTRLREARA (SEQ ID NO:29)

SHIQIPPGLTELLQGYTVEVLRQQPPDLVEFVVEYFTRLREARA (SEQ ID NO:30)

SHIQIPPGLTELLQGYTVEVLRQQPPDLVEFAVDYFTRLREARA (SEQ ID NO:31)

[060] Alto et al. (2003, Proc Natl Acad Sci USA 100:4445-50) performed a bioinformatic analysis of the AD sequence of various AKAP proteins to design an RII selective AD sequence called AKAP-IS (SEQ ID NO:3), 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 SEQ ID NO:3 below. The skilled artisan will realize that in designing sequence variants of the AD 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 DDD binding. **Table 2** shows potential conservative amino acid substitutions in the sequence of AKAP-IS (AD1, SEQ ID NO:3), similar to that shown for DDD1 (SEQ ID NO:1) in **Table 1** above.

[061] A limited number of such potential alternative AD moiety sequences are shown in SEQ ID NO:32 to SEQ ID NO:49 below. Again, a very large number of species within the genus of possible AD moiety sequences could be made, tested and used by the skilled artisan, based on the data of Alto et al. (2003). It is noted that Figure 2 of Alto (2003) shows an even large number of potential amino acid substitutions that may be made, while retaining binding activity to DDD moieties, based on actual binding experiments.

AKAP-IS

QIEYLAKQIVDNAIQQA (SEQ ID NO:3)

Table 2. Conservative Amino Acid Substitutions in AD1 (SEQ ID NO:3). Consensus sequence disclosed as SEQ ID NO:88.

Q	I	E	Y	L	A	K	Q	I	V	D	N	A	I	Q	Q	A
N	L V	D	F T S	I V		R	N			E	Q			N	N	L I V

NIEYLAKQIVDNAIQQA (SEQ ID NO:32)

QLEYLAKQIVDNAIQQA (SEQ ID NO:33)

QVEYLAKQIVDNAIQQA (SEQ ID NO:34)

QIDYLAKQIVDNAIQQA (SEQ ID NO:35)

QIEFLAKQIVDNAIQQA (SEQ ID NO:36)

QIETLAKQIVDNAIQQA (SEQ ID NO:37)

QIESLAKQIVDNAIQQA (SEQ ID NO:38)

QIEYIAKQIVDNAIQQA (SEQ ID NO:39)

QIEYVAKQIVDNAIQQA (SEQ ID NO:40)

QIEYLARQIVDNAIQQA (SEQ ID NO:41)

QIEYLAKNIVDNAIQQA (SEQ ID NO:42)

QIEYLAKQIVENAIQQA (SEQ ID NO:43)

QIEYLAKQIVDQAIQQA (SEQ ID NO:44)

QIEYLAKQIVDNAINQA (SEQ ID NO:45)

QIEYLAKQIVDNAIQNA (SEQ ID NO:46)

QIEYLAKQIVDNAIQQL (SEQ ID NO:47)

QIEYLAKQIVDNAIQQI (SEQ ID NO:48)

QIEYLAKQIVDNAIQQV (SEQ ID NO:49)

[062] Gold et al. (2006, Mol Cell 24:383-95) utilized crystallography and peptide screening to develop a SuperAKAP-IS sequence (SEQ ID NO:50), 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, which 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 DNL® constructs. Other alternative sequences that might be substituted for the AKAP-IS AD sequence are shown in SEQ ID NO:51-53. Substitutions relative to the AKAP-IS sequence are underlined. It is anticipated that, as with the AD2 sequence shown in SEQ ID NO:4, 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:50)

Alternative AKAP sequences

QIEYKAKQIVDHAIHQA (SEQ ID NO:51)

QIEYHAKQIVDHAIHQA (SEQ ID NO:52)

QIEYVAKQIVDHAIHQA (SEQ ID NO:53)

[063] 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:54)

AKAP79

LLIETASSLVKNAIQLSI (SEQ ID NO:55)

AKAP-Lbc

LIEEAASRIVDAVIEQVK (SEQ ID NO:56)

RI-Specific AKAPs

AKAPce

ALYQFADRFSELVISEAL (SEQ ID NO:57)

RIAD

LEQVANQLADQIIKEAT (SEQ ID NO:58)

PV38

FEELAWKIAKMIWSDVF (SEQ ID NO:59)

Dual-Specificity AKAPs

AKAP7

ELVRLSKRLVENAVLKAV (SEQ ID NO:60)

MAP2D

TAEEVSARIVQVVTAEAV (SEQ ID NO:61)

DAKAP1

QIKQAAFQLISQVILEAT (SEQ ID NO:62)

DAKAP2

LAWKIAKMIVSDVMQQ (SEQ ID NO:63)

[064] Stokka et al. (2006, Biochem J 400:493-99) also developed peptide competitors of AKAP binding to PKA, shown in SEQ ID NO:64-66. The peptide antagonists were designated as Ht31 (SEQ ID NO:64), RIAD (SEQ ID NO:65) and PV-38 (SEQ ID NO:66). 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:64)

RIAD

LEQYANQLADQIIKEATE (SEQ ID NO:65)

PV-38

FEELAWKIAKMIWSDVFQQC (SEQ ID NO:66)

[065] Hundsrucker et al. (2006, Biochem J 396:297-306) 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 are provided in Table 1 of Hundsrucker et al., reproduced in **Table 3** below. AKAPIS represents a synthetic RII subunit-binding peptide. All other peptides are derived from the RII-binding domains of the indicated AKAPs.

Table 3. AKAP Peptide sequences

	Peptide Sequence
AKAPIS	QIEYLAKQIVDNAIQQA (SEQ ID NO:3)
AKAPIS-P	QIEYLAKQIPDNAIQQA (SEQ ID NO:67)
Ht31	KGADLIEEAASRIVDAVIEQVKAAG (SEQ ID NO:68)
Ht31-P	KGADLIEEAASRIPDAPIEQVKAAG (SEQ ID NO:69)
AKAP7δ-wt-pep	PEDAELVRLSKRLVENAVLKAVQQY (SEQ ID NO:70)
AKAP7δ-L304T-pep	PEDAELVRTSKRLVENAVLKAVQQY (SEQ ID NO:71)
AKAP7δ-L308D-pep	PEDAELVRLSKRDVENAVLKAVQQY (SEQ ID NO:72)
AKAP7δ-P-pep	PEDAELVRLSKRLPENAVLKAVQQY (SEQ ID NO:73)
AKAP7δ-PP-pep	PEDAELVRLSKRLPENAPLKAVQQY (SEQ ID NO:74)

AKAP7δ-L314E-pep PEDAELVRLSKRLVENAVEKAVQQY (SEQ ID NO:75)

AKAP1-pep EEGLDRNEEIKRAAFQIISQVISEA (SEQ ID NO:76)

AKAP2-pep LVDDPLEYQAGLLVQNAIQQAIAEQ (SEQ ID NO:77)

AKAP5-pep QYETLLIETASSLVKNAIQLSIEQL (SEQ ID NO:78)

AKAP9-pep LEKQYQEQLEEEVAKVIVSMSIAFA (SEQ ID NO:79)

AKAP10-pep NTDEAQEELAWKIAKMIVSDIMQQA (SEQ ID NO:80)

AKAP11-pep VNLDKKAVLAEKIVAEAIEKAEREL (SEQ ID NO:81)

AKAP12-pep NGILELETKSSKLVQNIIQTAVDQF (SEQ ID NO:82)

AKAP14-pep TQDKNYEDELTQVALALVEDVINYA (SEQ ID NO:83)

Rab32-pep ETSAKDNINIEEAARFLVEKILVNH (SEQ ID NO:84)

[066] 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 (SEQ ID NO:3). 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).
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:3)

[067] Carr et al. (2001, J Biol Chem 276:17332-38) 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 of SEQ ID NO:1. 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. The skilled artisan will realize that in designing sequence variants of DDD, it would be most preferred to avoid changing the most conserved residues (italicized), and it would be preferred to also avoid changing the conserved residues (underlined), while

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conservative amino acid substitutions may be considered for residues that are neither underlined nor italicized..

SHIQ*IP*P*GL*TE*LL*QG*Y*T*VEVLR*QQ*PP*DL*VE*FA*VE*YF*TR*L*REA*R*A (SEQ ID NO:1)

[068] A modified set of conservative amino acid substitutions for the DDD1 (SEQ ID NO:1) sequence, based on the data of Carr et al. (2001) is shown in **Table 4**. Even with this reduced set of substituted sequences, there are over 65,000 possible alternative DDD moiety sequences that may be produced, tested and used by the skilled artisan without undue experimentation. The skilled artisan could readily derive such alternative DDD amino acid sequences as disclosed above for **Table 1** and **Table 2.**

Table 4. Conservative Amino Acid Substitutions in DDD1 (SEQ ID NO:1). Consensus sequence disclosed as SEQ ID NO:89.

S	H	I	Q	I	P	P	G	L	T	E	L	L	Q	G	Y	T	V	E	V	L	R
T			N						S								I L A				

Q	Q	P	P	D	L	V	E	F	A	V	E	Y	F	T	R	L	R	E	A	R	A
N										I L A	D			S	K		K		L I V		L I V

[069] The skilled artisan will realize that these and other amino acid substitutions in the DDD or AD amino acid sequences may be utilized to produce alternative species within the genus of AD or DDD moieties, using techniques that are standard in the field and only routine experimentation.

Amino Acid Substitutions

[070] In alternative embodiments, the disclosed methods and compositions may involve production and use of proteins or peptides with one or more substituted amino acid residues. For example, the DDD and/or AD sequences used to make DNL® constructs may be modified as discussed above.

[071] 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.

[072] 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.

[073] 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.

[074] 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).

[075] 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.

[076] 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.*)

[077] 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.

[078] 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.

Antibodies and Antibody Fragments

[079] Techniques for preparing monoclonal antibodies against virtually any target antigen, such as IL-6 or TNF-α, 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.

[080] 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).

[081] 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

[082] 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

[083] 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

[084] 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.

[085] 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.

[086] 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).

[087] 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. 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.

[088] 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.

[089] 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

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XENOMOUSE® system but may utilize any transgenic animal that has been genetically engineered to produce human antibodies.

Antibody Fragments

[090] 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.

[091] 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).

[092] Techniques for producing single domain antibodies are also known in the art, as disclosed for example in Cossins et al. (2006, Prot Express Purif 51:253-259). Single domain antibodies (VHH) may be obtained, for example, from camels, alpacas or llamas by standard immunization techniques. (See, e.g., Muyldermans et al., TIBS 26:230-235, 2001; Yau et al., J Immunol Methods 281:161-75, 2003; Maass et al., J Immunol Methods 324:13-25, 2007). The VHH may have potent antigen-binding capacity and can interact with novel epitopes that are inaccessible to conventional VH-VL pairs. (Muyldermans et al., 2001). Alpaca serum IgG contains about 50% camelid heavy chain only IgG antibodies (HCAbs) (Maass et al., 2007). Alpacas may be immunized with known antigens, such as TNF-α, and VHHs can be isolated that bind to and neutralize the target antigen (Maass et al., 2007). PCR primers that amplify virtually all alpaca VHH coding sequences have been identified and may be used to construct alpaca VHH phage display libraries, which can be used for antibody fragment isolation by standard biopanning techniques well known in the art (Maass et al., 2007). In certain embodiments, anti-pancreatic cancer VHH antibody fragments may be utilized in the claimed compositions and methods.

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[093] 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

[094] Various embodiments, for example in combination therapy, may involve the use of antibodies binding to target antigens besides IL-6 or TNF-α. A variety of antibodies are commercially available and/or known in the art. Antibodies of use may be commercially obtained, for example, 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; 6,720,155; 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 (see, e.g., U.S. Patent Nos. 7,531,327; 7,537,930; 7,608,425 and 7,785,880.

[095] Particular antibodies that may be of use for therapy of cancer within the scope of the claimed methods and compositions include, but are not limited to, LL1 (anti-CD74), LL2 and RFB4 (anti-CD22), RS7 (anti-epithelial glycoprotein-1 (EGP-1)), PAM4 and KC4 (both anti-mucin), MN-14 (anti-carcinoembryonic antigen (CEA, also known as CD66e), Mu-9 (anti-colon-specific antigen-p), Immu 31 (an anti-alpha-fetoprotein), TAG-72 (e.g., CC49), Tn, J591 or HuJ591 (anti-PSMA (prostate-specific membrane antigen)), AB-PG1-XG1-026 (anti-PSMA dimer), D2/B (anti-PSMA), G250 (anti-carbonic anhydrase IX), hL243 (anti-HLA-DR), alemtuzumab (anti-CD52), bevacizumab (anti-VEGF), cetuximab (anti-EGFR), gemtuzumab (anti-CD33), ibritumomab tiuxetan (anti-CD20); panitumumab (anti-EGFR); rituximab (anti-CD20); tositumomab (anti-CD20); GA101 (anti-CD20); and trastuzumab (anti-ErbB2). Such antibodies are known in the art (e.g., U.S. Patent Nos. 5,686,072; 5,874,540; 6,107,090; 6,183,744; 6,306,393; 6,653,104; 6,730.300; 6,899,864; 6,926,893; 6,962,702; 7,074,403; 7,230,084; 7,238,785; 7,238,786; 7,256,004; 7,282,567; 7,300,655; 7,312,318; 7,585,491; 7,612,180; 7,642,239; and U.S. Patent Application Publ. No. 20040202666 (now abandoned); 20050271671; and 20060193865.

Specific known antibodies of use include hPAM4 (U.S. Patent No. 7,282,567), hA20 (U.S. Patent No. 7,251,164), hA19 (U.S. Patent No. 7,109,304), hIMMU31 (U.S. Patent No. 7,300,655), hLL1 (U.S. Patent No. 7,312,318,), hLL2 (U.S. Patent No. 7,074,403), hMu-9 (U.S. Patent No. 7,387,773), hL243 (U.S. Patent No. 7,612,180), hMN-14 (U.S. Patent No. 6,676,924), hMN-15 (U.S. Patent No. 7,541,440), hR1 (U.S. Patent Application 12/772,645), hRS7 (U.S. Patent No. 7,238,785), hMN-3 (U.S. Patent No. 7,541,440), AB-PG1-XG1-026 (U.S. Patent Application 11/983,372, deposited as

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ATCC PTA-4405 and PTA-4406) and D2/B (WO 2009/130575).

[096] Anti-TNF-α antibodies are known in the art and may be of use to treat immune diseases, such as autoimmune disease, immune dysfunction (e.g., graft-versus-host disease, organ transplant rejection) or diabetes. Known antibodies against TNF-α include the human antibody CDP571 (Ofei et al., 2011, Diabetes 45:881-85); murine antibodies MTNFAI, M2TNFAI, M3TNFAI, M3TNFABI, M302B and M303 (Thermo Scientific, Rockford, IL); infliximab (Centocor, Malvern, PA); certolizumab pegol (UCB, Brussels, Belgium); and adalimumab (Abbott, Abbott Park, IL). These and many other known anti-TNF-α antibodies may be used in the claimed methods and compositions. Other antibodies of use for therapy of immune dysregulatory or autoimmune disease include, but are not limited to, anti-B-cell antibodies such as veltuzumab, epratuzumab, milatuzumab or hL243; tocilizumab (anti-IL-6 receptor); basiliximab (anti-CD25); daclizumab (anti-CD25); efalizumab (anti-CD11a); muromonab-CD3 (anti-CD3 receptor); anti-CD40L (UCB, Brussels, Belgium); natalizumab (anti-α4 integrin) and omalizumab (anti-IgE).

[097] Type-2 diabetes may be treated using known antibodies against B-cell antigens, such as CD22 (epratuzumab), CD74 (milatuzumab), CD19 (hA19), CD20 (veltuzumab) or HLA-DR (hL243) (*see, e.g.*, Winer et al., 2011, Nature Med 17:610-18). Anti-CD3 antibodies also have been proposed for therapy of type 1 diabetes (Cernea et al., 2010, Diabetes Metab Rev 26:602-05).

[098] Macrophage migration inhibitory factor (MIF) is an important regulator of innate and adaptive immunity and apoptosis. It has been reported that CD74 is the endogenous receptor for MIF (Leng et al., 2003, J Exp Med 197:1467-76). The therapeutic effect of antagonistic anti-CD74 antibodies on MIF-mediated intracellular pathways may be of use for treatment of a broad range of disease states, such as cancers of the bladder, prostate, breast, lung, colon and chronic lymphocytic leukemia (e.g., Meyer-Siegler et al., 2004, BMC Cancer 12:34; Shachar & Haran, 2011, Leuk Lymphoma 52:1446-54); autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (Morand & Leech, 2005, Front Biosci 10:12-22; Shachar & Haran, 2011, Leuk Lymphoma 52:1446-54); kidney diseases such as renal allograft rejection (Lan, 2008, Nephron Exp Nephrol. 109:e79-83); and numerous inflammatory diseases (Meyer-Siegler et al., 2009, Mediators Inflamm epub March 22, 2009; Takahashi et al., 2009, Respir Res 10:33; Milatuzumab (hLL1) is an exemplary anti-CD74 antibody of therapeutic use for treatment of MIF-mediated diseases.

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[099] Anti-CD3 antibodies have been reported to reduce development and progression of atherosclerosis (Steffens et al., 2006, Circulation 114:1977-84). Antibodies against oxidized LDL induced a regression of established atherosclerosis in a mouse model (Ginsberg, 2007, J Am Coll Cardiol 52:2319-21). Anti-ICAM-1 antibody was shown to reduce ischemic cell damage after cerebral artery occlusion in rats (Zhang et al., 1994, Neurology 44:1747-51).

Antibody Allotypes

[0100] Immunogenicity of therapeutic antibodies is associated with increased risk of infusion reactions and decreased duration of therapeutic response (Baert et al., 2003, N Engl J Med 348:602-08). The extent to which therapeutic antibodies induce an immune response in the host may be determined in part by the allotype of the antibody (Stickler et al., 2011, Genes and Immunity 12:213-21). Antibody allotype is related to amino acid sequence variations at specific locations in the constant region sequences of the antibody. The allotypes of IgG antibodies containing a heavy chain γ-type constant region are designated as Gm allotypes (1976, J Immunol 117:1056-59).

[0101] For the common IgG1 human antibodies, the most prevalent allotype is G1m1 (Stickler et al., 2011, Genes and Immunity 12:213-21). However, the G1m3 allotype also occurs frequently in Caucasians (*Id.*). It has been reported that G1m1 antibodies contain allotypic sequences that tend to induce an immune response when administered to non-G1m1 (nG1m1) recipients, such as G1m3 patients (*Id.*). Non-G1m1 allotype antibodies are not as immunogenic when administered to G1m1 patients (*Id.*).

[0102] The human G1m1 allotype comprises the amino acids aspartic acid at Kabat position 356 and leucine at Kabat position 358 in the CH3 sequence of the heavy chain IgG1. The nG1m1 allotype comprises the amino acids glutamic acid at Kabat position 356 and methionine at Kabat position 358. Both G1m1 and nG1m1 allotypes comprise a glutamic acid residue at Kabat position 357 and the allotypes are sometimes referred to as DEL and EEM allotypes. A non-limiting example of the heavy chain constant region sequences for G1m1 and nG1m1 allotype antibodies is shown for the exemplary antibodies rituximab (SEQ ID NO:85) and veltuzumab (SEQ ID NO:86).

Rituximab heavy chain variable region sequence (SEQ ID NO:85)

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK**KA**EPKSCDKTHTCPPCPAP
ELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK
TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE

PQVYTLPPSR**DEL**TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDG
SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Veltuzumab heavy chain variable region (SEQ ID NO:86

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK**RV**EPKSCDKTHTCPPCPAP
ELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK
TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE
PQVYTLPPSR**EEM**TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDG
SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

[0103] Jefferis and Lefranc (2009, mAbs 1:1-7) reviewed sequence variations characteristic of IgG allotypes and their effect on immunogenicity. They reported that the G1m3 allotype is characterized by an arginine residue at Kabat position 214, compared to a lysine residue at Kabat 214 in the G1m17 allotype. The nG1m1,2 allotype was characterized by glutamic acid at Kabat position 356, methionine at Kabat position 358 and alanine at Kabat position 431. The G1m1,2 allotype was characterized by aspartic acid at Kabat position 356, leucine at Kabat position 358 and glycine at Kabat position 431. In addition to heavy chain constant region sequence variants, Jefferis and Lefranc (2009) reported allotypic variants in the kappa light chain constant region, with the Km1 allotype characterized by valine at Kabat position 153 and leucine at Kabat position 191, the Km1,2 allotype by alanine at Kabat position 153 and leucine at Kabat position 191, and the Km3 allotype characterized by alanine at Kabat position 153 and valine at Kabat position 191.

[0104] With regard to therapeutic antibodies, veltuzumab and rituximab are, respectively, humanized and chimeric IgG1 antibodies against CD20, of use for therapy of a wide variety of hematological malignancies and/or autoimmune diseases. **Table 5** compares the allotype sequences of rituximab vs. veltuzumab. As shown in **Table 5**, rituximab (G1m17,1) is a DEL allotype IgG1, with an additional sequence variation at Kabat position 214 (heavy chain CH1) of lysine in rituximab vs. arginine in veltuzumab. It has been reported that veltuzumab is less immunogenic in subjects than rituximab (*see, e.g.,* Morchhauser et al., 2009, J Clin Oncol 27:3346-53; Goldenberg et al., 2009, Blood 113:1062-70; Robak & Robak, 2011, BioDrugs 25:13-25), an effect that has been attributed to the difference between humanized and chimeric antibodies. However, the difference in allotypes between the EEM and DEL allotypes likely also accounts for the lower immunogenicity of veltuzumab.

Table 5. Allotypes of Rituximab vs. Veltuzumab

		Heavy chain position and associated allotypes					
	Complete allotype	214 (allotype)		356/358 (allotype)		431 (allotype)	
Rituximab	G1m17,1	K	17	D/L	1	A	-
Veltuzumab	G1m3	R	3	E/M	-	A	-

[0105] In order to reduce the immunogenicity of therapeutic antibodies in individuals of nG1m1 genotype, it is desirable to select the allotype of the antibody to correspond to the G1m3 allotype, characterized by arginine at Kabat 214, and the nG1m1,2 null-allotype, characterized by glutamic acid at Kabat position 356, methionine at Kabat position 358 and alanine at Kabat position 431. Surprisingly, it was found that repeated subcutaneous administration of G1m3 antibodies over a long period of time did not result in a significant immune response. In alternative embodiments, the human IgG4 heavy chain in common with the G1m3 allotype has arginine at Kabat 214, glutamic acid at Kabat 356, methionine at Kabat 359 and alanine at Kabat 431. Since immunogenicity appears to relate at least in part to the residues at those locations, use of the human IgG4 heavy chain constant region sequence for therapeutic antibodies is also a preferred embodiment. Combinations of G1m3 IgG1 antibodies with IgG4 antibodies may also be of use for therapeutic administration.

Immunoconjugates

[0106] In certain embodiments, the antibodies or complexes 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.

[0107] 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.

[0108] 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. 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.

[0109] The Fc region may be absent if the antibody used as the antibody component 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. The engineered carbohydrate moiety is used to attach the therapeutic or diagnostic agent.

[0110] 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 No. 7,563,433.

[0111] 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

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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.

[0112] Chelates may be directly linked to antibodies or peptides, for example as disclosed in U.S. Patent 4,824,659. 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.

[0113] 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.

Therapeutic Agents

[0114] 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 antibody complexes or separately administered before, simultaneously with, or after the antibody complex. Drugs of use may possess a pharmaceutical property selected from the group consisting of antimitotic, kinase inhibitor, Bruton kinase inhibitor, alkylating, antimetabolite, antibiotic, alkaloid, anti-angiogenic, pro-apoptotic agents and combinations thereof.

[0115] Exemplary drugs of use include, but are not limited to, 5-fluorouracil, afatinib, aplidin, azaribine, anastrozole, anthracyclines, axitinib, AVL-101, AVL-291, bendamustine, bleomycin, bortezomib, bosutinib, bryostatin-1, busulfan, calicheamycin, camptothecin,

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carboplatin, 10-hydroxycamptothecin, carmustine, celecoxib, chlorambucil, cisplatin (CDDP), Cox-2 inhibitors, irinotecan (CPT-11), SN-38, carboplatin, cladribine, camptothecans, crizotinib, cyclophosphamide, cytarabine, dacarbazine, dasatinib, dinaciclib, docetaxel, dactinomycin, daunorubicin, doxorubicin, 2-pyrrolinodoxorubicine (2P-DOX), cyano-morpholino doxorubicin, doxorubicin glucuronide, epirubicin glucuronide, erlotinib, estramustine, epidophyllotoxin, entinostat, estrogen receptor binding agents, etoposide (VP16), etoposide glucuronide, etoposide phosphate, exemestane, fingolimod, floxuridine (FUdR), 3',5'-O-dioleoyl-FudR (FUdR-dO), fludarabine, flutamide, farnesyl-protein transferase inhibitors, flavopiridol, fostamatinib, ganetespib, GDC-0834, GS-1101, gefitinib, gemcitabine, hydroxyurea, ibrutinib, idarubicin, idelalisib, ifosfamide, imatinib, L-asparaginase, lapatinib, lenolidamide, leucovorin, LFM-A13, lomustine, mechlorethamine, melphalan, mercaptopurine, 6-mercaptopurine, methotrexate, mitoxantrone, mithramycin, mitomycin, mitotane, navelbine, neratinib, nilotinib, nitrosurea, olaparib, plicomycin, procarbazine, paclitaxel, PCI-32765, pentostatin, PSI-341, raloxifene, semustine, sorafenib, streptozocin, SU11248, sunitinib, tamoxifen, temazolomide (an aqueous form of DTIC), transplatinum, thalidomide, thioguanine, thiotepa, teniposide, topotecan, uracil mustard, vatalanib, vinorelbine, vinblastine, vincristine, vinca alkaloids and ZD1839.

[0116] 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.

[0117] Chemokines of use may include RANTES, MCAF, MIP1-alpha, MIP1-Beta and IP-10.

[0118] 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.

[0119] 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.

[0120] 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, ^{227}Th 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, Th-227 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. Radionuclides and other metals may be delivered, for example, using chelating groups attached to an antibody or conjugate. 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 for complexing ^{223}Ra, may be used.

[0121] 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.

[0122] 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

[0123] 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.

Therapeutic Use

[0124] In another aspect, the invention relates to a method of treating a subject, comprising administering a therapeutically effective amount of an antibody complex as described herein to a subject. Diseases that may be treated with the antibody complexes described herein include, but are not limited to immune diseases (e.g., SLE, RA, juvenile idiopathic arthritis, Crohn's disease, type 2 diabetes, Castleman's disease) or inflammatory diseases (e.g., sepsis, septic shock, inflammation, inflammatory bowel disease, inflammatory liver injury, acute pancreatitis). Such therapeutics can be given once or repeatedly, depending on the disease state and tolerability of the conjugate, and can also be used optimally in combination with other therapeutic modalities, such as immunomodulator therapy, immunotherapy, chemotherapy, antisense therapy, interference RNA therapy, gene therapy, and the like. Each combination will be adapted to patient condition and prior therapy, and other factors considered by the managing physician.

[0125] As used herein, the term "subject" refers to any animal (i.e., vertebrates and invertebrates) including, but not limited to mammals, including humans. It is not intended that the term be limited to a particular age or sex. Thus, adult and newborn subjects, as well as fetuses, whether male or female, are encompassed by the term.

[0126] In preferred embodiments, the antibodies that are used in the treatment of human disease are human or humanized (CDR-grafted) versions of antibodies; although murine and chimeric versions of antibodies can be used. Same species IgG molecules are mostly preferred to minimize immune responses. This is particularly important when considering

repeat treatments. For humans, a human or humanized IgG antibody is less likely to generate an anti-IgG immune response from patients.

[0127] In another preferred embodiment, diseases that may be treated using the antibody complexes include, but are not limited to immune dysregulation disease and related autoimmune diseases, including Class III autoimmune diseases such as immune-mediated thrombocytopenias, such as acute idiopathic thrombocytopenic purpura and chronic idiopathic thrombocytopenic purpura, dermatomyositis, Sjögren's syndrome, multiple sclerosis, 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, sarcoidosis, ulcerative colitis, erythema multiforme, IgA nephropathy, polyarteritis nodosa, ankylosing spondylitis, Goodpasture's syndrome, thromboangitis obliterans, Sjögren's syndrome, primary biliary cirrhosis, Hashimoto's thyroiditis, thyrotoxicosis, scleroderma, chronic active hepatitis, rheumatoid arthritis, polymyositis/dermatomyositis, polychondritis, pemphigus vulgaris, Wegener's granulomatosis, membranous nephropathy, amyotrophic lateral sclerosis, tabes dorsalis, giant cell arteritis/polymyalgia, pernicious anemia, rapidly progressive glomerulonephritis and fibrosing alveolitis, and also juvenile diabetes, as disclosed in U.S. Provisional Application Serial No. 60/360,259, filed March 1, 2002 (now expired). Antibodies that may be of use for combination therapy in these diseases include, but are not limited to, those reactive with HLA-DR antigens, B-cell and plasma-cell antigens (e.g., CD19, CD20, CD21, CD22, CD23, CD4, CD5, CD8, CD14, CD15, CD19, CD20, CD21, CD22, CD23, CD25, CD33, CD37, CD38, CD40, CD40L, CD46, CD52, CD54, CD74, CD80, CD126, CD138, B7, MUC1, Ia, HM1.24, and HLA-DR), IL-6, IL-17. Since many of these autoimmune diseases are affected by autoantibodies made by aberrant B-cell populations, depletion of these B-cells is a preferred method of autoimmune disease therapy. In a preferred embodiment, the anti-B-cell, anti-T-cell, or anti-macrophage or other such antibodies of use in the co-treatment of patients with autoimmune diseases also can be conjugated to result in more effective therapeutics to control the host responses involved in said autoimmune diseases, and can be given alone or in combination with other therapeutic agents, such as TNF inhibitors or anti-IL-6R antibodies and the like.

[0128] In a preferred embodiment, a more effective therapeutic agent can be provided by using multivalent, multispecific antibodies. Exemplary bivalent and bispecific antibodies are found in U.S. Patent Nos. 7,387,772; 7,300,655; 7,238,785; and 7,282,567.

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These multivalent or multispecific antibodies are particularly preferred in the targeting of disease associated cells which express multiple antigen targets and even multiple epitopes of the same antigen target, but which often evade antibody targeting and sufficient binding for immunotherapy because of insufficient expression or availability of a single antigen target on the cell. By targeting multiple antigens or epitopes, said antibodies show a higher binding and residence time on the target, thus affording a higher saturation with the drug being targeted in this invention.

Formulation and Administration

[0129] Suitable routes of administration of the conjugates include, without limitation, oral, parenteral, rectal, transmucosal, intestinal administration, intramuscular, subcutaneous, intramedullary, intrathecal, direct intraventricular, intravenous, intravitreal, intraperitoneal, intranasal, or intraocular injections. The preferred routes of administration are parenteral. Alternatively, one may administer the compound in a local rather than systemic manner, for example, via injection of the compound directly into a solid tumor.

[0130] Antibody complexes or immunoconjugates can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby the antibody complex or immunoconjugate 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.

[0131] The antibody complex or immunoconjugate can be formulated for intravenous administration via, for example, bolus injection or continuous infusion. Preferably, the antibody of the present invention 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.

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[0132] Additional pharmaceutical methods may be employed to control the duration of action of the antibody complex. Control release preparations can be prepared through the use of polymers to complex or adsorb the antibody complex. 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 of an antibody complex or immunoconjugate from such a matrix depends upon the molecular weight, the amount of antibody complex or immunoconjugate 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.

[0133] Generally, the dosage of an administered antibody complex or immunoconjugate 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 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.

[0134] Alternatively, an antibody complex or immunoconjugate may be administered as one dosage every 2 or 3 weeks, repeated for a total of at least 3 dosages. Or, 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.

Expression Vectors

[0135] Still other embodiments may concern DNA sequences comprising a nucleic acid encoding an antibody, antibody fragment, toxin or constituent fusion protein of an antibody complex, such as a DNL® construct. Fusion proteins may comprise an antibody or fragment or toxin attached to, for example, an AD or DDD moiety.

[0136] 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.

[0137] 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.

Kits

[0138] Various embodiments may concern kits containing components suitable for treating or diagnosing diseased tissue in a patient. Exemplary kits may contain one or more antibody complexes 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.

[0139] 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,

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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

[0140] In the working Examples below, the DOCK-AND-LOCK® (DNL®) technology was used to generate the first bispecific antibody (bsAb) with high potency to neutralize both TNF-α and IL-6. This prototype DNL® construct, designated cT*-(c6)-(c6), comprises a chimeric anti-TNF-α IgG linked at the carboxyl terminus of each light chain to a pair of dimerized Fab's derived from a chimeric anti-IL-6 antibody, thus featuring a hexavalent bsAb capable of blocking 2 and 4 molecules of TNF-α and IL-6, respectively, as well as a fully functional Fc. As discussed below, the exemplary anti-TNF-α/anti-IL-6 bispecific antibody showed potent activity in *in vitro* assays designed to test efficacy for immune diseases such as SLE or RA. However, the person of ordinary skill will realize that the subject complexes of use are not limited to the specific DNL® cT*-(c6)-(c6) complex discussed below, but more generally encompass bispecific antibodies and/or antigen-binding antibody fragments with at least one binding site for IL-6 an at least one binding site for TNF-α.

Example 1. Generation of Neutralizing Mouse Anti-Human IL-6 Monoclonal Antibody

[0141] The 2-3B2 mouse monoclonal antibody against human IL-6 was produced using standard immunological techniques, discussed below, that may be used to make anti-human IL-6 antibodies in general.

[0142] Recombinant human IL-6 (rhIL-6) was obtained from ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel). Multiple mice were initially immunized with 30 μg rhIL-6 administered i.p., followed by booster injections of 30 or 10 μg with or without adjuvant, according to a standard boosting schedule. Animals were tested for presence of anti-IL-6 antibodies by ELISA assay using rhIL-6 coated microtiter plates and serial dilutions of serum. Prior to fusion, the presence of neutralizing anti-IL-6 antibodies was detected by the ability to block IL-6 stimulated protein phosphorylation (of STAT3) using Western blotting (data not shown).

[0143] Cells secreting neutralizing anti-IL-6 antibodies were fused with the P3-X63.Ag8.653 myeloma cell line by PEG mediated cell fusion using standard techniques to generate

antibody-secreting hybridomas cell lines. The 2-3B2, 4-4F5 and 4-4E6 anti-IL-6 clones were obtained by selection on HAT medium, cloning and subcloning. Supernatants from isolated clones containing neutralizing anti-IL-6 were detected by the ability to block IL-6 stimulated STAT3 protein phosphorylation, determined by Western blotting (**FIG. 1**). Clones 2-3B2, 4-4E6 and control anti-IL-6 MAb206, but not clone 4-4F5, were able to block rhIL-6 induced phosphorylation (**FIG. 1**). Size exclusion HPLC of antibodies purified by protein A column chromatography demonstrated the presence of homogeneous antibodies, which was confirmed by SDS-PAGE (data not shown). Isotyping using an SBA CLONETYPING™ system showed that the anti-IL-6 antibodies were IgG1/κ murine isotypes.

[0144] Binding to human IL-6 was determined Western blotting against rhIL-6 (**FIG. 2**). Based on the intensity of labeling using identical concentrations of antibody, it was determined that the 2-3B2 clone (**FIG. 2A**) showed higher affinity for human IL-6 than the 4-4E6 clone (**FIG. 2B**) and 2-3B2 was selected for production of chimeric and humanized anti-IL-6 antibodies. Serial dilution demonstrated that the 2-3B2 antibody was about 100-fold more potent than 4-4E6 for inhibiting the IL-6 induced phosphorylation of STAT3 (**FIG. 2A-B**). Neither antibody bound to murine IL-6 (not shown).

Example 2. Generation of Neutralizing Mouse Anti-Human TNF-α Monoclonal Antibody

[0145] Monoclonal antibodies against human TNF-α were prepared using standard techniques, as discussed in Example 1 above for IL-6. Mice were immunized with recombinant human TNF-α obtained from ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel).Testing of serum from immunized mice for anti-TNF-α antibodies was performed by ELISA (data not shown).

[0146] Neutralizing antibodies were also detected by cytotoxicity assay. Briefly, WEHI 164 cells (mouse fibrosarcoma) were cultured in RPMI complete media. Cells were plated at a density of $1x10^4$ cells/well in 75 µL of medium in 96-well plates and kept in a 37°C incubator overnight before the assay. On the day of the assay, sera from the immunized mice were diluted 1:25, 1:125, 1:625, 1:3,125, 1: 15,625, and 1: 78,125 in RPMI complete medium containing 8 µg/mL of actinomycin-D and 0.4 ng/mL of rhTNF-α. Twenty-five µL of the diluted sera were added to the cells in the corresponding wells. The addition of the sera to the cells made the final dilutions of the sera as 1:100, 1:500, 1:2,500, 1:12,500, 1:62,500, and 1:312,500. The final concentration of actinomycin-D was 2 µg/mL and rhTNF-α was 0.1 ng/mL. Plates were incubated in a 37°C/5% CO2 incubator for 20 hours. After this incubation, 20 µL of MTS reagent was added to all the wells and the absorbance in each well

determined in a plate reader at 490nm after two hours. As a negative control, serum from a naive mouse (not immunized) was diluted in a like manner. One set of wells was incubated with only actinomycin-D and rhTNF-α to determine maximum growth inhibition. Another set of cells remained untreated (cells grown in media lacking actinomycin-D and rhTNF-α). Growth inhibition was measured as percent of untreated control cell growth.

[0147] The results of the cytotoxicity assay are shown in **FIG. 3**. Serum from each of the inoculated mice showed the ability to neutralize rhTNF-α mediated cytotoxicity. Serum from mouse #3 showed the greatest ability to inhibit rhTNF-α mediated cytotoxicity.

[0148] Hybridomas were produced from splenocytes of mice showing the presence of anti-TNF-α antibodies by PEG fusion, essentially as discussed above. Selection of fused hybridomas was performed using HAT medium. Neutralizing clones 4C9 and 4D3 were obtained from mouse #3. After further subcloning, antibodies were purified by chromatography on protein G columns. Purified antibodies were determined to be homogeneous by size separation HPLC and SDS-PAGE (data not shown). Isotype analysis, performed as discussed above, showed that 4C9 was IgG1/κ while 4D3 was IgG2a/κ.

[0149] The ability of anti-TNF-α antibodies from clones 4C9D11 and 4D3B11 to neutralize TNF-α-mediated cytotoxicity was determined (**FIG. 4**). WEHI 164 cells were seeded at 1×10^4 cells/well into 96-well plates and grown in 200 µL of RPMI complete medium overnight. On the day of the assay, supernatants from clones were collected and diluted 1:2. A further 1:5 dilution was made thereafter. Each dilution was made in RPMI complete medium containing a final concentration of actinomycin-D at 2 µg/mL and rhTNF-α at 0.1 ng/mL. Before addition of the diluted supernatant, the medium in the plate for WEHI 164 cells growth was removed, and replaced with the diluted supernatant in the corresponding wells, 100 µL/well. The plate was incubated for 20 hours at 37°C in a 5% CO2 incubator. After this incubation, 20 µL of MTS was added to all the wells and the absorbance in each well determined in a plate reader at 490nm after two hours. As a negative control, supernatant from a clone which stopped producing antibody (ELISA negative) was diluted in a like manner. One set of wells was incubated with only actinomycin-D and rhTNF- α to determine maximum growth inhibition. Another set of cells remained untreated (cells grown in media lacking actinomycin-D and rhTNF-α). Growth inhibition was measured as percent of untreated control cell growth. The antibody from clone 4D3B11 was more effective at blocking TNF-α mediated cytotoxicity in this assay (**FIG. 4**).

[0150] Antibody binding specificity was determined by Western blotting against rhTNF-α. Under reducing conditions, 4D3B11C4 and the anti-TNF-α antibody REMICADE®

(infliximab) showed no or weak binding to human TNF-α, with no binding to human TNF-β or murine TNF-α (not shown). Under the same reducing conditions, antibody 4C9D11G11 showed strong binding to human TNF-α, with no binding to human TNF-β or murine TNF-α (not shown).

[0151] Neutralization of rhTNF-α induced cytotoxicity by anti-TNF-α antibodies was determined in a different *in vitro* system (**FIG. 5**). L929 cells (mouse fibroblasts) were seeded at 2×10^4 cells/well into 96-well plates and grown in 90 µL of MEM medium overnight (10% horse serum complete medium). On the following day, the purified antibodies were diluted 1:5 in MEM medium (containing a final concentration of actinomycin-D at 20 µg/mL and rhTNF-α at 1 ng/mL) for an antibody concentration range of 10,000 to 3.2 ng/mL. The antibodies were pre-incubated with rhTNF-α at RT for one hour. After this pre-incubation, 10 µL of the diluted antibodies were then added to the 90 µL cells in the corresponding wells, that made the final concentration of the antibodies from 1000 to 0.32 ng/mL, with a final concentration of actinomycin-D and rhTNF-α at 2 µg/mL and 0.1 ng/mL, respectively. The plate was incubated for 20 hours at 37°C. Following this incubation, 20 µL of MTS was added to all the wells and the absorbance in each well determined in a plate reader at 490nm after two hours. As a negative control, an anti-hTNF-α antibody, 4C3 (non-neutralizing), was diluted in a like manner. REMICADE®, the commercial anti-TNF-α antibody was also diluted in a like manner as a positive control. One set of wells was incubated with only actinomycin-D and rhTNF-α to determine maximum growth inhibition.

[0152] Under these conditions, the 4C9D11G11 antibody (EC_{50} 11.2 ng/mL) was more effective than 4D3B11C4 (EC_{50} 22.1 ng/mL) at inhibiting TNF-α-induced cytotoxicity (**FIG. 5**). Neither monoclonal antibody was as effective as REMICADE® (EC_{50} 3.6 ng/mL) (**FIG. 5**).

[0153] An assay was performed for antibody based neutralization of rhTNF-α-induced cell surface expression of ICAM-1 (**FIG. 6**). ECV-304 cells (a derivative of T24, bladder cancer cell line) were seeded at $2x10^5$ cells/well into 6-well plates, grown in 10% FBS Medium 199 for 6 hours for attaching. Varying doses of the mAbs or REMICADE® (positive control) were mixed with constant amounts of rhTNF- α (10 ng/mL). The mixture of the antibodies and rhTNF-α was pre-incubated at 37 °C for two hours, and then pipetted into the appropriate corresponding wells in duplicate. Cells were then grown for 72 hours in a 37 °C incubator. After this incubation, supernatant was removed and cells were trypsinized and transferred to 15 mL tubes. Cells were washed with cold PBS/0.5% BSA two times, supernatant was

removed and the cell pellets were re-suspended in the residual wash buffer (~100 µL). An aliquot of 25 µL from the cell suspension from each sample was then transferred to 4 mL flow tubes. Cells were Fc-blocked by treatment with 1 µg of human IgG for 15 min at RT and then incubated with fluorescent-conjugated anti-CD54 reagent for 45 min at 4 °C. Cells were then washed with 4 mL of PBS/5% BSA for two times and re-suspended in 400 µL of PBS and then subjected to flow-cytometric analysis (FACS). One set of cells remained untreated as background fluorescent control. Another set of cells treated with only 10 ng/mL of rhTNF-α served as the positive control for obtaining maximum fluorescent (i.e. maximum ICAM-1 up-regulation).

[0154] The 4C9 clone again showed higher neutralizing activity than the 4D3 clone and 4C9 was selected for chimerization.

Example 3. Production of Chimeric Anti-IL-6 Antibody from 2-3B2 Hybridoma

[0155] Total RNA was extracted from hybridomas 2-3B2 cells by standard techniques and mRNA was separated from the total RNA fraction. The mRNA was used as a template for VH and VK cDNA synthesis, using a QIAGEN® OneStep RT-PCR kit. Primers used were as shown below (restriction sites are underlined).

Vk1 BACK (PNAS 86:3833-3837,1989)

GACATTCAGCTGACCCAGTCTCCA (SEQ ID NO:90)

CK3'-BH: (Biotechniques 15:286-291, 1993)

GCCGGATCCTCACTGGATGGTGGGAAGATGGATACA (SEQ ID NO:91)

VH1 BACK: (PNAS 86:3833-3837, 1989)

AGGTSMARCTGCAGSAGTCWGG (SEQ ID NO:92, S=C/G, M=A/C, R=A/G, W=A/T)

CH1-C: (Clinical Cancer Res 5:3095s-3100s, 1999)

AGCTGGGAAGGTGTGCAC (SEQ ID NO:93)

[0156] The VH and Vk cDNA sequences were cloned into the pGEMT vector for sequencing by the Sanger dideoxy technique, using an automated DNA sequencer. The putative VH (SEQ ID NO:94) and Vk (SEQ ID NO:96) murine amino sequences are shown in **FIG. 7** and **FIG. 8**. The locations of the heavy and light chain CDR sequences are proposed, based on homology with the known heavy and light chain antibody sequences of B34871 (SEQ ID NO:95) and AAB53778.1 (SEQ ID NO:97), respectively, from the NCBI protein sequence database. The indicated 2-3B2 heavy chain CDR sequences are CDR1 (GFTFSRFGMH,

SEQ ID NO:107), CDR2 (YIGRGSSTIYYADTVKG, SEQ ID NO:108) and CDR3 (SNWDGAMDY, SEQ ID NO:109). The 2-3B2 light chain CDR sequences are CDR1 (RASGNIHNFLA, SEQ ID NO:110), CDR2 (NAETLAD, SEQ ID NO:111) and CDR3 (QHFWSTPWT, SEQ ID NO:112).

[0157] The VH and VK sequences from the 2-3B2 anti-IL-6 antibody and the VH and VK sequences from the 4C9 anti-TNF-α antibody were used to make a cIL6/TNFα DVD (dual variable domain) antibody construct. (See, e.g., Wu et al., 2009, MAbs 1:339-47.) The resulting bispecific DVD construct was compared with the parent 2-3B2 anti-IL-6 antibody for the ability to inhibit IL-6 induced phosphorylation of STAT3 on HT-29 cells (**FIG. 9**). As shown in FIG. 9, the DVD construct showed the same efficacy as the parent anti-IL-6 antibody for inhibition of IL-6 mediated phosphorylation.

[0158] The sequences for restriction sites and leader peptides for cloning into vector pdHL2 were added to the VH and VK sequences of 2-3B2. The complete sequences were synthesized commercially (GenScript, Piscataway, NJ). The 2-3B2-VH-pUC57 and 2-3B2-VK-pUC57 vectors were produced by incorporating the VH sequence as a XhoI-HindIII insert and the VK sequence as a XbaI-BamH1 insert into corresponding sites in pUC57. A vector expressing chimeric 2-3B2 antibody was produced starting with the hA20-pdHL2-IgG vector (see, e.g., Goldenberg et al., 2002, Blood 100:11 Abstract 2260). The hA20-VH sequence was replaced with cIL6-VH and the hA20-VK sequence was replaced with cIL6-VK by restriction enzyme digestion and ligation. The resulting chimeric 2-3B2 antibody comprised the murine VH and VK sequences of 2-3B2, attached to human antibody constant region sequences. After transfection, screening and antibody purification on a protein A column, a chimeric anti-IL6 clone 1B5 (c-IL6-1B5) was obtained as a homogeneous antibody preparation, as confirmed by HPLC and SDS-PAGE (not shown). The final clone is identified as 1B5A9.

Example 4. Production of Chimeric Anti-TNF-α Antibody from 4C9 Hybridoma

[0159] Total RNA was extracted from hybridomas 4C9 cells by standard techniques and mRNA was separated from the total RNA fraction. The mRNA was used as a template for VH and VK cDNA synthesis, using a PHUSION® High Fidelity PCR kit (Thermo Scientific, Pittsburgh, PA). Primers used were as disclosed in Example 3 above.

[0160] The VH and Vk cDNA sequences were cloned into the pGEMT vector for sequencing by the Sanger dideoxy technique, using an automated DNA sequencer. The putative VH (SEQ ID NO:98) and Vk (SEQ ID NO:100) murine amino sequences are shown in **FIG. 10**

and **FIG. 11**. The locations of the heavy and light chain CDR sequences are proposed, based on homology with the known heavy and light chain antibody sequences of AAS66033.1 (SEQ ID NO:99) and AAS66032.1 (SEQ ID NO:101), respectively, from the NCBI protein sequence database. The indicated 4C9 heavy chain CDR sequences are CDR1 (GFWN, SEQ ID NO:113), CDR2 (YISYSGRTYYNPSLKS, SEQ ID NO:114) and CDR3 (DANYVLDY, SEQ ID NO:115). The 4C9 light chain CDR sequences are CDR1 (KSSQSLLNSSTQKNYLA, SEQ ID NO:116), CDR2 (FASARES, SEQ ID NO:117) and CDR3 (QQHYRTPFT, SEQ ID NO:118).

[0161] An optimized DNA sequence encoding the TNF-α VH, also comprising a 5' leader sequence and 3' flanking sequence, was designed as shown in SEQ ID NO:102 below and cloned into pdHL2. The optimized 4C9-VH sequence is underlined. The DNA sequence was synthesized by GenScript (Piscataway, NJ).

*CTCGAG*CACACAGGACCTCACCATGGGATGGAGCTGTATCATCCTCTTCTTGGTA
GCAACAGCTACAGGTAAGGGGCTCACAGTAGCAGGCTTGAGGTCTGGACATATA
TATGGGTGACAATGACATCCACTTTGCCTTTCTCTCCACAGGTGTCCACTCCGTGC
AGCTGCAGGAGAGCGGACCCTCCCTGGTGAAGCCTAGTCAGACCCTGAGCCTGA
CATGCTCCGTGACTGGGGACTCTATCACCAGTGGCTTCTGGAACTGGATTCGGAA
GTTCCCAGGAAACAAGTTTGAATACATGGGATATATCTCTTACAGTGGGCGCACA
TACTATAACCCCAGCCTGAAGTCCAGGCTGTCTATTACAAGAGACACTTCTAAAA
ACCAGTTTTATCTGCAGCTGAACAGCGTGACTGCCGAGGATACTGCTACCTACTA
TTGTGCCAGGGACGCTAATTATGTGCTGGATTACTGGGGCCAGGGAACCACACTG
ACCGTGAGCTCCGGTGAGTCCTTACAACCTCTCTCTTCTATTCAGCTTAAATAGAT
TTTACTGCATTTGTTGGGGGGGAAATGTGTGTATCTGAATTTCAGGTCATGAAGG
ACTAGGGACACCTTGGGAGTCAGAAAGGGTCATTGGG*AAGCTT* (SEQ ID NO:102)

[0162] An optimized DNA sequence encoding the TNF-α VK, also comprising a 5' leader sequence and 3' flanking sequence, was designed as shown in SEQ ID NO:103 below and cloned into pdHL2. The optimized 4C9-VK sequence is underlined. The DNA sequence was synthesized by GenScript (Piscataway, NJ).

TCTAGACACAGGACCTCACCATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGC
AACAGCTACAGGTAAGGGGCTCACAGTAGCAGGCTTGAGGTCTGGACATATATA
TGGGTGACAATGACATCCACTTTGCCTTTCTCTCCACAGGTGTCCACTCCGACATC
CAGCTGACCCAGAGCCCCAGCTCCCTGGCTATGTCCGTGGGACAGAAGGTGACA

ATGAACTGCAAATCTAGTCAGTCTCTGCTGAACAGCTCCACTCAGAAGAATTACC
TGGCTTGGTTCCAGCAGAAGCCCGGGCAGAGTCCTAAACTGCTGGTGTATTTTGC
CTCTGCTAGGGAGAGTGGCGTGCCAGACAGATTCATCGGCAGCGGCAGCGGGAC
CGATTTTACCCTGACAATTTCTAGTGTGCAGGCCGAGGACCTGGCTGATTACTTC
TGTCAGCAGCACTATCGGACTCCCTTCACCTTTGGCTCCGGAACAAAGCTGGAGA
TCAAGCGTGAGTAGAATTTAAACTTTGCTTCCTCAGTTGGATCC (SEQ ID NO:103)

[0163] The VH and VK coding sequences were inserted into pUC57 and then pdHL2 for expression of the chimeric 4C9 antibody as discussed in Example 3 above. The chimeric 4C9 was produced by transfection of pdHL2, screening for transfectants and antibody purification on a protein A column. The selected clone was designated 6A9. The purified antibody was determined to be homogeneous by HPLC and SDS-PAGE (not shown). A binding affinity assay for chimeric anti-TNF-α showed a dissociation constant (K_D) of 4.13 e^{-11} (not shown).

Example 5. Construction of CH1-DDD2-cFab-anti-IL-6-pGSHL

[0164] The hLL2-Fab-DDD2-pGSHL#2 plasmid (see, e.g., WO2013181087A2; Rossi et al., 2009, Blood 113:6161-71; U.S. Patent Publ. Nos. 20130323204, 20140212425) was used as a starting material for production of a DDD2 conjugated Fab anti-IL-6 antibody fragment. The hLL2-DDD2 plasmid was digested with XbaI/XhoI and the 6577 bp vector was isolated. cIL6-pdHL2 (Example 3) was digested with XbaI/XhoI and the 2604 bp cIL6 coding insert was isolated. The two were ligated to form the 9182 bp Vk-cIL6-Fab-DDD2-pGSHL vector. After screening by PstI digestion and electrophoresis, the 9182 bp vector was digested with XhoI/Hind3/Alkaline phosphatase and an 8536 vector was isolated. The cIL6-pdHL2 vector, comprising a 648 bp cIL6-VH coding insert was digested with XhoI/Hind3. The 648 bp VH encoding insert was ligated with the 8536 bp vector and VK insert to generate C_H1-DDD2-cFab-anti-IL-6-pGSHL. The final construct was then transfected, clones were picked and purified by Kappa-select (GE Healthcare Life Sciences, Piscataway, NJ). The purified antibody product of C_H1-DDD2-cFab-anti-IL-6 appeared homogeneous on HPLC and SDS-PAGE (not shown). The DDD2-derivatized cIL6-Fab showed equivalent activity to the underivatized cIL6 or an hR1-$(IL6)_4$ construct when assayed for inhibition of IL-6 induced STAT3 phosphorylation (not shown).

Example 6. Construction of C_K-AD2-cIgG-anti-TNF-α-pdHL2

[0165] The Ck-AD2-IgG-hA20-pdHL2 plasmid (see, e.g., WO201262583A1; Chang et al., 2012, PLoS ONE 7(8): e44235; U.S. Patent Publ. Nos. 20130323204, 20070140966) was used as a starting material for production of an AD2 conjugated IgG anti-TNF-α antibody. The Ck-AD2-hA20 plasmid was digested with BamHI/XhoI to obtain the Ck-AD2 coding

portion. Plasmid cIgG-anti-TNF-α-pdHL2 (Example 4) was digested with BamHI/XhoI to obtain ΔC_K-cIgG-anti-TNF-α-pdHL2. The two were ligated to form Ck-AD2-cIgG-anti-TNF-α-pdHL2 (see FIG. 12). The C_K-AD2-cIgG-anti-TNF-α-pdHL2 vector was used to transform DHFα competent cells. Colonies were picked and purified by mini-Prep. Plasmid DNA was analyzed by restriction endonuclease digestion and agarose gel electrophoresis (not shown). The plasmid DNA was purified by Maxi-Prep and the insert was DNA sequenced. The DNA sequences encoding cTNF-α-VH, AD2 and cTNF-α-VK are shown in SEQ ID NOs 104-106 below.

cTNF-α-VH

GTGCAGCTGCAGGAGAGCGGACCCTCCCTGGTGAAGCCTAGTCAGACCCTGAGC
CTGACATGCTCCGTGACTGGGGACTCTATCACCAGTGGCTTCTGGAACTGGATTC
GGAAGTTCCCAGGAAACAAGTTTGAATACATGGGATATATCTCTTACAGTGGGC
GCACATACTATAACCCCAGCCTGAAGTCCAGGCTGTCTATTACAAGAGACACTTC
TAAAAACCAGTTTTATCTGCAGCTGAACAGCGTGACTGCCGAGGATACTGCTACC
TACTATTGTGCCAGGGACGCTAATTATGTGCTGGATTACTGGGGCCAGGGAACCA
CACTGACCGTGAGCTCC (SEQ ID NO:104)

AD2

TGTGGCCAGATCGAGTACCTGGCCAAGCAGATCGTGGACAACGCCATCCAGCAG
GCCGGGTGC (SEQ ID NO:105)

cTNF-α-VK

GACATCCAGCTGACCCAGAGCCCCAGCTCCCTGGCTATGTCCGTGGGACAGAAG
GTGACAATGAACTGCAAATCTAGTCAGTCTCTGCTGAACAGCTCCACTCAGAAGA
ATTACCTGGCTTGGTTCCAGCAGAAGCCCGGGCAGAGTCCTAAACTGCTGGTGTA
TTTTGCCTCTGCTAGGGAGAGTGGCGTGCCAGACAGATTCATCGGCAGCGGCAGC
GGGACCGATTTTACCCTGACAATTTCTAGTGTGCAGGCCGAGGACCTGGCTGATT
ACTTCTGTCAGCAGCACTATCGGACTCCCTTCACCTTTGGCTCCGGAACAAAGCT
GGAGATCAAGCGTGAGTAGAATTTAAACTTTGCT (SEQ ID NO:106)

[0166] After transfection, screening, expression and antibody purification, clone 4A5 encoding C_K-AD2-cIgG-4A5 was obtained.

Example 7. Construction of cT*-(c6)-(c6) Anti-IL-6/Anti-TNF-α Bispecific DNL® Complex

[0167] The Ck-AD2-cIgG-4A5 and C_H1-DDD2-cFab-anti-IL-6 fusion proteins were used to make a DOCK-AND-LOCK® (DNL) ® complex, using techniques disclosed herein and in issued U.S. Patent Nos. 7,550,143; 7,521,056; 7,534,866; 7,527,787; 7,666,400; 7858,070; 7,871,622; 7,906,121; 7,906,118; 8,163,291; 7,901,680; 7,981,398; 8,003,111; 8,034,352; 8,562,988; 8,211,440; 8,491,914; 8,282,934; 8,246,960; 8,349,332; 8,277,817; 8,158,129; 8,475,794; 8,597,659; 8,481,041; 8,435,540 and 8,551,480.

[0168] The intact DNL® complex was formed by mixing the AD2 and DDD2 components together under reducing conditions and allowing the complementary sequences on the DDD moiety to form a dimer that binds to the AD moiety. Twenty five mg of C_K-AD2-cIgG-4A5 was mixed with 50 mg of C_H1-DDD2-cFab-anti-IL-6. A 1/10 volume of 1 M Tris, pH 7.5, 1 mM EDTA, 2 mM reduced glutathione was added to the reaction and the proteins were reduced overnight at room temperature. The complexes were then oxidized with 4 mM oxidized glutathione at room temperature for 3 hours to form disulfide bonds between the AD2 and DDD2 moieties to stabilize the complex.

[0169] Chromatography of the complex on a MABSELECT™ column was performed. After loading, the column was washed with 0.04 M PBS, pH 7.4 + 1 mM oxidized glutathione, followed by a PBS wash and elution with 0.1 M citrate (pH 3.5). The elution volume was 25 ml (2.5 ml of 3 M Tris, pH 8.6 + 22.5 ml of eluate). The concentration measured by OD280 was 2.3 mg/ml (57.5 mg total).

[0170] The product was dialyzed against two 5-L changes of 0.04 M PBS, pH 7.4. The final concentration by OD280 was 1.8 mg/ml (52.5 mg total). Purified complex was analyzed by SE-HPLC, which confirmed the presence of cT*-(c6)-(c6) as an apparently homogeneous peak (not shown). The results were confirmed by SDS-PAGE. The activity of the purified cT*-(c6)-(c6) bispecific antibody complex was then examined.

[0171] The cT*-(c6)-(c6) complex showed greater activity than the Fab-DDD2-cIL-6 protein for inhibiting IL-6 induced phosphorylation of STAT3 (**FIG. 13**). HT-29 cells were seeded at $2X10^6$ cells/well in 6-well plates, grown overnight. The indicated antibodies were pre-incubated with hIL6 at 37°C for 1 hour. Then media containing rhIL-6 alone or in combination with antibodies was added to the HT-29 cells for 30 min at 37°C. After the incubation, the supernatant was removed, cells were washed and lysed.

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[0172] Two SDS-PAGE gels were run, transferred to nitrocellulose membranes. Membranes were cut at 60 KDa, the upper portions was probed with either anti-p-STAT3 or anti-t-STAT3 (**FIG. 13**). The lower portions were probed with b-actin for loading control (**FIG. 13**). This assay showed that TNF-(IL6)-(IL6) neutralized IL-6 with similar or greater potency compared to anti-IL-6 Fab-DDD2.

[0173] **FIG. 14** shows that the cT*-(c6)-(c6) complex was able to neutralize natural IL-6 induced phosphorylation of STAT3 in HT-29 cells. HT-29 cells were seeded at $2X10^6$ cells/well in 6-well plates and grown overnight. TNF-(IL6)-(IL6) or chimeric anti-IL6 1B5A9 was pre-incubated with the supernatant containing 10 ng/mL of natural IL6 released from collagen Type II stimulated RA patient PBMCs at 37°C for 1 hour. At the end of the incubation, the supernatant containing natural IL-6 alone or in combination with antibodies was added to the HT-29 cells for 30 min at 37°C. After incubation, the supernatant was removed and cells were washed and lysed. Two SDS-PAGE gels were run, transferred to nitrocellulose membranes. Membranes were cut at 60 KDa, the upper portion was probed with either anti-p-STAT3 or anti-t-STAT3 (**FIG. 14**). The lower portions were probed with b-actin for loading control (**FIG. 14**).

[0174] The ability to neutralize TNF-α induced cell death was also examined for cT*-(c6)-(c6) compared to other anti-TNF-α antibody constructs (**FIG. 15**). In the presence of 2 μg/mL of actinomycin-D, recombinant human TNF-α at 0.1 ng/mL induced about 70% cell death in L929 cells. As shown, the TNF-α-IL6-IL6, chimeric anti-TNF-α clone 6A9 and Ck-AD2-cTNF-α-IgG clone 4A5 were able to neutralize the activity of rhTNF-α, and inhibit cell death in a dose-response manner (**FIG. 15**), just like their parent antibody 4C9.

[0175] TNF-α-(IL6)-(IL6) and Ck-AD2-cTNF-α were also able to neutralize cell death of L929 cells induced by natural human TNF-α (released from RA PBMCs) (**FIG. 16**). Upon stimulation by type II collagen for 5 days, natural human TNF-α is released from the cultured PBMCs isolated from a rheumatoid arthritis patient (S22). In the presence of 2 μg/mL of actinomycin-D, TNF-α at 0.1 ng/mL induced about 76% cell death. As shown in **FIG. 16**, TNF-α-IL6-IL6 and Ck-AD2-cTNF-α-IgG clone 4A5 were able to neutralize the activity of the natural human TNF-α, and inhibit cell death in a dose-response manner.

[0176] The ability of cT*-(c6)-(c6) to bind to IL-6 or TNF-α from rat, monkey or human was determined by ELISA. The results are summarized in **FIG. 17**, which shows that the affinity of cT*-(c6)-(c6) for IL-6 or TNF-α from different species was approximately the same as the

individual antibodies, and that the antibodies showed approximately similar dissociation constants for human, Cynomolgus monkey and canine antigens.

[0177] As can be seen in **FIG. 18,** STAT3 plays a central role in both TNF-α and IL-6 mediated pathways and disease processes and inhibition of STAT3 phosphorylation induced by TNF-α or IL-6 is a reasonable surrogate to determine the efficacy of anti-TNF-α or anti-IL-6 antibody complexes as moderators of such disease processes. Because TNF-α and IL-6 play pathogenic roles in the development of a variety of autoimmune, immune dysfunction or inflammatory diseases, including but not limited to systemic lupus erythematosus, rheumatoid arthritis, inflammatory bowel disease, type II diabetes, obesity, atherosclerosis and cachexia related to cancer, the presence results show that bispecific anti-IL-6/anti-TNF-α antibodies, such as cT*-(c6)-(c6), are of use for treatment of such TNF-α/IL-6 mediated diseases or conditions.

CLAIMS:

1. A bispecific antibody comprising at least one anti-tumor necrosis factor alpha (TNF-α) antibody or antigen-binding fragment thereof and at least one anti-interleukin 6 (IL-6) antibody or antigen-binding fragment thereof, wherein the anti-IL-6 antibody or fragment thereof comprises the heavy chain CDR sequences CDR1 (GFTFSRFGMH, SEQ ID NO:107), CDR2 (YIGRGSSTIYYADTVKG, SEQ ID NO:108) and CDR3 (SNWDGAMDY, SEQ ID NO:109) and the light chain CDR sequences CDR1 (RASGNIHNFLA, SEQ ID NO:110), CDR2 (NAETLAD, SEQ ID NO:111) and CDR3 (QHFWSTPWT, SEQ ID NO:112), and wherein the anti-TNF-α antibody or fragment thereof comprises the heavy chain CDR sequences CDR1 (GFWN, SEQ ID NO:113), CDR2 (YISYSGRTYYNPSLKS, SEQ ID NO:114) and CDR3 (DANYVLDY, SEQ ID NO:115) and the light chain CDR sequences CDR1 (KSSQSLLNSSTQKNYLA, SEQ ID NO:116), CDR2 (FASARES, SEQ ID NO:117) and CDR3 (QQHYRTPFT, SEQ ID NO:118).

2. The bispecific antibody of claim 1, wherein the bispecific antibody comprises a chimeric or humanized anti-TNF-α IgG antibody and one or more chimeric or humanized anti-IL-6 antigen-binding fragments.

3. The bispecific antibody of claim 1, wherein the bispecific antibody comprises a chimeric or humanized anti-TNF-α IgG antibody and four chimeric or humanized anti-IL-6 antigen-binding fragments.

4. The bispecific antibody of claim 1, wherein the antigen-binding fragments are selected from the group consisting of $F(ab')_2$, Fab', Fab, Fv, and scFv.

5. The bispecific antibody of claim 3, wherein the anti-IL-6 antigen-binding fragments are Fab antibody fragments.

6. The bispecific antibody of any one of claims 1 to 5, wherein the anti-TNF-α antibody or antigen-binding fragment thereof and the anti-IL-6 antibody or antigen-binding fragment thereof are fusion proteins.

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7. The bispecific antibody of any one of claims 1 to 6, wherein the bispecific antibody is conjugated to at least one diagnostic or therapeutic agent.

8. The bispecific antibody of claim 7, wherein the therapeutic agent is selected from the group consisting of a drug, an anti-angiogenic agent, a pro-apoptotic agent, an antibiotic, a hormone, a hormone antagonist, an immunomodulator, a cytokine, a chemokine, a prodrug, and an enzyme.

9. The bispecific antibody of any one of claims 1 to 8, wherein the antibody has an allotype selected from the group consisting of nGlm1, Glm3, nGlm1,2 and Km3.

10. The bispecific antibody of any one of claims 1 to 9, wherein the bispecific antibody is useful for treating a disease selected from the group consisting of autoimmune disease, immune system dysfunction and inflammatory disease.

11. The bispecific antibody of claim 10, wherein the disease is selected from the group consisting of systemic lupus erythematosus (SLE), rheumatoid arthritis, inflammatory bowel disease, type II diabetes, atherosclerosis, juvenile idiopathic arthritis, Castleman's disease, systemic sclerosis, polymyositis, vasculitis syndrome, giant cell arteritis, Takayasu arteritis, cryoglobulinemia, glomerulonephritis, rheumatoid vasculitis, non-septic hyperinflammatory disorder, nephritis, acute pancreatitis, acute respiratory distress syndrome, and graft-vs.-host disease.

12. A murine, chimeric, humanized or human anti-IL-6 antibody or antigen-binding fragment thereof comprising the heavy chain CDR sequences CDR1 (GFTFSRFGMH, SEQ ID NO:107), CDR2 (YIGRGSSTIYYADTVKG, SEQ ID NO:108) and CDR3 (SNWDGAMDY, SEQ ID NO:109) and the light chain CDR sequences CDR1 (RASGNIHNFLA, SEQ ID NO:110), CDR2 (NAETLAD, SEQ ID NO:111) and CDR3 (QHFWSTPWT, SEQ ID NO:112).

13. The anti-IL-6 antibody or antigen-binding fragment thereof of claim 12, wherein the antibody has an allotype selected from the group consisting of nGlm1, Glm3, nGlm1,2 and Km3.

14. The anti-IL-6 antibody or antigen-binding fragment thereof of claim 12, wherein the antibody or antigen-binding fragment is a naked antibody or antigen-binding fragment.

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15. The anti-IL-6 antibody or antigen-binding fragment thereof of claim 12, wherein the antibody or antigen-binding fragment is conjugated to at least one diagnostic or therapeutic agent.

16. The anti-IL-6 antibody or antigen-binding fragment thereof of claim 15, wherein the diagnostic agent is 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.

17. The anti-IL-6 antibody or antigen-binding fragment thereof of claim 15, wherein the therapeutic agent is selected from the group consisting of a drug, an anti-angiogenic agent, a pro-apoptotic agent, an antibiotic, a hormone, a hormone antagonist, an immunomodulator, a cytokine, a chemokine, a prodrug, and an enzyme.

18. The anti-IL-6 antibody or antigen-binding fragment thereof of claim 17, wherein the drug possesses a pharmaceutical property selected from the group consisting of antimitotic, antikinase, anti-tyrosine kinase, alkylating, antimetabolite, antibiotic, anti-angiogenic, pro-apoptotic, and immune modulatory.

19. The anti-IL-6 antibody or antigen-binding fragment thereof of claim 17, wherein the drug is selected from the group consisting of 5-fluorouracil, aplidin, azaribine, anastrozole, anthracyclines, bendamustine, bleomycin, bryostatin-1, busulfan, calicheamycin, camptothecin, carboplatin, carmustine, celecoxib, chlorambucil, cisplatinum, Cox-2 inhibitors, irinotecan (CPT-11), 7-Ethyl-10-hydroxycamptothecin (SN-38), carboplatin, cladribine, camptothecans, cyclophosphamide, cytarabine, dacarbazine, docetaxel, dactinomycin, daunorubicin, doxorubicin, 2-pyrrolinodoxorubicine (2P-DOX), pro-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, nitrosourea, plicomycin, procarbazine, paclitaxel, pentostatin, bortezomib (PSI-341), raloxifene, semustine, streptozocin, tamoxifen,

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temazolomide, transplatinum, thalidomide, thioguanine, thiotepa, teniposide, topotecan, uracil mustard, vinorelbine, vinblastine, vincristine and vinca alkaloids.

20. The anti-IL-6 antibody or antigen-binding fragment thereof of claim 17, wherein the chemokine is selected from the group consisting of regulated on activation, normal T cell expressed and secreted (RANTES), MBD1-containing chromatin-associated factor (MCAF), macrophage inflammatory protein-1 alpha (MIP1-alpha), macrophage inflammatory protein-1 beta (MIP1-Beta) and interferon gamma-induced protein 10 (IP-10).

21. The anti-IL-6 antibody or antigen-binding fragment thereof of claim 17, wherein the anti-angiogenic agent is selected from the group consisting of angiostatin, baculostatin, canstatin, maspin, anti-vascular endothelial growth factor (VEGF) antibody, anti-placental growth factor (PlGF) peptide, anti-vascular growth factor antibody, anti-fetal liver kinase-1 (Flk-1) antibody, anti-Flt-1 antibody, anti-Kras antibody, anti-tyrosine-protein kinase Met (cMET) antibody, anti-macrophage migration-inhibitory factor (MIF) antibody, laminin peptide, fibronectin peptide, plasminogen activator inhibitor, tissue metalloproteinase inhibitor, interferon, interleukin-12, IP-10, growth-regulated oncogene-beta (Gro-β), 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 and minocycline.

22. The anti-IL-6 antibody or antigen-binding fragment thereof of claim 17, wherein the immunomodulator is selected from the group consisting of a cytokine, a stem cell growth factor, a lymphotoxin, a hematopoietic factor, a colony stimulating factor (CSF), an interferon (IFN), erythropoietin, and thrombopoietin.

23. The anti-IL-6 antibody or antigen-binding fragment thereof of claim 17, wherein the therapeutic agent is selected from the group consisting of human growth hormone, N-methionyl human growth hormone, bovine growth hormone, parathyroid hormone, thyroxine, insulin, proinsulin, relaxin, prorelaxin, follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), luteinizing hormone (LH), hepatic growth factor, prostaglandin, fibroblast growth factor,

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prolactin, placental lactogen, OB protein, tumor necrosis factor-α, tumor necrosis factor-β, mullerian-inhibiting substance, mouse gonadotropin-associated peptide, inhibin, activin, vascular endothelial growth factor, integrin, thrombopoietin (TPO), nerve growth factor beta (NGF-β), platelet-growth factor, transforming growth factor-α (TGF-α), TGF-β, insulin-like growth factor-I, insulin-like growth factor-II, interferon-α, interferon-β, interferon-γ, interferon-λ, macrophage-CSF, interleukin-1 (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, leukemia inhibitory factor (LIF), fms like tyrosine kinase 3 (FLT-3), angiostatin, thrombospondin, endostatin, and LT (lymphotoxin).

24. The anti-IL-6 antibody or antigen-binding fragment thereof of any one of claims 12 to 23, wherein the anti-IL-6 antibody or fragment thereof is useful for treating a disease selected from the group consisting of autoimmune disease, immune system dysfunction and inflammatory disease.

25. The anti-IL-6 antibody or antigen-binding fragment thereof of claim 24, wherein the disease is selected from the group consisting of systemic lupus erythematosus (SLE), rheumatoid arthritis, inflammatory bowel disease, type II diabetes, atherosclerosis, juvenile idiopathic arthritis, Castleman's disease, systemic sclerosis, polymyositis, vasculitis syndrome, giant cell arteritis, Takayasu arteritis, cryoglobulinemia, glomerulonephritis, rheumatoid vasculitis, non-septic hyperinflammatory disorder, nephritis, acute pancreatitis, acute respiratory distress syndrome, and graft-vs.-host disease.

26. A murine, chimeric, humanized or human anti-TNF-α antibody or antigen-binding fragment thereof comprising the heavy chain CDR sequences CDR1 (GFWN, SEQ ID NO:113), CDR2 (YISYSGRTYYNPSLKS, SEQ ID NO:114) and CDR3 (DANYVLDY, SEQ ID NO:115) and the light chain CDR sequences CDR1 (KSSQSLLNSSTQKNYLA, SEQ ID NO:116), CDR2 (FASARES, SEQ ID NO:117) and CDR3 (QQHYRTPFT, SEQ IDNO:118).

27. The anti-TNF-α antibody or antigen-binding fragment thereof of claim 26, wherein the antibody has an allotype selected from the group consisting of nGlm1, Glm3, nGlm1,2 and Km3.

28. The anti-TNF-α antibody or antigen-binding fragment thereof of claim 26, wherein the antibody or antigen-binding fragment is a naked antibody or antigen-binding fragment.

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29. The anti-TNF-α antibody or antigen-binding fragment thereof of claim 26, wherein the antibody or antigen-binding fragment is conjugated to at least one diagnostic or therapeutic agent.

30. The anti-TNF-α antibody or antigen-binding fragment thereof of claim 29, wherein the diagnostic agent is 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.

31. The anti-TNF-α antibody or antigen-binding fragment thereof of claim 29, wherein the therapeutic agent is selected from the group consisting of a drug, an anti-angiogenic agent, a pro-apoptotic agent, an antibiotic, a hormone, a hormone antagonist, an immunomodulator, a cytokine, a chemokine, a prodrug, and an enzyme.

32. The anti-TNF-α antibody or antigen-binding fragment thereof of claim 31, wherein the drug possesses a pharmaceutical property selected from the group consisting of antimitotic, antikinase, anti-tyrosine kinase, alkylating, antimetabolite, antibiotic, anti-angiogenic, pro-apoptotic, and immune modulatory.

33. The anti-TNF-α antibody or antigen-binding fragment thereof of claim 31, wherein the drug is selected from the group consisting of 5-fluorouracil, aplidin, azaribine, anastrozole, anthracyclines, bendamustine, bleomycin, bryostatin-1, busulfan, calicheamycin, camptothecin, carboplatin, carmustine, celecoxib, chlorambucil, cisplatinum, Cox-2 inhibitors, irinotecan (CPT-11), 7-Ethyl-10-hydroxycamptothecin (SN-38), carboplatin, cladribine, camptothecans, cyclophosphamide, cytarabine, dacarbazine, docetaxel, dactinomycin, daunorubicin, doxorubicin, 2-pyrrolinodoxorubicine (2P-DOX), pro-2P-DOX, cyan-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, nitrosourea, plicomycin, procarbazine, paclitaxel, pentostatin, bortezomib (PSI-341), raloxifene, semustine, streptozocin, tamoxifen,

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temazolomide, transplatinum, thalidomide, thioguanine, thiotepa, teniposide, topotecan, uracil mustard, vinorelbine, vinblastine, vincristine and ulna alkaloids.

34. The anti-TNF-α antibody or antigen-binding fragment thereof of claim 31, wherein the chemokine is selected from the group consisting of RANTES, MCAF, MIP1-alpha, MIP1-Beta and IP-10.

35. The anti-TNF-α antibody or antigen-binding fragment thereof of claim 31, wherein the anti-angiogenic agent is selected from the group consisting of angiostatin, baculostatin, canstatin, maspin, anti-VEGF antibody, anti-PlGF peptide, anti-vascular growth factor antibody, anti-Flk-1 antibody, anti-Flt-1 antibody, anti-Kras antibody, anti-cMET antibody, anti-MIF (macrophage migration-inhibitory factor) antibody, laminin peptide, fibronectin peptide, plasminogen activator inhibitor, tissue metalloproteinase inhibitor, interferon, interleukin-12, IP-10, Gro-β, 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 and minocycline.

36. The anti-TNF-α antibody or antigen-binding fragment thereof of claim 31, wherein the immunomodulator is selected from the group consisting of a cytokine, a stem cell growth factor, a lymphotoxin, a hematopoietic factor, a colony stimulating factor (CSF), an interferon (IFN), erythropoietin, and thrombopoietin.

37. The anti-TNF-α antibody or antigen-binding fragment thereof of claim 31, wherein the therapeutic agent is selected from the group consisting of human growth hormone, N-methionyl human growth hormone, bovine growth hormone, parathyroid hormone, thyroxine, insulin, proinsulin, relaxin, prorelaxin, follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), luteinizing hormone (LH), hepatic growth factor, prostaglandin, fibroblast growth factor, prolactin, placental lactogen, OB protein, tumor necrosis factor-α, tumor necrosis factor-β, mullerian-inhibiting substance, mouse gonadotropin-associated peptide, inhibin, activin, vascular endothelial growth factor, integrin, thrombopoietin (TPO), NGF-β, platelet-growth factor,

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transforming growth factor-α (TGF-α), TGF-β, insulin-like growth factor-I, insulin-like growth factor-II, interferon-α, interferon-β, interferon-γ, interferon-λ, macrophage-CSF, interleukin-1 (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, IL18, IL-21, IL-25, LIF, FLT-3, angiostatin, thrombospondin, endostatin, and LT (lymphotoxin).

38. The anti-TNF-α antibody or antigen-binding fragment thereof of any one of claims 26 to 37, wherein the anti-TNF- α antibody or fragment thereof is useful for treating a disease selected from the group consisting of autoimmune disease, immune system dysfunction and inflammatory disease.

39. The anti-TNF-α antibody or antigen-binding fragment thereof of claim 38, wherein the disease is selected from the group consisting of systemic lupus erythematosus (SLE), rheumatoid arthritis, inflammatory bowel disease, type II diabetes, atherosclerosis, juvenile idiopathic arthritis, Castleman's disease, systemic sclerosis, polymyositis, vasculitis syndrome, giant cell arteritis, Takayasu arteritis, cryoglobulinemia, glomerulonephritis, rheumatoid vasculitis, non-septic hyperinflammatory disorder, nephritis, acute pancreatitis, acute respiratory distress syndrome, and graft-vs.-host disease.

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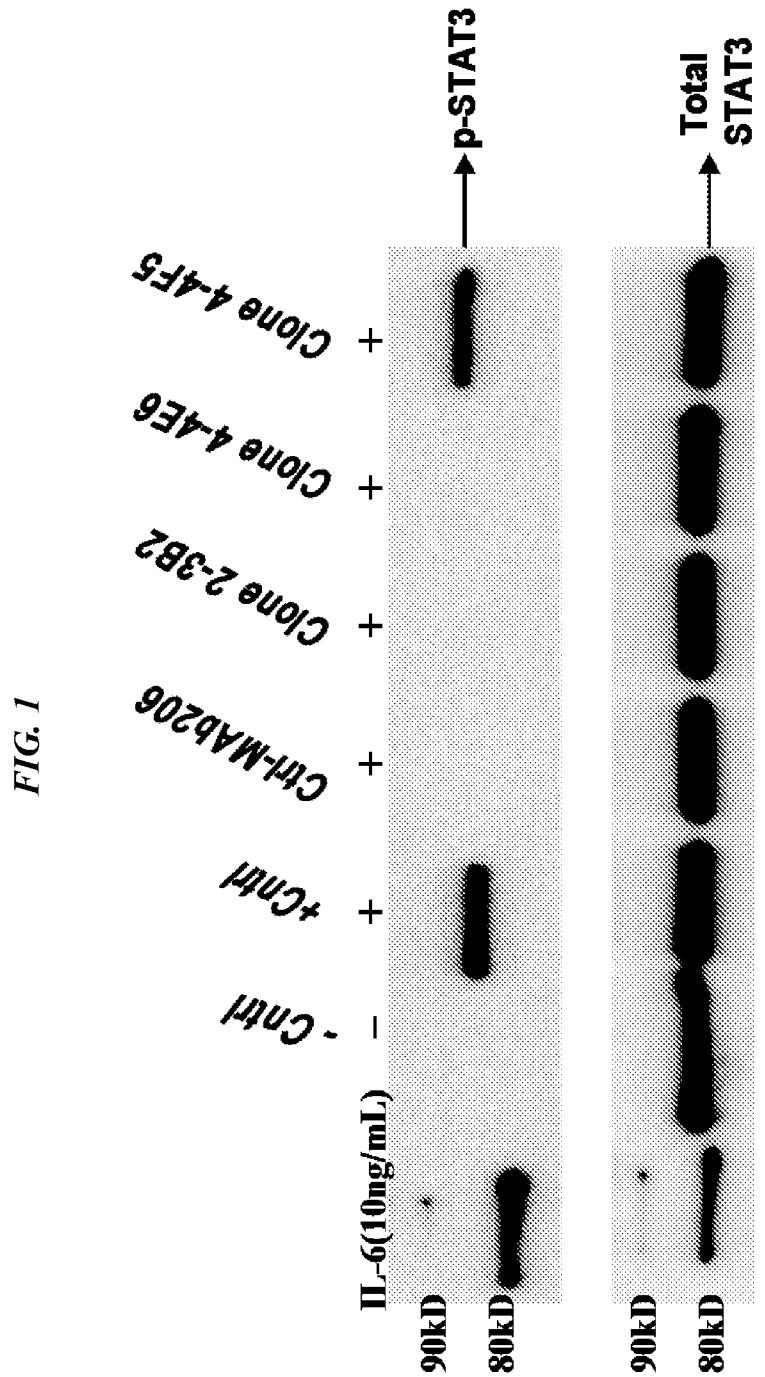
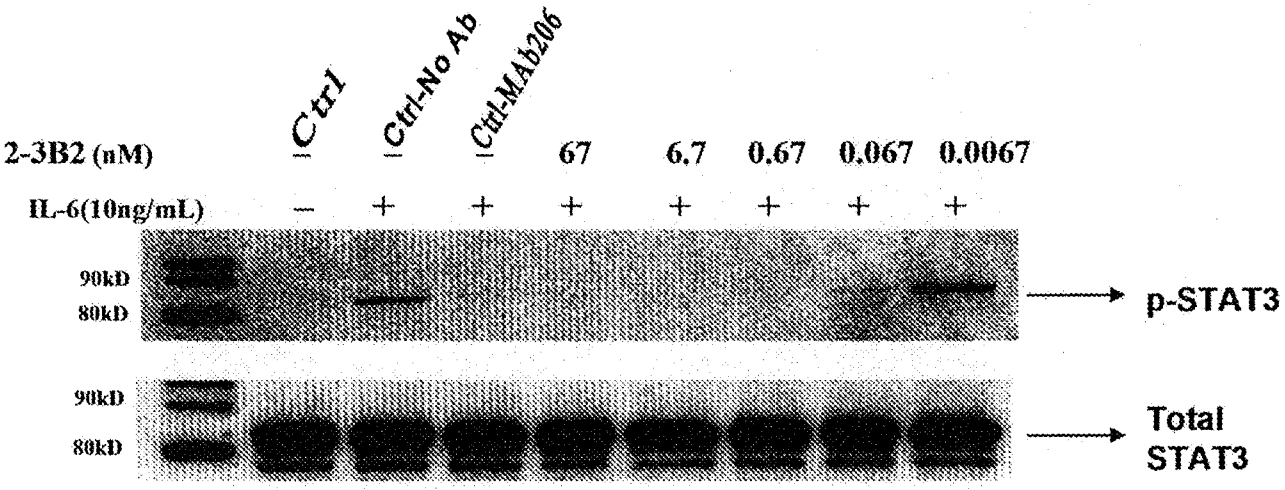

p-STAT3
Total STAT3
Clone 4-4F5
Clone 4-4E6
Clone 2-3B2
Cltr-MAb206
+Cntfr
-Cntfr
IL-6(10ng/mL)
90kD
80kD
90kD
80kD

FIG. 1

FIG. 2A



Neutralization Assay with clone 2-3B2

FIG. 2B

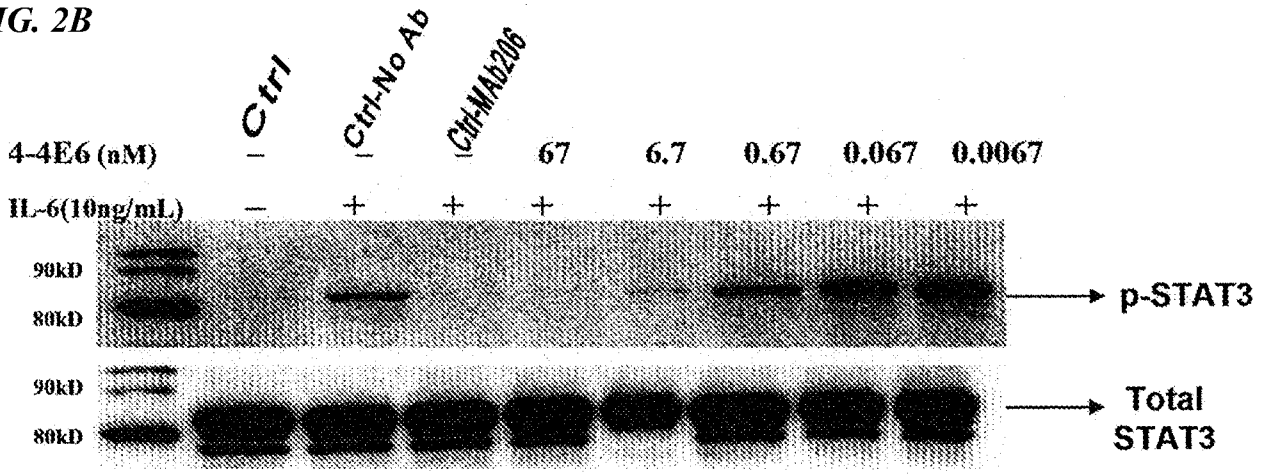


FIG. 3

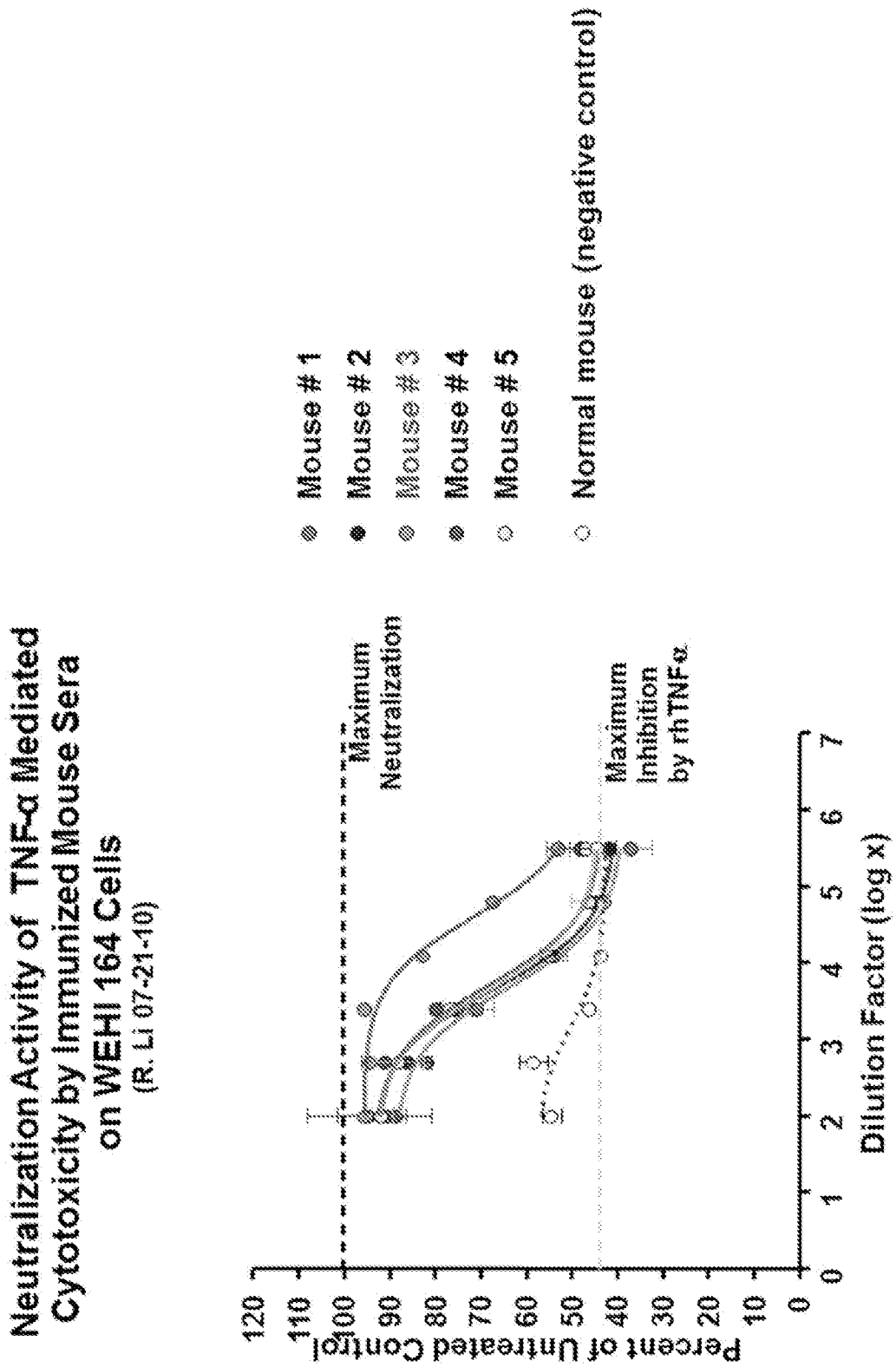

Neutralization Activity of TNF-α Mediated Cytotoxicity by Immunized Mouse Sera on WEHI 164 Cells
(R. Li 07-21-10)
Mouse # 1
Mouse # 2
Mouse # 3
Mouse # 4
Mouse # 5
Normal mouse (negative control)
Maximum Neutralization
Maximum Inhibition by rhTNF-α
Percent of Untreated Control
Dilution Factor (log x)
120
110
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7

FIG. 4

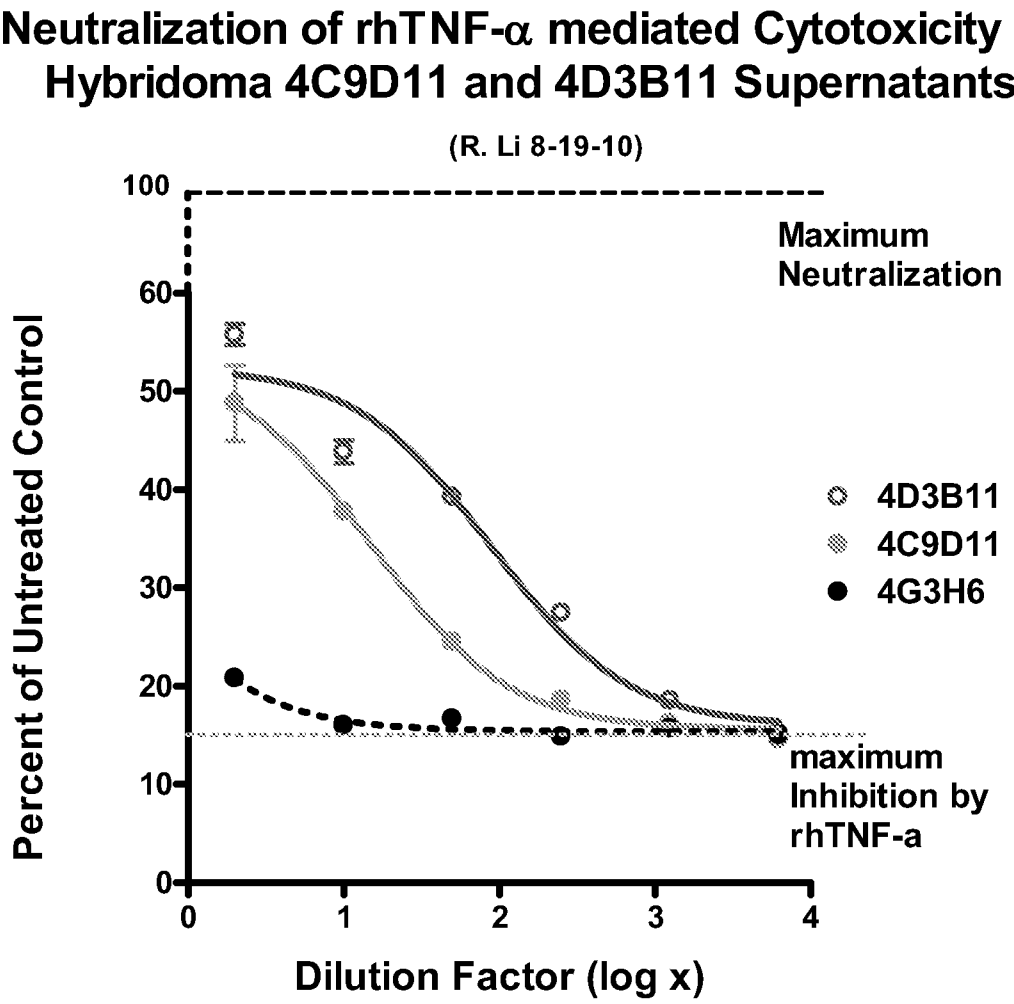
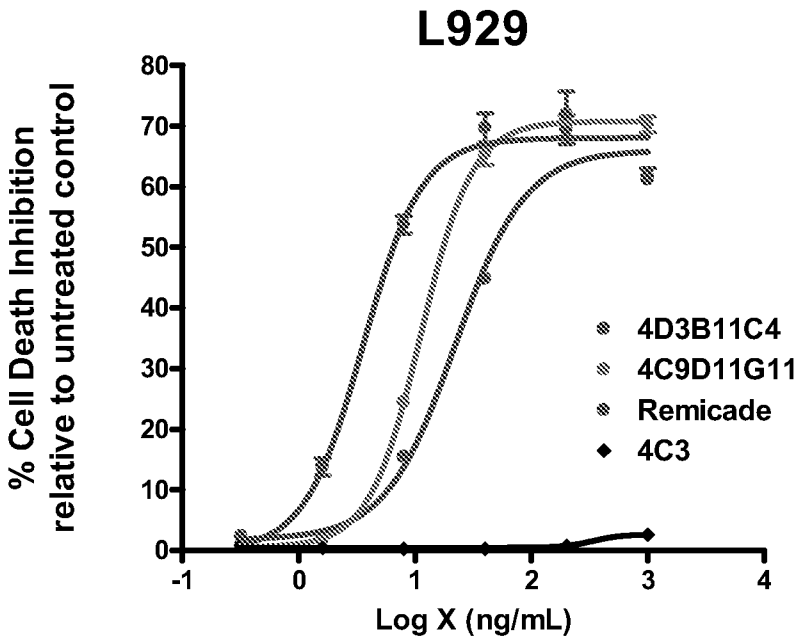

Neutralization of rhTNF-α mediated Cytotoxicity by Hybridoma 4C9D11 and 4D3B11 Supernatants
(R. Li 8-19-10)
Percent of Untreated Control
100
60
50
40
30
20
10
0
0
1
2
3
4
Dilution Factor (log x)
Maximum Neutralization
4D3B11
4C9D11
4G3H6
maximum Inhibition by rhTNF-a

FIG. 5



Antibody	EC_{50}–value (ng/mL)	95% C.I. (ng/mL)	R^2
4D3B11C4	22.1	15.1 to 32.5	0.98
4C9D11G11	11.2	10.2 to 12.3	0.99
Remicade	3.6	2.4 to 5.4	0.98

FIG. 6

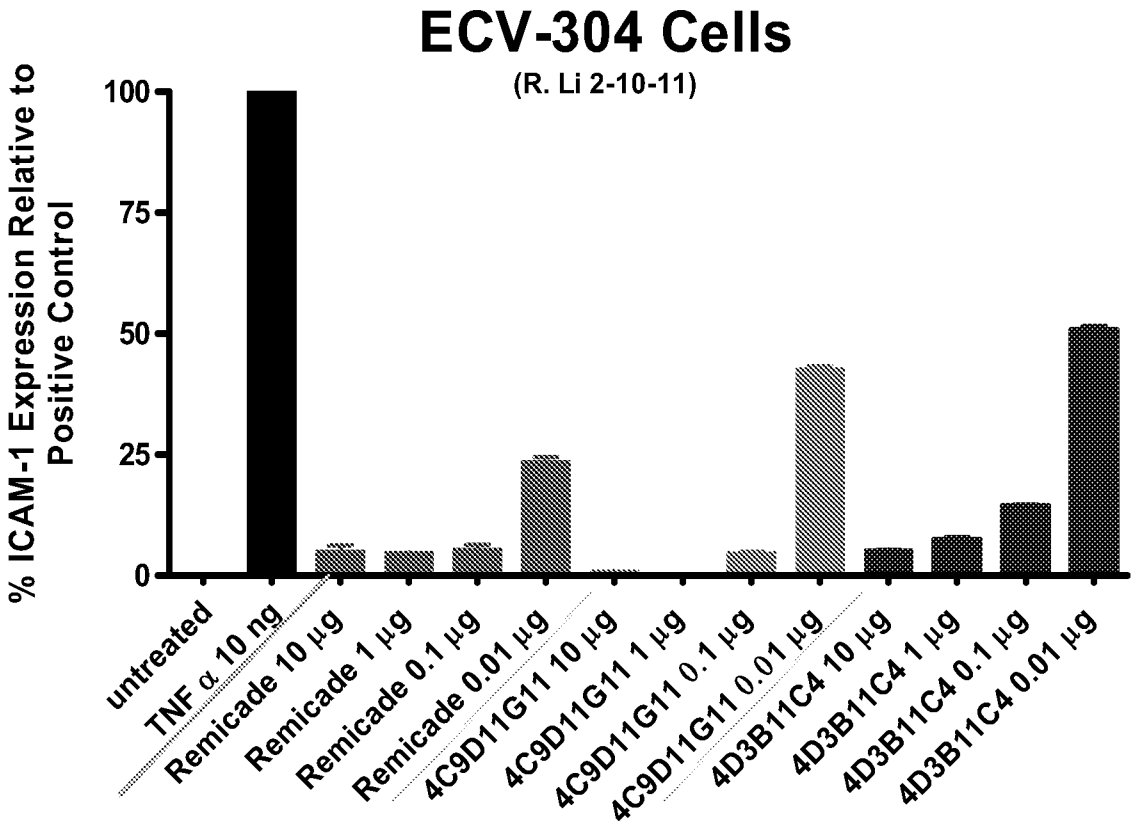

ECV-304 Cells
(R. Li 2-10-11)
% ICAM-1 Expression Relative to Positive Control
100
75
50
25
0
untreated
TNF α 10 ng
Remicade 10 μg
Remicade 1 μg
Remicade 0.1 μg
Remicade 0.01 μg
4C9D11G11 10 μg
4C9D11G11 1 μg
4C9D11G11 0.1 μg
4C9D11G11 0.01 μg
4D3B11C4 10 μg
4D3B11C4 1 μg
4D3B11C4 0.1 μg
4D3B11C4 0.01 μg

FIG. 7

CDR1 CDR2

2-3B2-VH VKLQESGGGLVQPGGSRKLSCAASGFTFSRFGMHWVRQAPEKGLEWVAYIGRGSSTIYYA
NCBI sequence VQLVESGGGLVQPGGSRQLSCAASGFTFSSFGMHWVRQAPEKGLEWVAYISRGGNTIYYA

CDR3

2-3B2-VH DTVKGRFTISRDNPKNTLFLQMTSLRSEDTAMYYCARSNWDG----AMDYWGQGTSVTVSS
NCBI sequence NTVKGRFTISRDNPKNTLFLQMTSLRSDDTAMYYCARSHYYGYFYAMDYWGQGTTLTVSS

FIG. 8

CDR1 CDR2

B2-VK DIQ LTQSPASLSASVGETVTITCRASGNIHNFLAWYQQKQGKSPQLLVYNAETLADGVPS
BI sequence DIQMTQSPASLSASVGETVTITCRASGNIHNYLAWYQQKQGKSPQLLVYNAKTLADGVPS

CDR3

B2-VK RFSGSGSGTQYSLKINSLQPEDFGSYYCQHFWSTPWTFGGGTKLEIKRADAAPTVSIFPPSSE
BI sequence RFSGSGSGTQYSLKINSLQPEDFGTYYCHHFWSTPWTFGGGTKLEVKRADAAPTVSILPPSSE

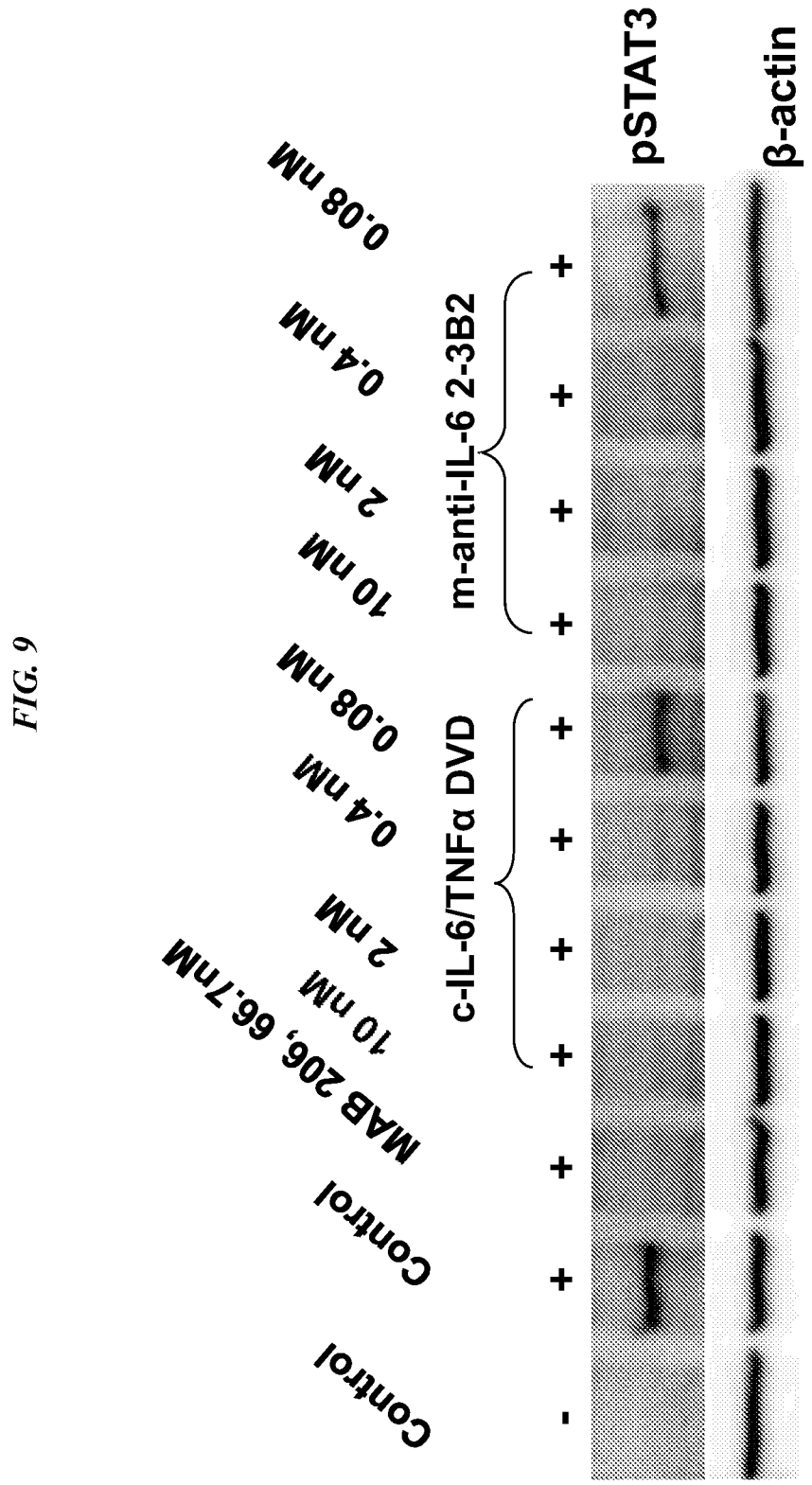

pSTAT3
β-actin
Control
Control
MAB 206, 66.7nM
10 nM
2 nM
0.4 nM
0.08 nM
10 nM
2 nM
0.4 nM
0.08 nM
c-IL-6/TNFα DVD
m-anti-IL-6 2-3B2
-
+
+
+
+
+
+
+
+
+

FIG. 9

FIG. 10

CDR1 CDR2

4C9-VH VQLQESGPSLVKPSQTLSLTCSVTGDSITSG-FWNWIRKFPGNKFEYMGYISYSGRTYYN

NCBI LQESGPGLVKPSQSLSLTCSVSGYSITSGYFWNWIRQFSGNKLEWMGYISYDGSNNYN

CDR3

4C9-VH PSLKSRLSITRDTSKNQFYLQLNSVTAEDTATYYCARDANYVLDYWGQGTTLTVSS

NCBI PSLKNRI SITRDTSKNQFFLKLNSVTPEDTATYYCARDGDYYFDYWGQGTTVTVSS

FIG. 11

CDR1 CDR2

4C9-VK DIQLTQSPSSLAMSVGQKVTMNCKSSQSLLNSSTQKNYLAWFQQKPGQSPKLLVYFASARES
NCBI LTQSPSSLAMSVGQKVTMNCKSSQSLLNSYTQKNYLAWYQQKPGQSPKLLVYFASTRES

CDR3

4C9-VK GVPDRFI GSGSGTDFTLTISSVQAEDLADYFCQQHYRTPFTFGSGTKLEIKR
NCBI GVPDRFMGSGSGTDFTLTISSVQTEDLADYFCQQHYRI PFTFGSGTKLEI

FIG. 12

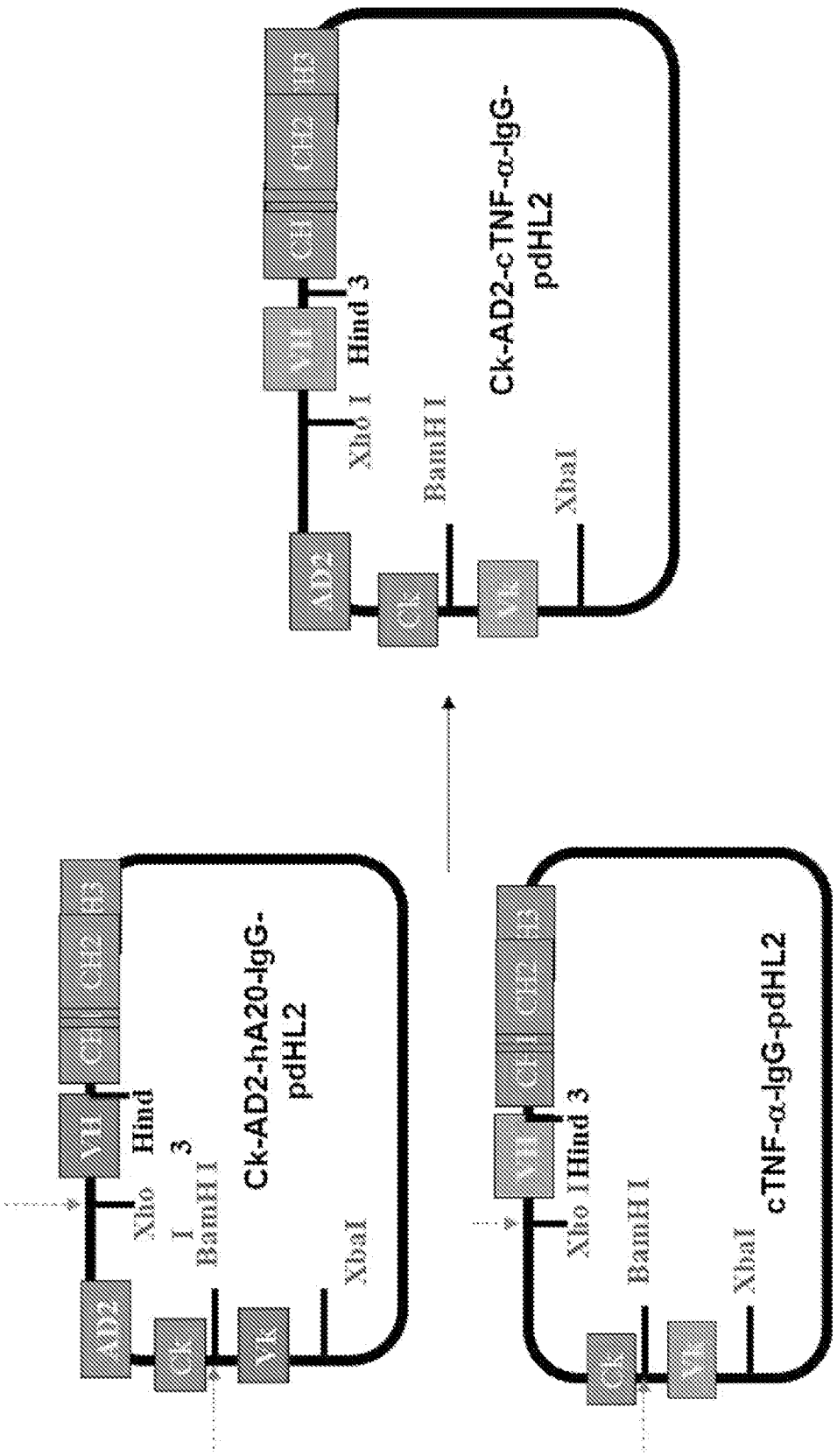

Ck-AD2-hA20-IgG-pdHL2
Xho I
Hind 3
BamH I
XbaI
Ck-AD2-cTNF-α-IgG-pdHL2
cTNF-α-IgG-pdHL2

FIG. 13

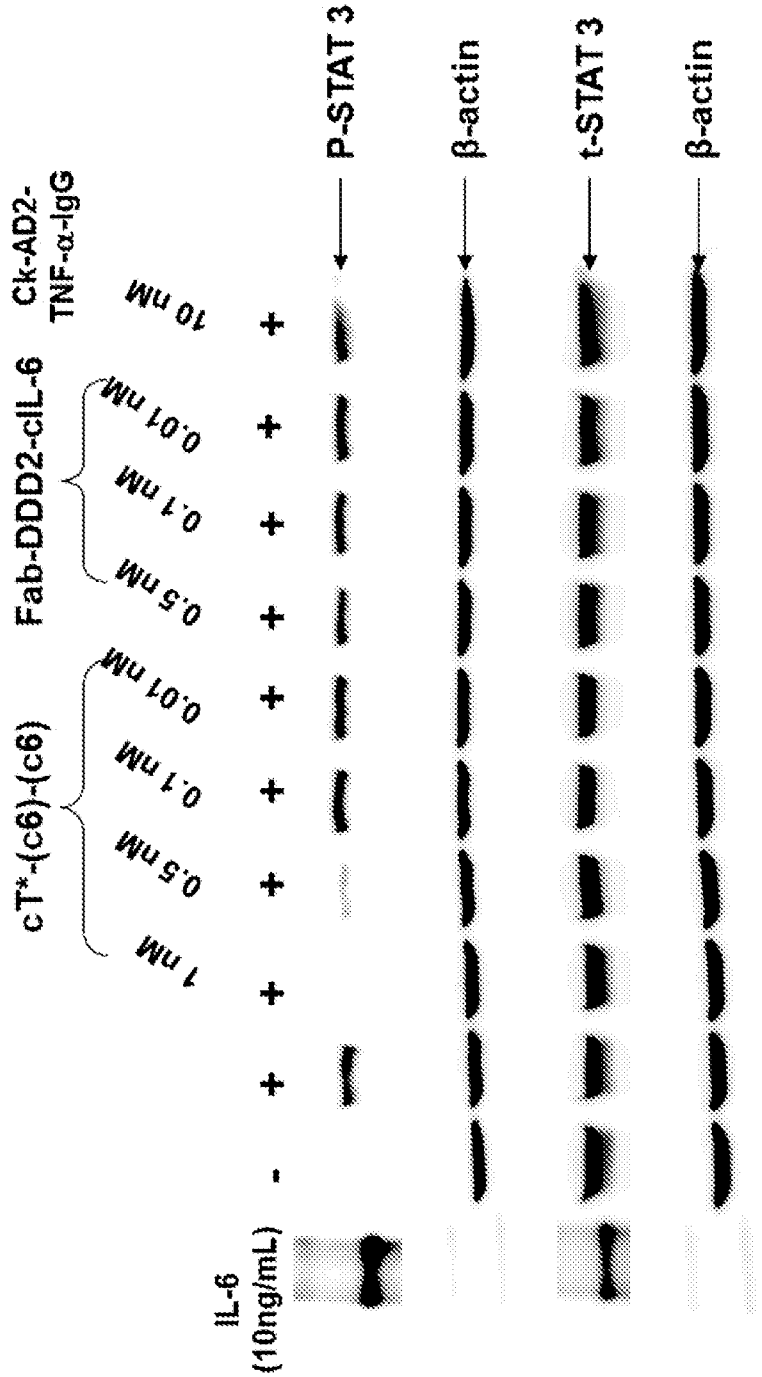

IL-6 (10ng/mL)
-
+
+
+
+
+
+
+
+
+
cT*-(c6)-(c6)
7 nM
0.5 nM
0.1 nM
0.01 nM
Fab-DDD2-cIL-6
0.5 nM
0.1 nM
0.01 nM
Ck-AD2-TNF-α-IgG
10 nM
P-STAT 3
β-actin
t-STAT 3
β-actin

FIG. 14

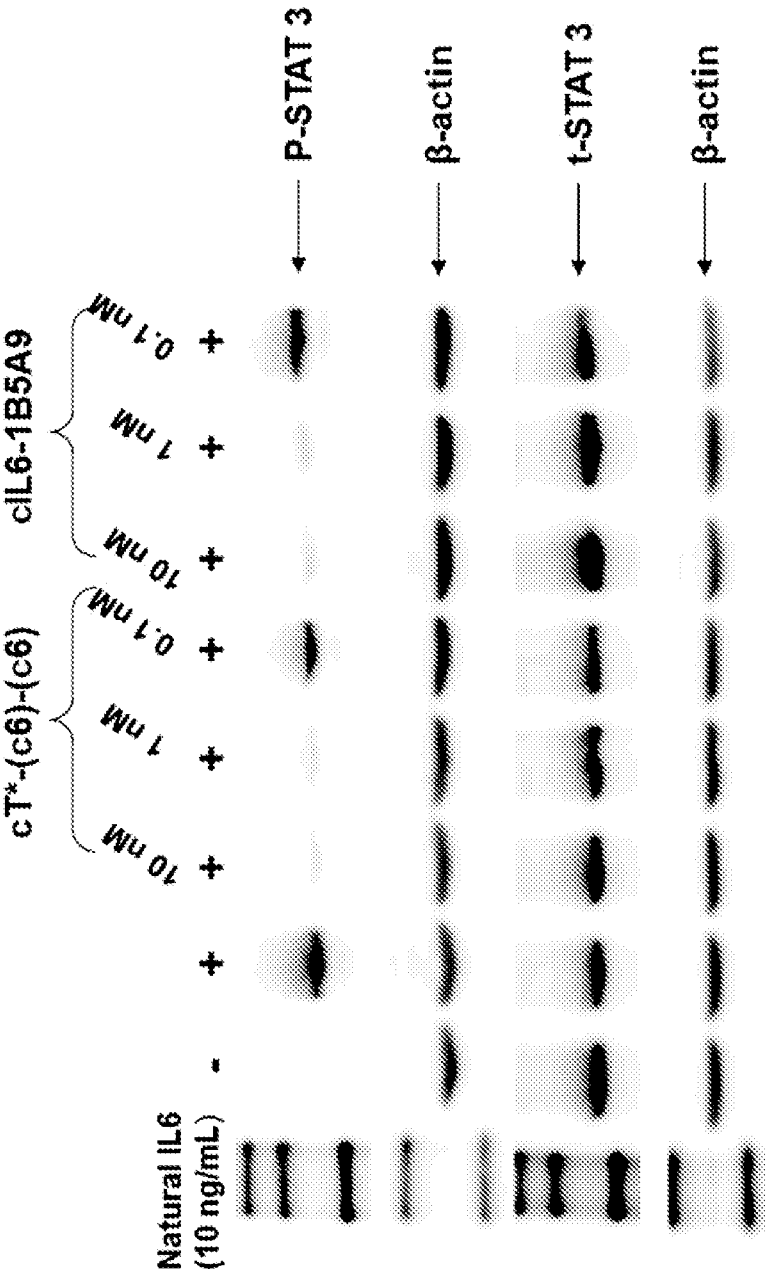

P-STAT 3
β-actin
t-STAT 3
β-actin
cIL6-1B5A9
0.1 nM
1 nM
10 nM
cT*-(c6)-(c6)
0.1 nM
1 nM
10 nM
Natural IL6 (10 ng/mL)
+
+
+
+
+
+
+
-

FIG. 15

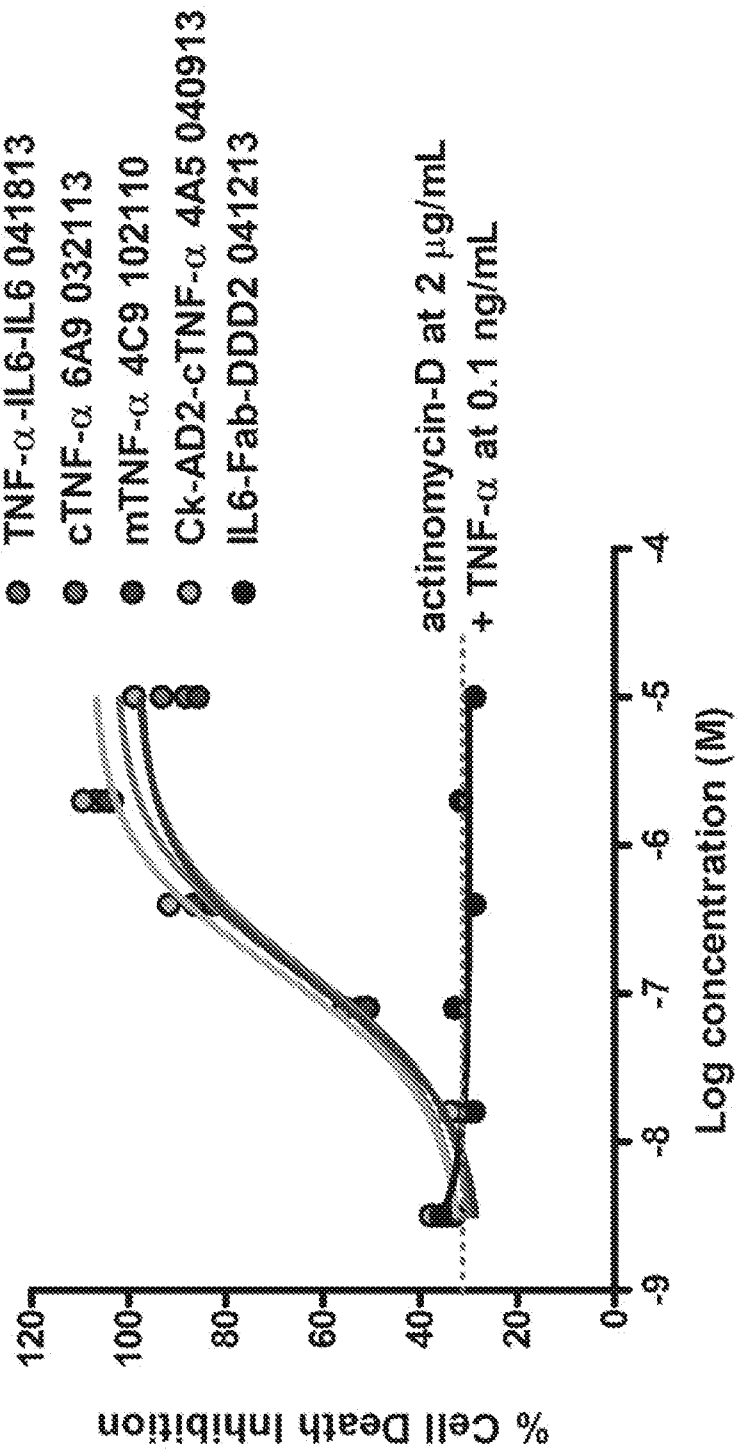
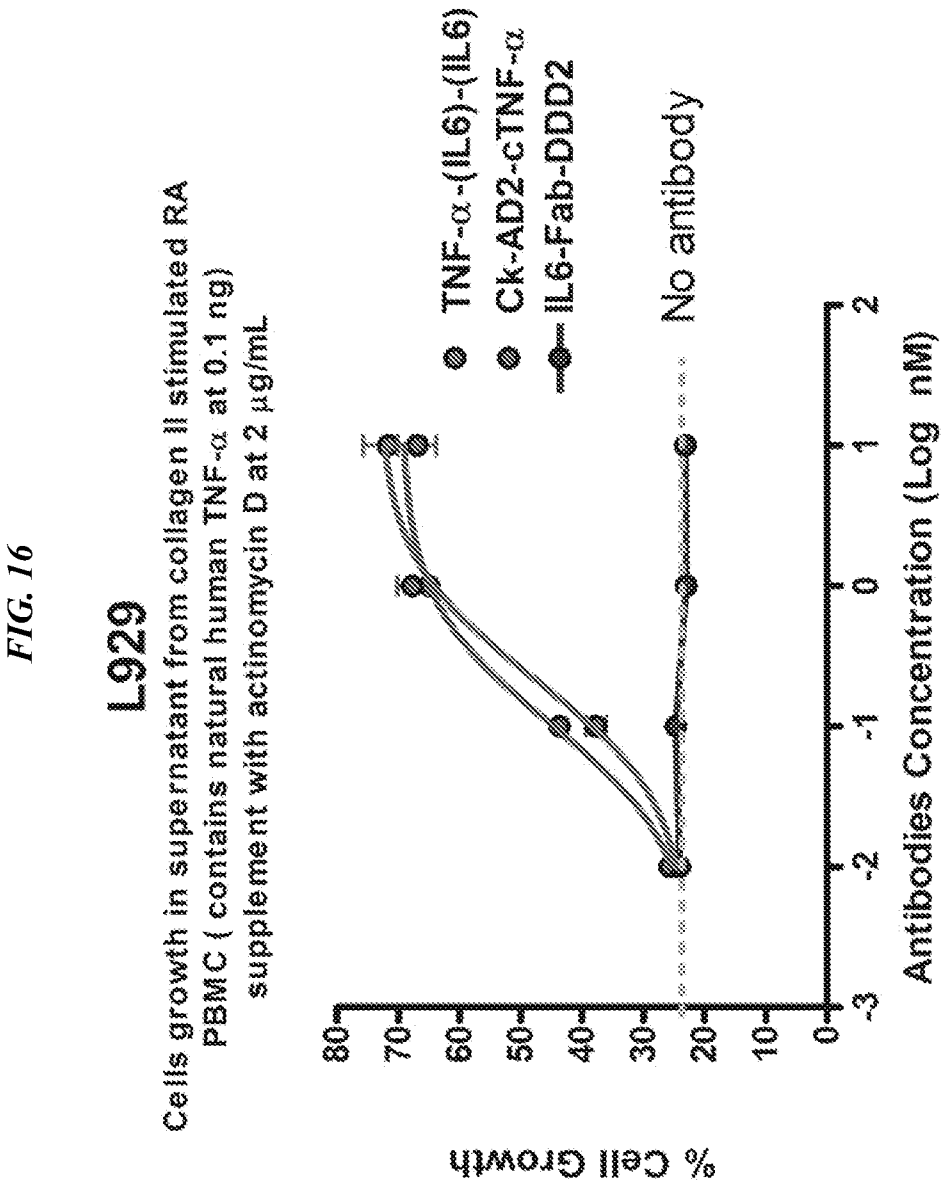

L929 Cells
TNF-α-IL6-IL6 041813
cTNF-α 6A9 032113
mTNF-α 4C9 102110
Ck-AD2-cTNF-α 4A5 040913
IL6-Fab-DDD2 041213
actinomycin-D at 2 μg/mL
+ TNF-α at 0.1 ng/mL
% Cell Death Inhibition
120
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
Log concentration (M)

FIG. 16


L929
Cells growth in supernatant from collagen II stimulated RA PBMC (contains natural human TNF-α at 0.1 ng) supplement with actinomycin D at 2 μg/mL
TNF-α-(IL6)-(IL6)
Ck-AD2-cTNF-α
IL6-Fab-DDD2
No antibody
% Cell Growth
80
70
60
50
40
30
20
10
0
-3
-2
-1
0
1
2
Antibodies Concentration (Log nM)

		c-anti-IL-6	c-anti-TNF-α	cT*-(c6)-(c6)
		cIL-6	cTNF	cT*-(c6)-(c6)
		1B5A9	6A9	DNL
rIL-6	Human	2.71; 2.57		2.67; 1.61
rIL-6	Cynomolgus	6.19		5.34
rIL-6	Canine	2.3		0.46
rTNF-α	Human		0.287; 0.283	0.699; 0.493
rTNF-α	Cynomolgus		0.308	0.732
rTNF-α	Canine		0.254	0.52

FIG. 17

FIG. 18A

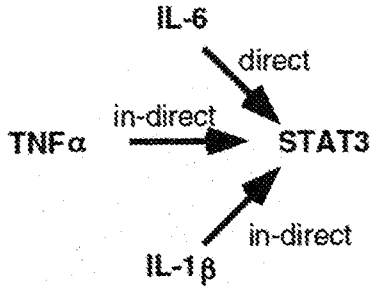
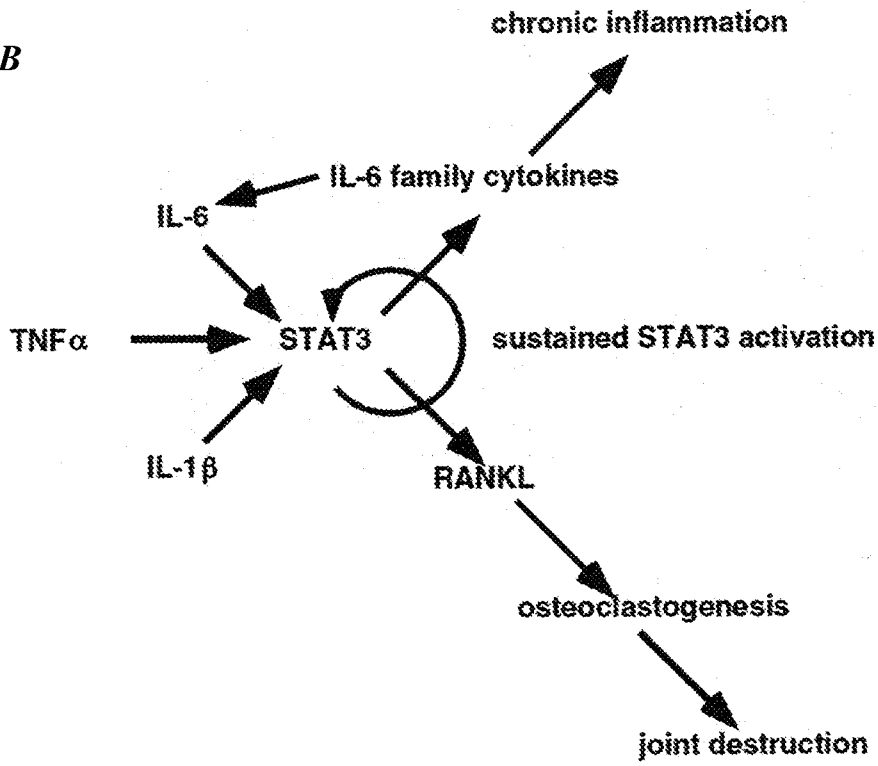
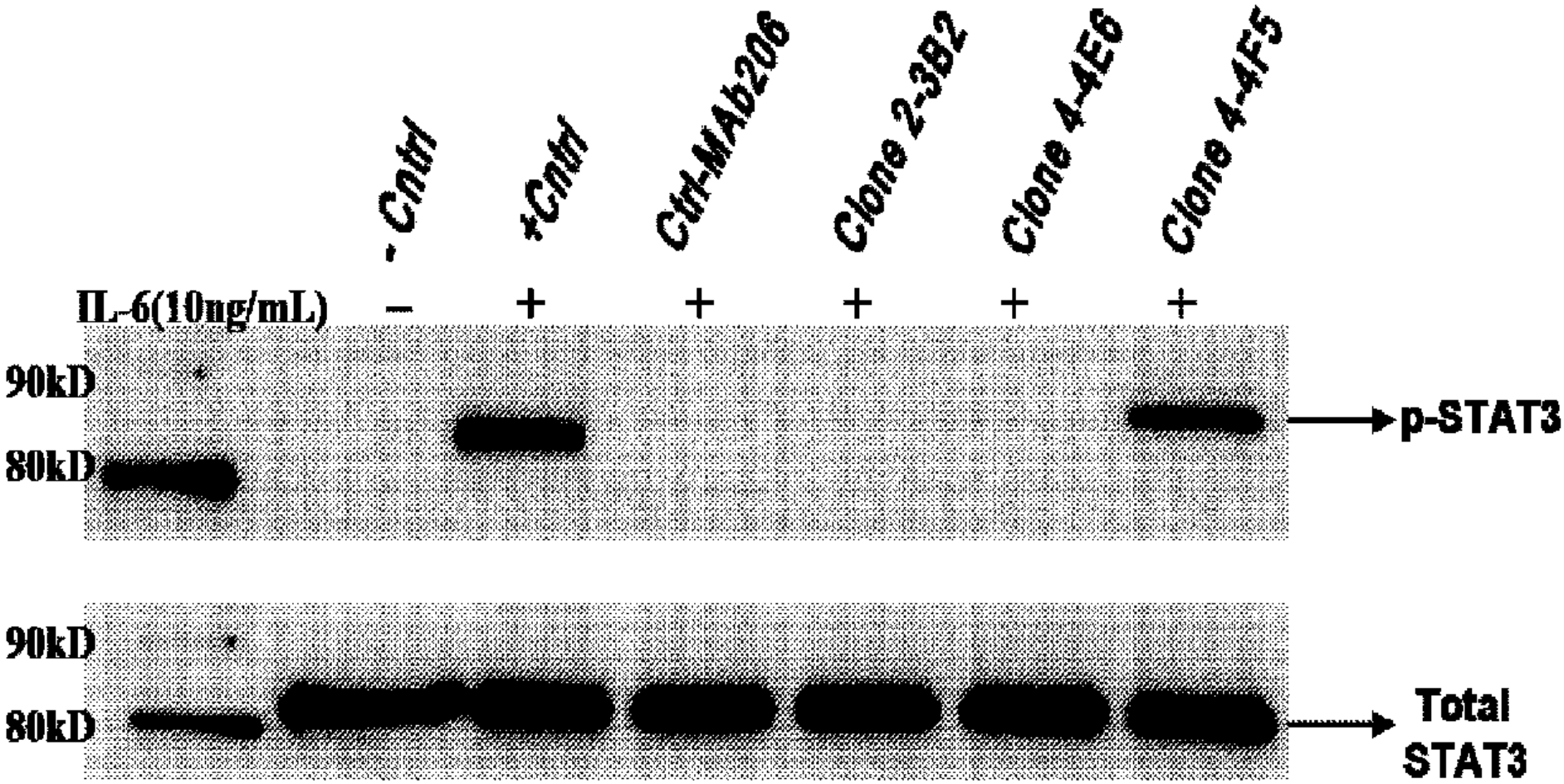


FIG. 18B




- Cntrl
+Cntrl
Ctrl-MAb206
Clone 2-3B2
Clone 4-4E6
Clone 4-4F5
IL-6(10ng/mL)
–
+
+
+
+
+
90kD
80kD
p-STAT3
90kD
80kD
Total
STAT3