# METHODS OF USING DEATH RECEPTOR LIGANDS AND CD20 ANTIBODIES

## RELATED APPLICATIONS

This application claims priority under Section 119(e) to provisional application number 60/607,909 filed September 8, 2004 and to provisional application number 60/666,553 filed March 30, 2005.

## FIELD OF THE INVENTION

The present invention relates to methods of using death receptor ligands and CD20 antibodies. More particularly, the invention relates to methods of using Apo-2 ligand/TRAIL or death receptor antibodies in combination with CD20 antibodies to treat various pathological disorders, such as cancer and immune related diseases.

## BACKGROUND OF THE INVENTION

Various ligands and receptors belonging to the tumor necrosis factor (TNF) superfamily have been identified in the art. Included among such ligands are tumor necrosis factor-alpha ("TNF-alpha"), tumor necrosis factor-beta ("TNF-beta" or "lymphotoxin-alpha"), lymphotoxin-beta ("LT-beta"), CD30 ligand, CD27 ligand, CD40 ligand, OX-40 ligand, 4-1BB ligand, LIGHT, Apo-1 ligand (also referred to as Fas ligand or CD95 ligand), Apo-2 ligand (also referred to as Apo2L or TRAIL), Apo-3 ligand (also referred to as TWEAK), APRIL, OPG ligand (also referred to as RANK ligand, ODF, or TRANCE), and TALL-1 (also referred to as BlyS, BAFF or THANK) (See, e.g., Ashkenazi, Nature Review, 2:420-430 (2002); Ashkenazi and Dixit, Science, 281:1305-1308 (1998); Ashkenazi and Dixit, Curr. Opin. Cell Biol., 11:255-260 (2000); Golstein, Curr. Biol., 7:750-753 (1997) Wallach, Cytokine Reference, Academic Press, 2000, pages 377-411; Locksley et al., Cell, 104:487-501 (2001); Gruss and Dower, Blood, 85:3378-3404 (1995); Schmid et al., Proc. Natl. Acad. Sci., 83:1881 (1986); Dealtry et al., Eur. J. Immunol., 17:689 (1987); Pitti et al., J. Biol. Chem., 271:12687-12690 (1996); Wiley et al., Immunity, 3:673-682 (1995); Browning et al., Cell, 72:847-856 (1993); Armitage et al. Nature, 357:80-82 (1992); WO 97/01633 published January 16, 1997; WO 97/25428 published July 17, 1997; Marsters et al., Curr. Biol., 8:525-528 (1998); Chicheportiche et al., Biol. Chem., 272:32401-32410 (1997); Hahne et al., J. Exp. Med., 188:1185-1190 (1998); WO98/28426 published July 2, 1998; WO98/46751 published October 22, 1998; WO/98/18921 published May 7, 1998; Moore et al., Science, 285:260-263 (1999); Shu et al., J. Leukocyte Biol., 65:680 (1999); Schneider et al., J. Exp. Med., 189:1747-1756 (1999); Mukhopadhyay et al., J. Biol. Chem., 274:15978-15981 (1999)).

Induction of various cellular responses mediated by such TNF family ligands is typically initiated by their binding to specific cell receptors.

Some, but not all, TNF family ligands bind to, and induce various biological activity through, cell surface "death receptors" to activate caspases, or enzymes that carry out the cell death or apoptosis pathway (Salvesen et al., Cell, 91:443-446 (1997). Included among the members of the TNF receptor superfamily identified to date are TNFR1, TNFR2, TACI, GITR,, CD27, OX-40, CD30, CD40, HVEM, Fas (also referred to as Apo-1 or CD95), DR4 (also referred to as TRAIL-R1), DR5 (also referred to as Apo-2 or TRAIL-R2), DcR1, DcR2, osteoprotegerin (OPG), RANK and Apo-3 (also referred to as DR3 or TRAMP) (see, e.g., Ashkenazi, Nature Reviews, 2:420-430 (2002); Ashkenazi and Dixit, Science, 281:1305-1308 (1998); Ashkenazi and Dixit, Curr. Opin. Cell Biol., 11:255-260 (2000); Golstein, Curr. Biol., 7:750-753 (1997); Wallach, Cytokine Reference, Academic Press, 2000, pages 377-411; Locksley et al., Cell, 104:487-501 (2001); Gruss and Dower, Blood, 85:3378-3404 (1995); Hohman et al., J. Biol. Chem., 264:14927-14934 (1989); Brockhaus et al., Proc. Natl. Acad. Sci., 87:3127-3131 (1990); EP 417,563, published March 20, 1991; Loetscher et al., Cell, 61:351 (1990); Schall et al., Cell, 61:361 (1990); Smith et al., Science, 248:1019-1023 (1990); Lewis et al., Proc. Natl. Acad. Sci., 88:2830-2834 (1991); Goodwin et al., Mol. Cell. Biol., 11:3020-3026 (1991); Stamenkovic et al., EMBO J., 8:1403-1410 (1989); Mallett et al., EMBO J., 9:1063-1068 (1990); Anderson et al., Nature, 390:175-179 (1997); Chicheportiche et al., J. Biol. Chem., 272:32401-32410 (1997); Pan et al., Science, 276:111-113 (1997); Pan et al., Science, 277:815-818 (1997); Sheridan et al., Science, 277:818-821 (1997); Degli-Esposti et al., J. Exp. Med., 186:1165-1170 (1997); Marsters et al., Curr. Biol., 7:1003-1006 (1997); Tsuda et al., BBRC, 234:137-142 (1997); Nocentini et al., Proc. Natl. Acad. Sci., 94:6216-6221 (1997); vonBulow et al., Science, 278:138-141 (1997)).

Most of these TNF receptor family members share the typical structure of cell surface receptors including extracellular, transmembrane and intracellular regions, while others are found naturally as soluble proteins lacking a transmembrane and intracellular domain. The extracellular portion of typical TNFRs contains a repetitive amino acid sequence pattern of multiple cysteine-rich domains (CRDs), starting from the $NH_2$-terminus.

The ligand referred to as Apo-2L or TRAIL was identified several years ago as a member of the TNF family of cytokines. (see, e.g., Wiley et al., Immunity, 3:673-682 (1995); Pitti et al., J. Biol. Chem., 271:12697-12690 (1996); WO 97/01633; WO 97/25428; US Patent 5,763,223 issued June 9, 1998; US Patent 6,284,236 issued September 4, 2001). The full-length native sequence human Apo2L/TRAIL polypeptide is a 281 amino acid long, Type II transmembrane protein. Some cells can produce a natural soluble form of the polypeptide, through enzymatic cleavage of the polypeptide's extracellular region (Mariani et al., J. Cell. Biol., 137:221-229 (1997)). Crystallographic studies of soluble forms of Apo2L/TRAIL reveal a homotrimeric structure similar to the structures of TNF and other related proteins (Hymowitz et al., Molec. Cell, 4:563-571 (1999); Cha et al.,

Immunity, 11:253-261 (1999); Mongkolsapaya et al., Nature Structural Biology, 6:1048 (1999); Hymowitz et al., Biochemistry, 39:633-644 (2000)). Apo2L/TRAIL, unlike other TNF family members however, was found to have a unique structural feature in that three cysteine residues (at position 230 of each subunit in the homotrimer) together coordinate a zinc atom, and that the zinc binding is important for trimer stability and biological activity. (Hymowitz et al., supra; Bodmer et al., J. Biol. Chem., 275:20632-20637 (2000)).

It has been reported in the literature that Apo2L/TRAIL may play a role in immune system modulation, including autoimmune diseases such as rheumatoid arthritis [see, e.g., Thomas et al., J. Immunol., 161:2195-2200 (1998); Johnsen et al., Cytokine, 11:664-672 (1999); Griffith et al., J. Exp. Med., 189:1343-1353 (1999); Song et al., J. Exp. Med., 191:1095-1103 (2000)].

Soluble forms of Apo2L/TRAIL have also been reported to induce apoptosis in a variety of cancer cells, including colon, lung, breast, prostate, bladder, kidney, ovarian and brain tumors, as well as melanoma, leukemia, and multiple myeloma (see, e.g., Wiley et al., supra; Pitti et al., supra; US Patent 6,030,945 issued February 29, 2000; US Patent 6,746,668 issued June 8, 2004; Rieger et al., FEBS Letters, 427:124-128 (1998); Ashkenazi et al., J. Clin. Invest., 104:155-162 (1999); Walczak et al., Nature Med., 5:157-163 (1999); Keane et al., Cancer Research, 59:734-741 (1999); Mizutani et al., Clin. Cancer Res., 5:2605-2612 (1999); Gazitt, Leukemia, 13:1817-1824 (1999); Yu et al., Cancer Res., 60:2384-2389 (2000); Chinnaiyan et al., Proc. Natl. Acad. Sci., 97:1754-1759 (2000)). *In vivo* studies in murine tumor models further suggest that Apo2L/TRAIL, alone or in combination with chemotherapy or radiation therapy, can exert substantial anti-tumor effects (see, e.g., Ashkenazi et al., supra; Walzcak et al., supra; Gliniak et al., Cancer Res., 59:6153-6158 (1999); Chinnaiyan et al., supra; Roth et al., Biochem. Biophys. Res. Comm., 265:1999 (1999); PCT Application US/00/15512; PCT Application US/01/23691). In contrast to many types of cancer cells, most normal human cell types appear to be resistant to apoptosis induction by certain recombinant forms of Apo2L/TRAIL (Ashkenazi et al., supra; Walzcak et al., supra). Jo et al. has reported that a polyhistidine-tagged soluble form of Apo2L/TRAIL induced apoptosis *in vitro* in normal isolated human, but not non-human, hepatocytes (Jo et al., Nature Med., 6:564-567 (2000); see also, Nagata, Nature Med., 6:502-503 (2000)). It is believed that certain recombinant Apo2L/TRAIL preparations may vary in terms of biochemical properties and biological activities on diseased versus normal cells, depending, for example, on the presence or absence of a tag molecule, zinc content, and % trimer content (See, Lawrence et al., Nature Med., Letter to the Editor, 7:383-385 (2001); Qin et al., Nature Med., Letter to the Editor, 7:385-386 (2001)).

Apo2L/TRAIL has been found to bind at least five different receptors. At least two of the receptors which bind Apo2L/TRAIL contain a functional, cytoplasmic death domain. One such receptor has been referred to as "DR4" (and alternatively as TR4 or TRAIL-R1) (Pan et al., Science, 276:111-113 (1997); see also WO98/32856 published July 30, 1998; WO99/37684 published July 29, 1999; WO 00/73349 published December 7, 2000; US 6,433,147 issued August 13, 2002; US 6,461,823 issued October 8, 2002, and US 6,342,383 issued January 29, 2002).

Another such receptor for Apo2L/TRAIL has been referred to as DR5 (it has also been alternatively referred to as Apo-2; TRAIL-R or TRAIL-R2, TR6, Tango-63, hAPO8, TRICK2 or KILLER) (see, e.g., Sheridan et al., Science, 277:818-821 (1997); Pan et al., Science, 277:815-818 (1997); WO98/51793 published November 19, 1998; WO98/41629 published September 24, 1998; Screaton et al., Curr. Biol., 7:693-696 (1997); Walczak et al., EMBO J., 16:5386-5387 (1997); Wu et al., Nature Genetics, 17:141-143 (1997); WO98/35986 published August 20, 1998; EP870,827 published October 14, 1998; WO98/46643 published October 22, 1998; WO99/02653 published January 21, 1999; WO99/09165 published February 25, 1999; WO99/11791 published March 11, 1999; US 2002/0072091 published August 13, 2002; US 2002/0098550 published December 7, 2001; US 6,313,269 issued December 6, 2001; US 2001/0010924 published August 2, 2001; US 2003/01255540 published July 3, 2003; US 2002/0160446 published October 31, 2002; US 2002/0048785 published April 25, 2002; US 6,342,369 issued February, 2002; US 6,569,642 issued May 27, 2003; US 6,072,047 issued June 6, 2000; US 6,642,358 issued November 4, 2003; US 6,743,625 issued June 1, 2004). Like DR4, DR5 is reported to contain a cytoplasmic death domain and be capable of signaling apoptosis upon ligand binding (or upon binding a molecule, such as an agonist antibody, which mimics the activity of the ligand). The crystal structure of the complex formed between Apo-2L/TRAIL and DR5 is described in Hymowitz et al., Molecular Cell, 4:563-571 (1999).

Upon ligand binding, both DR4 and DR5 can trigger apoptosis independently by recruiting and activating the apoptosis initiator, caspase-8, through the death-domain-containing adaptor molecule referred to as FADD/Mort1 [Kischkel et al., Immunity, 12:611-620 (2000); Sprick et al., Immunity, 12:599-609 (2000); Bodmer et al., Nature Cell Biol., 2:241-243 (2000)].

Apo2L/TRAIL has been reported to also bind those receptors referred to as DcR1, DcR2 and OPG, which believed to function as inhibitors, rather than transducers of signaling (see., e.g., DCR1 (also referred to as TRID, LIT or TRAIL-R3) [Pan et al., Science, 276:111-113 (1997); Sheridan et al., Science, 277:818-821 (1997); McFarlane et al., J. Biol. Chem., 272:25417-25420 (1997); Schneider et al., FEBS Letters, 416:329-334 (1997); Degli-Esposti et al., J. Exp. Med., 186:1165-1170 (1997); and Mongkolsapaya et al., J. Immunol., 160:3-6 (1998); DCR2 (also called TRUNDD or TRAIL-R4) [Marsters et al., Curr. Biol., 7:1003-1006 (1997); Pan et al., FEBS

Letters, 424:41-45 (1998); Degli-Esposti et al., Immunity, 7:813-820 (1997)], and OPG [Simonet et al., supra]. In contrast to DR4 and DR5, the DcR1 and DcR2 receptors do not signal apoptosis.

Certain antibodies which bind to the DR4 and/or DR5 receptors have been reported in the literature. For example, anti-DR4 antibodies directed to the DR4 receptor and having agonistic or apoptotic activity in certain mammalian cells are described in, e.g., WO 99/37684 published July 29, 1999; WO 00/73349 published July 12, 2000; WO 03/066661 published August 14, 2003. See, also, e.g., Griffith et al., J. Immunol., 162:2597-2605 (1999); Chuntharapai et al., J. Immunol., 166:4891-4898 (2001); WO 02/097033 published December 2, 2002; WO 03/042367 published May 22, 2003; WO 03/038043 published May 8, 2003; WO 03/037913 published May 8, 2003. Certain anti-DR5 antibodies have likewise been described, see, e.g., WO 98/51793 published November 8, 1998; Griffith et al., J. Immunol., 162:2597-2605 (1999); Ichikawa et al., Nature Med., 7:954-960 (2001); Hylander et al., "An Antibody to DR5 (TRAIL-Receptor 2) Suppresses the Growth of Patient Derived Gastrointestinal Tumors Grown in SCID mice", Abstract, 2d International Congress on Monoclonal Antibodies in Cancers, Aug. 29-Sept. 1, 2002, Banff, Alberta, Canada; WO 03/038043 published May 8, 2003; WO 03/037913 published May 8, 2003. In addition, certain antibodies having cross-reactivity to both DR4 and DR5 receptors have been described (see, e.g., US patent 6,252,050 issued June 26, 2001).

The CD20 antigen (also called human B-lymphocyte-restricted differentiation antigen, Bp35) is a hydrophobic transmembrane protein with a molecular weight of approximately 35 kD located on pre-B and mature B lymphocytes (Valentine *et al. J. Biol. Chem.* 264(19):11282-11287 (1989); and Einfeld *et al. EMBO J.* 7(3):711-717 (1988)). The antigen is also expressed on greater than 90% of B cell non-Hodgkin's lymphomas (NHL) (Anderson *et al. Blood* 63(6):1424-1433 (1984)), but is not found on hematopoietic stem cells, pro-B cells, normal plasma cells or other normal tissues (Tedder *et al. J. Immunol.* 135(2):973-979 (1985)). CD20 regulates an early step(s) in the activation process for cell cycle initiation and differentiation (Tedder *et al., supra*) and possibly functions as a calcium ion channel (Tedder *et al. J. Cell. Biochem.* 14D:195 (1990)). Given the expression of CD20 in B cell lymphomas, this antigen may serve as a candidate for "targeting" of such lymphomas.

The rituximab (RITUXAN®) antibody is a genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen. Rituximab is the antibody called "C2B8" in US Patent No. 5,736,137 issued April 7, 1998 (Anderson *et al.*). RITUXAN® is indicated for the treatment of patients with relapsed or refractory low-grade or follicular, CD20 positive, B cell non-Hodgkin's lymphoma. *In vitro* mechanism of action studies have demonstrated that RITUXAN® binds human complement and lyses lymphoid B cell lines through complement-dependent cytotoxicity (CDC)

(Reff *et al. Blood* 83(2):435-445 (1994); Cragg and Marlin, Blood, 103: 2738-2743 (2004). Additionally, it has significant activity in assays for antibody-dependent cell-mediated cytotoxicity (ADCC). More recently, RITUXAN® has been shown to have anti-proliferative effects in tritiated thymidine incorporation assays and to induce apoptosis directly, while other anti-CD19 and CD20 antibodies do not (Maloney *et al. Blood* 88(10):637a (1996)). Synergy between RITUXAN® and certain chemotherapies and toxins has also been observed experimentally. In particular, RITUXAN® sensitizes drug-resistant human B cell lymphoma cell lines to the cytotoxic effects of doxorubicin, CDDP, VP-16, diphtheria toxin and ricin (Demidem *et al. Cancer Chemotherapy & Radiopharmaceuticals* 12(3):177-186 (1997)). *In vivo* preclinical studies have shown that RITUXAN® depletes B cells from the peripheral blood, lymph nodes, and bone marrow of cynomolgus monkeys, presumably through complement and cell-mediated processes (Reff *et al. Blood* 83(2):435-445 (1994)).

## SUMMARY OF THE INVENTION

Methods for using death receptor ligands, such as Apo-2 ligand/TRAIL polypeptides or death receptor antibodies, and CD20 antibodies are provided herein. Embodiments of the invention include methods of treating cancer, comprising exposing cancer cells to an effective amount of Apo2L/TRAIL and CD20 antibody. Optionally, the cancer cells are exposed to an effective amount of death receptor antibody, such as an agonist DR4 antibody or agonist DR5 antibody, and CD20 antibody. Optionally, the amount of Apo2L/TRAIL or death receptor antibody and CD20 antibodies employed in the methods are effective to achieve synergy therapeutically, e.g., their combined anti-cancer effect is greater than the anti-cancer effect achieved when the Apo2L/TRAIL or antibodies are employed individually as a single therapeutic agent. The methods may entail in vitro use or in vivo use wherein the Apo2L/TRAIL or death receptor antibody and CD20 antibody are administered to a mammal (patient). Optionally, in the methods, the cancer cells treated with Apo2L/TRAIL or death receptor antibody and CD20 antibody are lymphoma cells.

Further embodiments of the invention include methods of treating an immune-related disease, comprising administering to a mammal an effective amount of Apo2L/TRAIL and CD20 antibody. Optionally, an effective amount of death receptor antibody, such as an agonist DR4 antibody or agonist DR5 antibody, and CD20 antibody is administered to the mammal. Optionally, the amount of Apo2L/TRAIL or death receptor antibody and CD20 antibodies employed in the methods are effective to achieve synergy therapeutically, e.g., their combined effect in treating the immune-related disease is greater than the effect achieved when the Apo2L/TRAIL or antibodies are employed individually as a single therapeutic agent. Optionally, in the

methods, the immune-related disease is rheumatoid arthritis or multiple sclerosis.

Methods of the invention include methods of treating a disorder in a mammal, such as an immune-related disease or cancer, comprising steps of obtaining tissue or a cell sample from the mammal, examining the tissue or cells for expression of CD20, DR4, and/or DR5, and upon determining said tissue or cell sample expresses said one or more receptors, administering an effective amount of Apo2L/TRAIL or death receptor antibody and CD20 antibody to said mammal. The steps in the methods for examining expression of one or more of such receptors may be conducted in a variety of assay formats, including assays detecting mRNA expression and immunohistochemistry assays.

Optionally, the methods of the invention comprise, in addition to administering an effective amount of Apo2L/TRAIL and/or death receptor antibody and CD20 antibody, administering chemotherapeutic agent(s) or radiation therapy to said mammal.

More embodiments of the invention are illustrated by way of example in the following claims:

1. A method of treating cancer cells, comprising exposing mammalian cancer cells to a synergistic effective amount of Apo2L/TRAIL polypeptide and CD20 antibody.

2. The method of claim 1 wherein said Apo2L/TRAIL polypeptide comprises amino acids 1-281 of Fig. 1 (SEQ ID NO:1) or a fragment or variant thereof.

3. The method of claim 1 wherein said Apo2L/TRAIL polypeptide comprises amino acids 114-281 of Fig. 1 (SEQ ID NO:1).

4. The method of claim 1 wherein said cancer cells are exposed to said synergistic effective amount of Apo2L/TRAIL polypeptide and CD20 antibody *in vivo*.

5. The method of claim 1 wherein said cancer cells are lymphoma cells.

6. The method of claim 1 further comprising exposing the cancer cells to one or more growth inhibitory agents.

7. The method of claim 1 further comprising exposing the cells to radiation.

8. The method of claim 1 wherein said Apo2L/TRAIL polypeptide is expressed in a recombinant host cell selected from the group consisting of a CHO cell, yeast cell and *E. coli*.

9. The method of claim 1 wherein said Apo2L/TRAIL polypeptide is linked to a polyethylene glycol molecule.

10. The method of claim 1 wherein said CD20 antibody is a monoclonal antibody.

11. The method of claim 10 wherein said CD20 antibody is the antibody Rituximab.

12. A method of treating an immune related disease, comprising administering to a mammal a synergistic effective amount of Apo2L/TRAIL polypeptide and CD20 antibody.

13. The method of claim 12 wherein said Apo2L/TRAIL polypeptide comprises amino acids 1-281 of Fig. 1 (SEQ ID NO:1) or a fragment or variant thereof.

14. The method of claim 12 wherein said Apo2L/TRAIL polypeptide comprises amino acids 114-281 of Fig. 1 (SEQ ID NO:1).

15. The method of claim 12 wherein said Apo2L/TRAIL polypeptide is expressed in a recombinant host cell selected from the group consisting of a CHO cell, yeast cell and *E. coli*.

16. The method of claim 12 wherein said Apo2L/TRAIL polypeptide is linked to a polyethylene glycol molecule.

17. The method of claim 12 wherein said immune related disease is rheumatoid arthritis or multiple sclerosis.

18. The method of claim 12 wherein said CD20 antibody is a monoclonal antibody.

19. The method of claim 18 wherein said CD20 antibody is the antibody Rituximab.

20. The method of claim 1 or 12 wherein said Apo2L/TRAIL polypeptide and CD20 antibody are administered sequentially.

21. The method of claim 1 or 12 wherein said Apo2L/TRAIL polypeptide

and CD20 antibody are administered concurrently.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows the nucleotide sequence of human Apo-2 ligand cDNA (SEQ ID NO:2) and its derived amino acid sequence (SEQ ID NO:1). The "N" at nucleotide position 447 is used to indicate the nucleotide base may be a "T" or "G".

Figures 2A and 2B show the nucleotide sequence of a cDNA (SEQ ID NO:4) for full length human DR4 and its derived amino acid sequence (SEQ ID NO:3). The respective nucleotide and amino acid sequences for human DR4 are also reported in Pan et al., Science, 276:111 (1997).

Figure 3A shows the 411 amino acid sequence of human DR5 (SEQ ID NO:5) as published in WO 98/51793 on November 19, 1998. A transcriptional splice variant of human DR5 is known in the art. This DR5 splice variant encodes the 440 amino acid sequence of human DR5 (SEQ ID NO:6) shown in Figures 3B and 3C as published in WO 98/35986 on August 20, 1998.

Figure 4 illustrates the expression of Apo2L/TRAIL receptors in B lymphoma cell lines.

Figure 5 illustrates expression of CD20 in B lymphoma cell lines.

Figure 6 shows the effects of Apo2L/TRAIL, RITUXAN®, or combination treatment on the growth of pre-established subcutaneous BJAB lymphoma tumor xenografts in SCID mice.

Figure 7 shows further results on the effects of Apo2L/TRAIL, RITUXAN®, or combination treatment of Apo2L/TRAIL and RITUXAN® on the growth of pre-established subcutaneous BJAB lymphoma tumor xenografts in SCID mice.

Figure 8 shows the effects of Apo2L/TRAIL, RITUXAN®, or combination treatment of Apo2L/TRAIL and RITUXAN® on caspase processing in pre-established subcutaneous BJAB lymphoma tumor xenografts grown in SCID mice.

Figure 9 shows the effects of DR5 agonistic antibody, RITUXAN®, or combination treatment on the growth of pre-established subcutaneous BJAB lymphoma tumor xenografts in SCID mice.

Figure 10 shows the effects of DR5 agonistic antibody, RITUXAN®, or combination treatment on caspase processing in pre-established subcutaneous BJAB lymphoma tumor xenografts grown in SCID mice.

Figure 11 illustrates the expression of CD20 and Apo2L/TRAIL receptors in NHL cell lines.

Figure 12 shows the effects of Apo2L/TRAIL, Rituximab, or combination treatment on the growth of pre-established subcutaneous Ramos RA1 tumor xenografts in SCID mice.

Figure 13 shows the effects of Apo2L/TRAIL, Rituximab, or combination treatment on the growth of pre-established DOHH-2 follicullar lymphoma xenografts in SCID mice.

Figure 14 illustrates the effects and mechanisms of cell killing by Apo2L/TRAIL and Rituximab or combination treatments on BJAB cells.

Figure 15 shows the effects of Apo2L/TRAIL, Rituximab, or combination treatment on the growth of Ramos T1 tumor xenografts in SCID mice.

Figure 16 shows the effects of Apo2L/TRAIL, Rituximab, or combination treatment on the BJAB-Luc tumor xenografts in SCID mice.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Unless otherwise defined, all terms of art, notations and other scientific terminology used herein are intended to have the meanings commonly understood by those of skill in the art to which this invention pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what is generally understood in the art. The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized molecular cloning methodologies described in Sambrook et al., Molecular Cloning: A Laboratory Manual 2nd. edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. As appropriate, procedures involving the use of commercially available kits and reagents are generally carried out in accordance with manufacturer defined protocols and/or parameters unless otherwise noted.

Before the present methods, kits and uses therefor are described, it is to be understood that this invention is not limited to the particular methodology, protocols, cell lines, animal species or genera, constructs, and reagents described as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

It must be noted that as used herein and in the appended claims, the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise.

All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. Publications cited herein are cited for their disclosure prior to the filing date of the present application. Nothing here is to be construed as an admission that the inventors are not entitled to antedate the publications by virtue of an earlier priority date or prior date of invention. Further the actual publication dates may be different from those shown and require independent verification.

**Definitions**

The terms "Apo-2 ligand", "Apo-2L", "Apo2L", "Apo2L/TRAIL", "Apo-2 ligand/TRAIL" and "TRAIL" are used herein interchangeably to refer to a polypeptide sequence which includes amino acid residues 114-281, inclusive, 95-281, inclusive, residues 92-281, inclusive, residues 91-281, inclusive, residues 41-281, inclusive, residues 39-281, inclusive, residues 15-281, inclusive, or residues 1-281, inclusive, of the amino acid sequence shown in Figure 1, as well as biologically active fragments, deletional, insertional, or substitutional variants of the above sequences. In one embodiment, the polypeptide sequence comprises residues 114-281 of Figure 1. Optionally, the polypeptide sequence comprises residues 92-281 or residues 91-281 of Figure 1. The Apo-2L polypeptides may be encoded by the native nucleotide sequence shown in Figure 1. Optionally, the codon which encodes residue Pro119 (Figure 1) may be "CCT" or "CCG". Optionally, the fragments or variants are biologically active and have at least about 80% amino acid sequence identity, more preferably at least about 90% sequence identity, and even more preferably, at least 95%, 96%, 97%, 98%, or 99% sequence identity with any one of the above sequences. The definition encompasses substitutional variants of Apo-2 ligand in which at least one of its native amino acids are substituted by another amino acid such as an alanine residue. Optional substitutional variants include one or more of the residue substitutions. Optional variants may comprise an amino acid sequence which differs from the native sequence Apo-2 ligand polypeptide sequence of Figure 1 and has one or more of the following amino acid substitutions at the residue position(s) in Figure 1: S96C; S101C; S111C; R170C; K179C. The definition also encompasses a native sequence Apo-2 ligand isolated from an Apo-2 ligand source or prepared by recombinant or synthetic methods. The Apo-2 ligand of the invention includes the polypeptides referred to as Apo-2 ligand or TRAIL disclosed in WO97/01633 published January 16, 1997, WO97/25428 published July 17, 1997, WO99/36535 published July 22, 1999, WO 01/00832 published January 4, 2001, WO02/09755 published February 7, 2002, and WO 00/75191 published December 14, 2000. The terms are used to refer generally to forms of the Apo-2 ligand which include monomer, dimer, trimer, hexamer or hight oligomer forms of the polypeptide. All numbering of amino acid residues referred to in the Apo-2L sequence use the numbering according to Figure 1, unless specifically stated otherwise. For instance, "D203" or "Asp203" refers to the aspartic acid residue at position 203 in the sequence provided in Figure 1.

The term "Apo-2 ligand selective variant" as used herein refers to an Apo-2 ligand polypeptide which includes one or more amino acid mutations in a native Apo-2 ligand sequence and has selective binding affinity for either the DR4 receptor or the DR5 receptor. In one embodiment, the Apo-2 ligand variant has a selective binding affinity for the DR4 receptor and

includes one or more amino acid substitutions in any one of positions 189, 191, 193, 199, 201 or 209 of a native Apo-2 ligand sequence. In another embodiment, the Apo-2 ligand variant has a selective binding affinity for the DR5 receptor and includes one or more amino acid substitutions in any one of positions 189, 191, 193, 264, 266, 267 or 269 of a native Apo-2 ligand sequence. Preferred Apo-2 ligand selective variants include one or more amino acid mutations and exhibit binding affinity to the DR4 receptor which is equal to or greater ($\geq$) than the binding affinity of native sequence Apo-2 ligand to the DR4 receptor, and even more preferably, the Apo-2 ligand variants exhibit less binding affinity ($<$) to the DR5 receptor than the binding affinity exhibited by native sequence Apo-2 ligand to DR5. When binding affinity of such Apo-2 ligand variant to the DR4 receptor is approximately equal (unchanged) or greater than (increased) as compared to native sequence Apo-2 ligand, and the binding affinity of the Apo-2 ligand variant to the DR5 receptor is less than or nearly eliminated as compared to native sequence Apo-2 ligand, the binding affinity of the Apo-2 ligand variant, for purposes herein, is considered "selective" for the DR4 receptor. Preferred DR4 selective Apo-2 ligand variants of the invention will have at least 10-fold less binding affinity to DR5 receptor (as compared to native sequence Apo-2 ligand), and even more preferably, will have at least 100-fold less binding affinity to DR5 receptor (as compared to native sequence Apo-2 ligand). The respective binding affinity of the Apo-2 ligand variant may be determined and compared to the binding properties of native Apo-2L (such as the 114-281 form) by ELISA, RIA, and/or BIAcore assays, known in the art. Preferred DR4 selective Apo-2 ligand variants of the invention will induce apoptosis in at least one type of mammalian cell (preferably a cancer cell), and such apoptotic activity can be determined by known art methods such as the alamar blue or crystal violet assay. The DR4 selective Apo-2 ligand variants may or may not have altered binding affinities to any of the decoy receptors for Apo-2L, those decoy receptors being referred to in the art as DcR1, DcR2 and OPG.

Further preferred Apo-2 ligand selective variants include one or more amino acid mutations and exhibit binding affinity to the DR5 receptor which is equal to or greater ($\geq$) than the binding affinity of native sequence Apo-2 ligand to the DR5 receptor, and even more preferably, such Apo-2 ligand variants exhibit less binding affinity ($<$) to the DR4 receptor than the binding affinity exhibited by native sequence Apo-2 ligand to DR4. When binding affinity of such Apo-2 ligand variant to the DR5 receptor is approximately equal (unchanged) or greater than (increased) as compared to native sequence Apo-2 ligand, and the binding affinity of the Apo-2 ligand variant to the DR4 receptor is less than or nearly eliminated as compared to native sequence Apo-2 ligand, the binding affinity of the Apo-2 ligand variant, for purposes herein, is considered "selective" for the DR5 receptor. Preferred DR5 selective Apo-2 ligand variants of the invention will have at least 10-fold less binding affinity to DR4 receptor (as

compared to native sequence Apo-2 ligand), and even more preferably, will have at least 100-fold less binding affinity to DR4 receptor (as compared to native sequence Apo-2 ligand). The respective binding affinity of the Apo-2 ligand variant may be determined and compared to the binding properties of native Apo2L (such as the 114-281 form) by ELISA, RIA, and/or BIAcore assays, known in the art. Preferred DR5 selective Apo-2 ligand variants of the invention will induce apoptosis in at least one type of mammalian cell (preferably a cancer cell), and such apoptotic activity can be determined by known art methods such as the alamar blue or crystal violet assay. The DR5 selective Apo-2 ligand variants may or may not have altered binding affinities to any of the decoy receptors for Apo-2L, those decoy receptors being referred to in the art as DcR1, DcR2 and OPG.

Amino acid identification may use the single-letter alphabet or three-letter alphabet of amino acids, i.e.,

| | | | | | |
|---|---|---|---|---|---|
| Asp | D | Aspartic acid | Ile | I | Isoleucine |
| Thr | T | Threonine | Leu | L | Leucine |
| Ser | S | Serine | Tyr | Y | Tyrosine |
| Glu | E | Glutamic acid | Phe | F | Phenylalanine |
| Pro | P | Proline | His | H | Histidine |
| Gly | G | Glycine | Lys | K | Lysine |
| Ala | A | Alanine | Arg | R | Arginine |
| Cys | C | Cysteine | Trp | W | Tryptophan |
| Val | V | Valine | Gln | Q | Glutamine |
| Met | M | Methionine | Asn | N | Asparagine |

The term "Apo2L/TRAIL extracellular domain" or "Apo2L/TRAIL ECD" refers to a form of Apo2L/TRAIL which is essentially free of transmembrane and cytoplasmic domains. Ordinarily, the ECD will have less than 1% of such transmembrane and cytoplasmic domains, and preferably, will have less than 0.5% of such domains. It will be understood that any transmembrane domain(s) identified for the polypeptides of the present invention are identified pursuant to criteria routinely employed in the art for identifying that type of hydrophobic domain. The exact boundaries of a transmembrane domain may vary but most likely by no more than about 5 amino acids at either end of the domain as initially identified. In preferred embodiments, the ECD will consist of a soluble, extracellular domain sequence of the polypeptide which is free of the transmembrane and cytoplasmic or intracellular domains (and is not membrane bound). Particular extracellular domain sequences of Apo-2L/TRAIL are described in PCT Publication Nos. WO97/01633 and WO97/25428.

The term "Apo2L/TRAIL monomer" or "Apo2L monomer" refers to a covalent chain of an extracellular domain sequence of Apo2L.

The term "Apo2L/TRAIL dimer" or "Apo2L dimer" refers to two Apo-2L monomers joined in a covalent linkage via a disulfide bond. The term as used herein includes free standing Apo2L dimers and Apo2L dimers that are

within trimeric forms of Apo2L (i.e., associated with another, third Apo2L monomer).

The term "Apo2L/TRAIL trimer" or "Apo2L trimer" refers to three Apo2L monomers that are non-covalently associated.

The term "Apo2L/TRAIL aggregate" is used to refer to self-associated higher oligomeric forms of Apo2L/TRAIL, such as Apo2L/TRAIL trimers, which form, for instance, hexameric and nanomeric forms of Apo2L/TRAIL. Determination of the presence and quantity of Apo2L/TRAIL monomer, dimer, or trimer (or other aggregates) may be made using methods and assays known in the art (and using commercially available materials), such as native size exclusion HPLC ("SEC"), denaturing size exclusion using sodium dodecyl sulphate ("SDS-SEC"), reverse phase HPLC and capillary electrophoresis.

"Apo-2 ligand receptor" includes the receptors referred to in the art as "DR4" and "DR5" whose polynucleotide and polypeptide sequences are shown in Figures 2 and 3 respectively. Pan et al. have described the TNF receptor family member referred to as "DR4" (Pan et al., Science, 276:111-113 (1997); see also WO98/32856 published July 30, 1998; WO 99/37684 published July 29, 1999; WO 00/73349 published December 7, 2000; US 6,433,147 issued August 13, 2002; US 6,461,823 issued October 8, 2002, and US 6,342,383 issued January 29, 2002). Sheridan et al., Science, 277:818-821 (1997) and Pan et al., Science, 277:815-818 (1997) described another receptor for Apo2L/TRAIL (see also, WO98/51793 published November 19, 1998; WO98/41629 published September 24, 1998). This receptor is referred to as DR5 (the receptor has also been alternatively referred to as Apo-2; TRAIL-R, TR6, Tango-63, hAPO8, TRICK2 or KILLER; Screaton et al., Curr. Biol., 7:693-696 (1997); Walczak et al., EMBO J., 16:5386-5387 (1997); Wu et al., Nature Genetics, 17:141-143 (1997); WO98/35986 published August 20, 1998; EP870,827 published October 14, 1998; WO98/46643 published October 22, 1998; WO99/02653 published January 21, 1999; WO99/09165 published February 25, 1999; WO99/11791 published March 11, 1999; US 2002/0072091 published August 13, 2002; US 2002/0098550 published December 7, 2001; US 6,313,269 issued December 6, 2001; US 2001/0010924 published August 2, 2001; US 2003/01255540 published July 3, 2003; US 2002/0160446 published October 31, 2002, US 2002/0048785 published April 25, 2002; US 6,569,642 issued May 27, 2003, US 6,072,047 issued June 6, 2000, US 6,642,358 issued November 4, 2003). As described above, other receptors for Apo-2L include DcR1, DcR2, and OPG (see, Sheridan et al., supra; Marsters et al., supra; and Simonet et al., supra). The term "Apo-2L receptor" when used herein encompasses native sequence receptor and receptor variants. These terms encompass Apo-2L receptor expressed in a variety of mammals, including humans. Apo-2L receptor may be endogenously expressed as occurs naturally in a variety of human tissue lineages, or may be expressed by recombinant or synthetic methods. A "native sequence Apo-2L receptor" comprises a polypeptide having the same amino acid sequence as an Apo-2L receptor derived from nature. Thus, a native sequence Apo-2L receptor can have the amino acid

sequence of naturally-occurring Apo-2L receptor from any mammal. Such native sequence Apo-2L receptor can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence Apo-2L receptor" specifically encompasses naturally-occurring truncated or secreted forms of the receptor (*e.g.*, a soluble form containing, for instance, an extracellular domain sequence), naturally-occurring variant forms (*e.g.*, alternatively spliced forms) and naturally-occurring allelic variants. Receptor variants may include fragments or deletion mutants of the native sequence Apo-2L receptor. Figure 3A shows the 411 amino acid sequence of human DR5 as published in WO 98/51793 on November 19, 1998. A transcriptional splice variant of human DR5 is known in the art. This DR5 splice variant encodes the 440 amino acid sequence of human DR5 shown in Figures 3B and 3C as published in WO 98/35986 on August 20, 1998.

"Death receptor antibody" is used herein to refer generally to antibody or antibodies directed to a receptor in the tumor necrosis factor receptor superfamily and containing a death domain capable of signalling apoptosis, and such antibodies include DR5 antibody and DR4 antibody.

"DR5 receptor antibody", "DR5 antibody", or "anti-DR5 antibody" is used in a broad sense to refer to antibodies that bind to at least one form of a DR5 receptor or extracellular domain thereof. Optionally the DR5 antibody is fused or linked to a heterologous sequence or molecule. Preferably the heterologous sequence allows or assists the antibody to form higher order or oligomeric complexes. Optionally, the DR5 antibody binds to DR5 receptor but does not bind or cross-react with any additional Apo-2L receptor (e.g. DR4, DcR1, or DcR2). Optionally the antibody is an agonist of DR5 signalling activity.

Optionally, the DR5 antibody of the invention binds to a DR5 receptor at a concentration range of about 0.1 nM to about 20 mM as measured in a BIAcore binding assay. Optionally, the DR5 antibodies of the invention exhibit an Ic 50 value of about 0.6 nM to about 18 mM as measured in a BIAcore binding assay.

"DR4 receptor antibody", "DR4 antibody", or "anti-DR4 antibody" is used in a broad sense to refer to antibodies that bind to at least one form of a DR4 receptor or extracellular domain therof. Optionally the DR4 antibody is fused or linked to a heterologous sequence or molecule. Preferably the heterologous sequence allows or assists the antibody to form higher order or oligomeric complexes. Optionally, the DR4 antibody binds to DR4 receptor but does not bind or cross-react with any additional Apo-2L receptor (e.g. DR5, DcR1, or DcR2). Optionally the antibody is an agonist of DR4 signalling activity.

Optionally, the DR4 antibody of the invention binds to a DR4 receptor at a concentration range of about 0.1 nM to about 20 mM as measured in a BIAcore binding assay. Optionally, the DR5 antibodies of the invention

exhibit an Ic 50 value of about 0.6 nM to about 18 mM as measured in a BIAcore binding assay.

The term "agonist" is used in the broadest sense, and includes any molecule that partially or fully enhances, stimulates or activates one or more biological activities of Apo2L/TRAIL, DR4 or DR5, *in vitro*, *in situ*, or *in vivo*. Examples of such biological activities binding of Apo2L/TRAIL to DR4 or DR5, include apoptosis as well as those further reported in the literature. An agonist may function in a direct or indirect manner. For instance, the agonist may function to partially or fully enhance, stimulate or activate one or more biological activities of DR4 or DR5, *in vitro*, *in situ*, or *in vivo* as a result of its direct binding to DR4 or DR5, which causes receptor activation or signal transduction. The agonist may also function indirectly to partially or fully enhance, stimulate or activate one or more biological activities of DR4 or DR5, *in vitro*, *in situ*, or *in vivo* as a result of, e.g., stimulating another effector molecule which then causes DR4 or DR5 activation or signal transduction. It is contemplated that an agonist may act as an enhancer molecule which functions indirectly to enhance or increase DR4 or DR5 activation or activity. For instance, the agonist may enhance activity of endogenous Apo-2L in a mammal. This could be accomplished, for example, by pre-complexing DR4 or DR5 or by stabilizing complexes of the respective ligand with the DR4 or DR5 receptor (such as stabilizing native complex formed between Apo-2L and DR4 or DR5).

The term "DR4" and "DR4 receptor" as used herein refers to full length and soluble, extracellular domain forms of the receptor described in Pan et al., Science, 276:111-113 (1997); WO98/32856 published July 30, 1998; US Patent 6,342,363 issued January 29, 2002; and WO99/37684 published July 29, 1999. The full length amino acid sequence of DR4 receptor is provided herein in Fig. 2.

The term "DR5" and "DR5 receptor" as used herein refers to the full length and soluble, extracellular domain forms of the receptor described in Sheridan et al., Science, 277:818-821 (1997); Pan et al., Science, 277:815-818 (1997), US Patent 6,072,047 issued June 6, 2000; US Patent 6,342,369, WO98/51793 published November 19, 1998; WO98/41629 published September 24, 1998; Screaton et al., Curr. Biol., 7:693-696 (1997); Walczak et al., EMBO J., 16:5386-5387 (1997); Wu et al., Nature Genetics, 17:141-143 (1997); WO98/35986 published August 20, 1998; EP870,827 published October 14, 1998; WO98/46643 published October 22, 1998; WO99/02653 published January 21, 1999; WO99/09165 published February 25, 1999; WO99/11791 published March 11, 1999. The DR5 receptor has also been referred to in the art as Apo-2; TRAIL-R, TR6, Tango-63, hAPO8, TRICK2 or KILLER. The term DR5 receptor used herein includes the full length 411 amino acid polypeptide provided in Fig. 3A and the full length 440 amino acid polypeptide provided in Figs. 3B-C.

The term "polyol" when used herein refers broadly to polyhydric alcohol compounds. Polyols can be any water-soluble poly(alkylene oxide) polymer for example, and can have a linear or branched chain. Preferred polyols include those substituted at one or more hydroxyl positions with a chemical group, such as an alkyl group having between one and four carbons. Typically, the polyol is a poly(alkylene glycol), preferably poly(ethylene glycol) (PEG). However, those skilled in the art recognize that other polyols, such as, for example, poly(propylene glycol) and polyethylene-polypropylene glycol copolymers, can be employed using the techniques for conjugation described herein for PEG. The polyols of the invention include those well known in the art and those publicly available, such as from commercially available sources.

The term "conjugate" is used herein according to its broadest definition to mean joined or linked together. Molecules are "conjugated" when they act or operate as if joined.

The term "extracellular domain" or "ECD" refers to a form of ligand or receptor which is essentially free of transmembrane and cytoplasmic domains. Ordinarily, the soluble ECD will have less than 1% of such transmembrane and cytoplasmic domains, and preferably, will have less than 0.5% of such domains.

The term "divalent metal ion" refers to a metal ion having two positive charges. Examples of divalent metal ions for use in the present invention include but are not limited to zinc, cobalt, nickel, cadmium, magnesium, and manganese. Particular forms of such metals that may be employed include salt forms (e.g., pharmaceutically acceptable salt forms), such as chloride, acetate, carbonate, citrate and sulfate forms of the above mentioned divalent metal ions. Divalent metal ions, as described herein, are preferably employed in concentrations or amounts (e.g., effective amounts) which are sufficient to, for example, (1) enhance storage stability of Apo-2L trimers over a desired period of time, (2) enhance production or yield of Apo-2L trimers in a recombinant cell culture or purification method, (3) enhance solubility (or reduce aggregation) of Apo-2L trimers, or (4) enhance Apo-2L trimer formation.

"Isolated," when used to describe the various proteins disclosed herein, means protein that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the protein, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the protein will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated protein includes protein in situ within recombinant cells, since at least one component of

the protein's natural environment will not be present. Ordinarily, however, isolated protein will be prepared by at least one purification step.

An "isolated" nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the nucleic acid. An isolated Apo-2 ligand nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated Apo-2 ligand nucleic acid molecules therefore are distinguished from the Apo-2 ligand nucleic acid molecule as it exists in natural cells. However, an isolated Apo-2 ligand nucleic acid molecule includes Apo-2 ligand nucleic acid molecules contained in cells that ordinarily express Apo-2 ligand where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

"Percent (%) amino acid sequence identity" with respect to the sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the Apo-2 ligand sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art can determine appropriate parameters for measuring alignment, including assigning algorithms needed to achieve maximal alignment over the full-length sequences being compared. For purposes herein, percent amino acid identity values can be obtained using the sequence comparison computer program, ALIGN-2, which was authored by Genentech, Inc. and the source code of which has been filed with user documentation in the US Copyright Office, Washington, DC, 20559, registered under the US Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available through Genentech, Inc., South San Francisco, CA. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome

binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

A "B cell" is a lymphocyte that matures within the bone marrow, and includes a naive B cell, memory B cell, or effector B cells (plasma cells). The B cell herein may be a normal or non-malignant B cell.

The "CD20" antigen is a ~35 kDa, non-glycosylated phosphoprotein found on the surface of greater than 90% of B cells from peripheral blood or lymphoid organs. CD20 is present on both normal B cells as well as malignant B cells, but is not expressed on stem cells. Other names for CD20 in the literature include "B-lymphocyte-restricted antigen" and "Bp35". The CD20 antigen is described in Clark *et al. PNAS (USA)* 82:1766 (1985), for example.

Examples of antibodies which bind the CD20 antigen include: "C2B8" which is now called "Rituximab" ("RITUXAN®") (US Patent No. 5,736,137); the yttrium-[90]-labeled 2B8 murine antibody designated "Y2B8" or "Ibritumomab Tiuxetan" ZEVALIN® commercially available from Idec Pharmaceuticals, Inc. (US Patent No. 5,736,137; 2B8 deposited with ATCC under accession no. HB11388 on June 22, 1993); murine IgG2a "B1," also called "Tositumomab," optionally labeled with $^{131}I$ to generate the "$^{131}I$-B1" antibody (iodine I131 tositumomab, BEXXAR™) commercially available from Corixa. (see, also, US Patent No. 5,595,721); murine monoclonal antibody "1F5" (Press *et al. Blood* 69(2):584-591 (1987)) and variants thereof including "framework patched" or humanized 1F5 (WO 2003/002607, Leung, ATCC Deposit HB-96450); murine 2H7 and chimeric 2H7 antibody (US Patent No. 5,677,180); humanized 2H7; HUMAX-CD20™ fully human, high-affinity antibody targeted at the CD20 molecule in the cell membrane of B-cells (Genmab, Denmark; see, for example, Glennie and van de Winkel, *Drug Discovery Today* 8: 503-510 (2003) and Cragg *et al.*, *Blood* 101: 1045-1052 (2003)); the human monoclonal antibodies set forth in WO04/035607 (Teeling *et al.*); AME-133™ antibodies (Applied Molecular Evolution); A20 antibody or variants thereof such as chimeric or humanized A20 antibody (cA20, hA20, respectively) (US 2003/0219433, Immunomedics); and monoclonal antibodies L27, G28-2, 93-1B3, B-C1 or NU-B2 available from the International Leukocyte Typing Workshop (Valentine *et al.*, In: *Leukocyte Typing* III (McMichael, Ed., p. 440, Oxford University Press (1987)). The preferred CD20 antibodies herein are chimeric, humanized, or human CD20 antibodies, more preferably rituximab, humanized 2H7, chimeric or humanized A20 antibody (Immunomedics), and HUMAX-CD20™ human CD20 antibody (Genmab).

The terms "rituximab" or "RITUXAN®" herein refer to the genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen and designated "C2B8" in US Patent No. 5,736,137, including fragments thereof which retain the ability to bind CD20.

Purely for the purposes herein and unless indicated otherwise, "humanized 2H7" refers to a humanized CD20 antibody, or an antigen-binding fragment thereof, wherein the antibody is effective to deplete primate B cells *in vivo*, the antibody comprising in the H chain variable region ($V_H$) thereof at least a CDR H3 sequence from an anti-human CD20 antibody and substantially the human consensus framework (FR) residues of the human heavy- chain subgroup III ($V_H$III).

A preferred humanized 2H7 is an intact antibody or antibody fragment comprising the variable light chain sequence:
DIQMTQSPSSLSASVGDRVTITCRASSSVSYMHWYQQKPGKAPKPLIYAPSNLASGVPSRFSGSGSGTDFTLTIS
SLQPEDFATYYCQQWSFNPPTFGQGTKVEIKR (SEQ ID NO:7);
and the variable heavy chain sequence:
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGAIYPGNGDTSYNQKFKGRFTISVDKS
KNTLYLQMNSLRAEDTAVYYCARVVYYSNSYWYFDVWGQGTLVTVSS (SEQ ID NO:8).

Where the humanized 2H7 antibody is an intact antibody, preferably it comprises the light chain amino acid sequence:
DIQMTQSPSSLSASVGDRVTITCRASSSVSYMHWYQQKPGKAPKPLIYAPSNLASGVPSRFSGSGSGTDFTLTIS
SLQPEDFATYYCQQWSFNPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVD
NALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID
NO:9);
and the heavy chain amino acid sequence:
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGAIYPGNGDTSYNQKFKGRFTISVDKS
KNTLYLQMNSLRAEDTAVYYCARVVYYSNSYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVK
GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK (SEQ ID NO:10)
or the heavy chain amino acid sequence:
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGAIYPGNGDTSYNQKFKGRFTISVDKS
KNTLYLQMNSLRAEDTAVYYCARVVYYSNSYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNATYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIAATISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVK
GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK (SEQ ID NO:11).

"Antibody-dependent cell-mediated cytotoxicity" and "ADCC" refer to a cell-mediated reaction in which nonspecific cytotoxic cells that express Fc receptors (FcRs) (*e.g.* Natural Killer (NK) cells, neutrophils, and

macrophages) recognize bound antibody on a target cell and subsequently cause lysis of the target cell. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells in summarized is Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an *in vitro* ADCC assay, such as that described in US Patent No. 5,500,362 or 5,821,337 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, *e.g.*, in a animal model such as that disclosed in Clynes *et al. PNAS (USA)* 95:652-656 (1998).

"Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. Preferably, the cells express at least FcγRIII and carry out ADCC effector function. Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with PBMCs and NK cells being preferred.

The terms "Fc receptor" or "FcR" are used to describe a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and Fcγ RIII subclasses, including allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (see Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991); Capel *et al.*, *Immunomethods* 4:25-34 (1994); and de Haas *et al.*, *J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer *et al.*, *J. Immunol.* 117:587 (1976) and Kim *et al.*, *J. Immunol.* 24:249 (1994)). FcRs herein include polymorphisms such as the genetic dimorphism in the gene that encodes FcγRIIIa resulting in either a phenylalanine (F) or a valine (V) at amino acid position 158, located in the region of the receptor that binds to IgG1. The homozygous valine FcγRIIIa (FcγRIIIa-158V) has been shown to have a higher affinity for human IgG1 and mediate increased ADCC *in vitro* relative to homozygous phenylalanine FcγRIIIa (FcγRIIIa-158F) or heterozygous (FcγRIIIa-158F/V) receptors.

"Complement dependent cytotoxicity" or "CDC" refer to the ability of a molecule to lyse a target in the presence of complement. The complement activation pathway is initiated by the binding of the first component of the complement system (C1q) to a molecule (*e.g.* an antibody) complexed with a cognate antigen. To assess complement activation, a CDC assay, *e.g.* as described in Gazzano-Santoro *et al.*, *J. Immunol. Methods* 202:163 (1996), may be performed.

The term "antibody" herein is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (*e.g.* bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of an intact antibody, preferably comprising the antigen-binding or variable region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

"Native antibodies" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain ($V_H$) followed by a number of constant domains. Each light chain has a variable domain at one end ($V_L$) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FRs). The variable domains of native heavy and light chains each comprise four FRs, largely adopting a β-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of

antibodies (see Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cell-mediated cytotoxicity (ADCC).

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-binding sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and antigen-binding site. This region consists of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the $V_H$-$V_L$ dimer. Collectively, the six hypervariable regions confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear at least one free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa ($\kappa$) and lambda ($\lambda$), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, antibodies can be assigned to different classes. There are five major classes of intact antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.*, IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of antibodies are called $\alpha$, $\delta$, $\varepsilon$, $\gamma$, and $\mu$, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

"Single-chain Fv" or "scFv" antibody fragments comprise the $V_H$ and $V_L$ domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the $V_H$ and $V_L$ domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain ($V_H$) connected to a light-chain variable domain ($V_L$) in the same polypeptide chain ($V_H$ - $V_L$). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993).

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al.*, *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson *et al.*, *Nature*, 352:624-628 (1991) and Marks *et al.*, *J. Mol. Biol.*, 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies

derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; Morrison *et al., Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)). Chimeric antibodies of interest herein include "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (*e.g.* Old World Monkey, such as baboon, rhesus or cynomolgus monkey) and human constant region sequences (US Pat No. 5,693,780).

"Humanized" forms of non-human (*e.g.*, murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones *et al., Nature* 321:522-525 (1986); Riechmann *et al., Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (*e.g.* residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat *et al., Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) and/or those residues from a "hypervariable loop" (*e.g.* residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

An antibody "which binds" an antigen of interest, *e.g.* CD20 or DR4 or DR5, is one capable of binding that antigen with sufficient affinity and/or avidity such that the antibody is useful as a therapeutic agent for targeting a cell expressing the antigen.

For the purposes herein, "immunotherapy" will refer to a method of treating a mammal (preferably a human patient) with an antibody, wherein the antibody may be an unconjugated or "naked" antibody, or the antibody may be conjugated or fused with heterologous molecule(s) or agent(s), such as one or more cytotoxic agent(s), thereby generating an "immunoconjugate".

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antagonist or antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody *in situ* within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

The expression "effective amount" refers to an amount of the Apo2L/TRAIL or death receptor antibody and CD20 antibody which is effective for preventing, ameliorating or treating the disease or condition in question.

The term "immunosuppressive agent" as used herein for adjunct therapy refers to substances that act to suppress or mask the immune system of the mammal being treated herein. This would include substances that suppress cytokine production, downregulate or suppress self-antigen expression, or mask the MHC antigens. Examples of such agents include 2-amino-6-aryl-5-substituted pyrimidines (see U.S. Pat. No. 4,665,077, the disclosure of which is incorporated herein by reference); nonsteroidal antiinflammatory drugs (NSAIDs); azathioprine; cyclophosphamide; bromocryptine; danazol; dapsone; glutaraldehyde (which masks the MHC antigens, as described in U.S. Pat. No. 4,120,649); anti-idiotypic antibodies for MHC antigens and MHC fragments; cyclosporin A; steroids such as glucocorticosteroids, *e.g.*, prednisone, methylprednisolone, dexamethasone, and hydrocortisone; methotrexate (oral or subcutaneous); hydroxycloroquine; sulfasalazine; leflunomide; cytokine or cytokine receptor antagonists including anti-interferon-γ, -β, or -α antibodies, anti-tumor necrosis factor-α antibodies (infliximab or adalimumab), anti-TNFα immunoahesin (etanercept), anti-tumor

necrosis factor-β antibodies, anti-interleukin-2 antibodies and anti-IL-2 receptor antibodies; anti-LFA-1 antibodies, including anti-CD11a and anti-CD18 antibodies; anti-L3T4 antibodies; heterologous anti-lymphocyte globulin; pan-T antibodies, preferably anti-CD3 or anti-CD4/CD4a antibodies; soluble peptide containing a LFA-3 binding domain (WO 90/08187 published 7/26/90); streptokinase; TGF-β; streptodornase; RNA or DNA from the host; FK506; RS-61443; deoxyspergualin; rapamycin; T-cell receptor (Cohen *et al.*, U.S. Pat. No. 5,114,721); T-cell receptor fragments (Offner *et al.*, *Science*, 251: 430-432 (1991); WO 90/11294; Ianeway, *Nature*, 341: 482 (1989); and WO 91/01133); and T cell receptor antibodies (EP 340,109) such as T10B9.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (*e.g.* $At^{211}$, $I^{131}$, $I^{125}$, $Y^{90}$, $Re^{186}$, $Re^{188}$, $Sm^{153}$, $Bi^{212}$, $P^{32}$ and radioactive isotopes of Lu), chemotherapeutic agents, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

"Synergistic activity" or "synergy" or "synergistic effect" or "synergistic effective amount" for the purposes herein means that the effect observed when employing a combination of Apo2L/TRAIL or death receptor antibody and CD20 antibody is (1) greater than the effect achieved when that Apo2L/TRAIL, death receptor antibody or CD20 antibody is employed alone (or individually) and (2) greater than the sum added (additive) effect for that Apo2L/TRAIL or death receptor antibody and CD20 antibody. Such synergy or synergistic effect can be determined by way of a variety of means known to those in the art. For example, the synergistic effect of Apo2L/TRAIL or death receptor antibody and CD20 antibody can be observed in in vitro or in vivo assay formats examining reduction of tumor cell number or tumor mass.

The terms "apoptosis" and "apoptotic activity" are used in a broad sense and refer to the orderly or controlled form of cell death in mammals that is typically accompanied by one or more characteristic cell changes, including condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. This activity can be determined and measured using well known art methods, for instance, by cell viability assays, FACS analysis or DNA electrophoresis, binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). Assays which determine the ability of an antibody (*e.g.* Rituximab) to induce apoptosis have been described in Shan *et al.* *Cancer Immunol Immunther* 48:673-83 (2000); Pedersen *et al.* *Blood* 99:1314-9 (2002); Demidem *et al.*

*Cancer Chemotherapy & Radiopharmaceuticals* 12(3):177-186 (1997), for example.

The terms "cancer", "cancerous", and "malignant" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma including adenocarcinoma, lymphoma, blastoma, melanoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, Hodgkin's and non-Hodgkin's lymphoma, pancreatic cancer, glioblastoma, glioma, cervical cancer, ovarian cancer, liver cancer such as hepatic carcinoma and hepatoma, bladder cancer, breast cancer, colon cancer, colorectal cancer, endometrial carcinoma, myeloma (such as multiple myeloma), salivary gland carcinoma, kidney cancer such as renal cell carcinoma and Wilms' tumors, basal cell carcinoma, melanoma, prostate cancer, vulval cancer, thyroid cancer, testicular cancer, esophageal cancer, and various types of head and neck cancer.

The term "immune related disease" means a disease in which a component of the immune system of a mammal causes, mediates or otherwise contributes to a morbidity in the mammal. Also included are diseases in which stimulation or intervention of the immune response has an ameliorative effect on progression of the disease. Included within this term are autoimmune diseases, immune-mediated inflammatory diseases, non-immune-mediated inflammatory diseases, infectious diseases, and immunodeficiency diseases. Examples of immune-related and inflammatory diseases, some of which are immune or T cell mediated, which can be treated according to the invention include systemic lupus erythematosis, rheumatoid arthritis, juvenile chronic arthritis, spondyloarthropathies, systemic sclerosis (scleroderma), idiopathic inflammatory myopathies (dermatomyositis, polymyositis), Sjogren's syndrome, systemic vasculitis, sarcoidosis, autoimmune hemolytic anemia (immune pancytopenia, paroxysmal nocturnal hemoglobinuria), autoimmune thrombocytopenia (idiopathic thrombocytopenic purpura, immune-mediated thrombocytopenia), thyroiditis (Grave's disease, Hashimoto's thyroiditis, juvenile lymphocytic thyroiditis, atrophic thyroiditis), diabetes mellitus, immune-mediated renal disease (glomerulonephritis, tubulointerstitial nephritis), demyelinating diseases of the central and peripheral nervous systems such as multiple sclerosis, idiopathic demyelinating polyneuropathy or Guillain-Barré syndrome, and chronic inflammatory demyelinating polyneuropathy, hepatobiliary diseases such as infectious hepatitis (hepatitis A, B, C, D, E and other non-hepatotropic viruses), autoimmune chronic active hepatitis, primary biliary cirrhosis, granulomatous hepatitis, and sclerosing cholangitis, inflammatory and fibrotic lung diseases such as inflammatory bowel disease (ulcerative colitis: Crohn's disease), gluten-sensitive enteropathy, and Whipple's disease, autoimmune or immune-mediated skin diseases including bullous skin diseases, erythema multiforme and contact

dermatitis, psoriasis, allergic diseases such as asthma, allergic rhinitis, atopic dermatitis, food hypersensitivity and urticaria, immunologic diseases of the lung such as eosinophilic pneumonias, idiopathic pulmonary fibrosis and hypersensitivity pneumonitis, transplantation associated diseases including graft rejection and graft-versus-host-disease. Infectious diseases include AIDS (HIV infection), hepatitis A, B, C, D, and E, bacterial infections, fungal infections, protozoal infections and parasitic infections.

A "B cell malignancy" is a malignancy involving B cells. Examples include Hodgkin's disease, including lymphocyte predominant Hodgkin's disease (LPHD); non-Hodgkin's lymphoma (NHL); follicular center cell (FCC) lymphoma; acute lymphocytic leukemia (ALL); chronic lymphocytic leukemia (CLL); hairy cell leukemia; plasmacytoid lymphocytic lymphoma; mantle cell lymphoma; AIDS or HIV-related lymphoma; multiple myeloma; central nervous system (CNS) lymphoma; post-transplant lymphoproliferative disorder (PTLD); Waldenstrom's macroglobulinemia (lymphoplasmacytic lymphoma); mucosa-associated lymphoid tissue (MALT) lymphoma; and marginal zone lymphoma/leukemia.

Non-Hodgkin's lymphoma (NHL) includes, but is not limited to, low grade/follicular NHL, relapsed or refractory NHL, front line low grade NHL, Stage III/IV NHL, chemotherapy resistant NHL, small lymphocytic (SL) NHL, intermediate grade/follicular NHL, intermediate grade diffuse NHL, diffuse large cell lymphoma, aggressive NHL (including aggressive front-line NHL and aggressive relapsed NHL), NHL relapsing after or refractory to autologous stem cell transplantation, high grade immunoblastic NHL, high grade lymphoblastic NHL, high grade small non-cleaved cell NHL, bulky disease NHL, etc.

An "autoimmune disease" herein is a disease or disorder arising from and directed against an individual's own tissues or a co-segregate or manifestation thereof or resulting condition therefrom. Examples of autoimmune diseases or disorders include, but are not limited to arthritis (rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis, and ankylosing spondylitis), psoriasis, dermatitis including atopic dermatitis; chronic idiopathic urticaria, including chronic autoimmune urticaria, polymyositis/dermatomyositis, toxic epidermal necrolysis, systemic scleroderma and sclerosis, responses associated with inflammatory bowel disease (IBD) (Crohn's disease, ulcerative colitis), and IBD with co-segregate of pyoderma gangrenosum, erythema nodosum, primary sclerosing cholangitis, and/or episcleritis), respiratory distress syndrome, including adult respiratory distress syndrome (ARDS), meningitis, IgE-mediated diseases such as anaphylaxis and allergic rhinitis, encephalitis such as Rasmussen's encephalitis, uveitis, colitis such as microscopic colitis and collagenous colitis, glomerulonephritis (GN) such as membranous GN, idiopathic membranous GN, membranous proliferative GN (MPGN), including Type I and Type II, and rapidly progressive GN, allergic

conditions, eczema, asthma, conditions involving infiltration of T cells and chronic inflammatory responses, atherosclerosis, autoimmune myocarditis, leukocyte adhesion deficiency, systemic lupus erythematosus (SLE) such as cutaneous SLE, lupus (including nephritis, cerebritis, pediatric, non-renal, discoid, alopecia), juvenile onset diabetes, multiple sclerosis (MS) such as spino-optical MS, allergic encephalomyelitis, immune responses associated with acute and delayed hypersensitivity mediated by cytokines and T-lymphocytes, tuberculosis, sarcoidosis, granulomatosis including Wegener's granulomatosis, agranulocytosis, vasculitis (including Large Vessel vasculitis (including Polymyalgia Rheumatica and Giant Cell (Takayasu's) Arteritis), Medium Vessel vasculitis (including Kawasaki's Disease and Polyarteritis Nodosa), CNS vasculitis, and ANCA-associated vasculitis , such as Churg-Strauss vasculitis or syndrome (CSS)), aplastic anemia, Coombs positive anemia, Diamond Blackfan anemia, immune hemolytic anemia including autoimmune hemolytic anemia (AIHA), pernicious anemia, pure red cell aplasia (PRCA), Factor VIII deficiency, hemophilia A, autoimmune neutropenia, pancytopenia, leukopenia, diseases involving leukocyte diapedesis, CNS inflammatory disorders, multiple organ injury syndrome, myasthenia gravis, antigen-antibody complex mediated diseases, anti-glomerular basement membrane disease, anti-phospholipid antibody syndrome, allergic neuritis, Bechet disease, Castleman's syndrome, Goodpasture's Syndrome, Lambert-Eaton Myasthenic Syndrome, Reynaud's syndrome, Sjorgen's syndrome, Stevens-Johnson syndrome, solid organ transplant rejection (including pretreatment for high panel reactive antibody titers, IgA deposit in tissues, and rejection arising from renal transplantation, liver transplantation, intestinal transplantation, cardiac transplantation, etc.), graft versus host disease (GVHD), pemphigoid bullous, pemphigus (including vulgaris, foliaceus, and pemphigus mucus-membrane pemphigoid), autoimmune polyendocrinopathies, Reiter's disease, stiff-man syndrome, immune complex nephritis, IgM polyneuropathies or IgM mediated neuropathy, idiopathic thrombocytopenic purpura (ITP), thrombotic throbocytopenic purpura (TTP), thrombocytopenia (as developed by myocardial infarction patients, for example), including autoimmune thrombocytopenia, autoimmune disease of the testis and ovary including autoimune orchitis and oophoritis, primary hypothyroidism; autoimmune endocrine diseases including autoimmune thyroiditis, chronic thyroiditis (Hashimoto's Thyroiditis), subacute thyroiditis, idiopathic hypothyroidism, Addison's disease, Grave's disease, autoimmune polyglandular syndromes (or polyglandular endocrinopathy syndromes), Type I diabetes also referred to as insulin-dependent diabetes mellitus (IDDM), including pediatric IDDM, and Sheehan's syndrome; autoimmune hepatitis, Lymphoid interstitial pneumonitis (HIV), bronchiolitis obliterans (non-transplant) vs NSIP, Guillain-Barré Syndrome, Berger's Disease (IgA nephropathy), primary biliary cirrhosis, celiac sprue (gluten enteropathy), refractory sprue with co-segregate dermatitis herpetiformis, cryoglobulinemia, amylotrophic lateral sclerosis (ALS; Lou

Gehrig's disease), coronary artery disease, autoimmune inner ear disease (AIED), autoimmune hearing loss, opsoclonus myoclonus syndrome (OMS), polychondritis such as refractory polychondritis, pulmonary alveolar proteinosis, amyloidosis, giant cell hepatitis, scleritis, monoclonal gammopathy of uncertain/unknown significance (MGUS), peripheral neuropathy, paraneoplastic syndrome, channelopathies such as epilepsy, migraine, arrhythmia, muscular disorders, deafness, blindness, periodic paralysis, and channelopathies of the CNS; autism, inflammatory myopathy, and focal segmental glomerulosclerosis (FSGS).

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to cancer cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, *e.g.*, Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella *et al.*, "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt *et al.*, (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, beta-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described below.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (*e.g.* $At^{211}$, $I^{131}$, $I^{125}$, $Y^{90}$, $Re^{186}$, $Re^{188}$, $Sm^{153}$, $Bi^{212}$, $P^{32}$ and radioactive isotopes of Lu), chemotherapeutic agents, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof.

A "chemotherapeutic agent" is A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and CYTOXAN® cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethiylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin,

carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; antibiotics such as the enediyne antibiotics (e. g., calicheamicin, especially calicheamicin gamma1I and calicheamicin omega1I (see, e.g., Agnew, Chem Intl. Ed. Engl., 33: 183-186 (1994)); dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN® doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2- ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, OR); razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g., TAXOL® paclitaxel (Bristol- Myers Squibb Oncology, Princeton, N.J.), ABRAXANE™ Cremophor-free, albumin-engineered nanoparticle formulation of

paclitaxel (American Pharmaceutical Partners, Schaumberg, Illinois), and TAXOTERE® doxetaxel (Rhône- Poulenc Rorer, Antony, France); chloranbucil; GEMZAR® gemcitabine; 6- thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; NAVELBINE® vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluorometlhylornithine (DMFO); retinoids such as retinoic acid; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen (including NOLVADEX® tamoxifen), raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and FARESTON· toremifene; aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles, aminoglutethimide, MEGASE® megestrol acetate, AROMASIN® exemestane, formestanie, fadrozole, RIVISOR® vorozole, FEMARA® letrozole, and ARIMIDEX® anastrozole; and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; as well as troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); antisense oligonucleotides, particularly those which inhibit expression of genes in signaling pathways implicated in abherant cell proliferation, such as, for example, PKC-alpha, Ralf and H-Ras; ribozymes such as a VEGF expression inhibitor (e.g., ANGIOZYME® ribozyme) and a HER2 expression inhibitor; vaccines such as gene therapy vaccines, for example, ALLOVECTIN® vaccine, LEUVECTIN® vaccine, and VAXID® vaccine; PROLEUKIN® rIL-2; LURTOTECAN® topoisomerase 1 inhibitor; ABARELIX® rmRH; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, either *in vitro* or *in vivo*. Thus, the growth inhibitory agent is one which significantly reduces the percentage of cells overexpressing such genes in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), taxol, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogens, and antineoplastic

drugs" by Murakami *et al.* (WB Saunders: Philadelphia, 1995), especially p. 13.

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone 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; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-α and -β; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors; platelet-growth factor; transforming growth factors (TGFs) such as TGF-α and TGF-β; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α, -β, and -gamma; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

A "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications, other therapeutic products to be combined with the packaged product, and/or warnings concerning the use of such therapeutic products, *etc.*

The terms "treating", "treatment" and "therapy" as used herein refer to curative therapy, prophylactic therapy, and preventative therapy.

The term "mammal" as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats. In a preferred embodiment of the invention, the mammal is a human.

**II. Compositions and Methods of the Invention**

A cytokine related to the TNF ligand family, the cytokine identified herein as "Apo-2 ligand" or "TRAIL" has been described. The predicted mature amino acid sequence of native human Apo-2 ligand contains 281 amino acids, and has a calculated molecular weight of approximately 32.5 kDa. The absence of a signal sequence and the presence of an internal hydrophobic region suggest that Apo-2 ligand is a type II transmembrane protein. Soluble extracellular domain Apo-2 ligand polypeptides have also

been described. See, e.g., WO97/25428 published July 17, 1997. Apo-2L substitutional variants have further been described. Alanine scanning techniques have been utilized to identify various substitutional variant molecules having biological activity. Particular substitutional variants of the Apo-2 ligand include those in which at least one amino acid is substituted by another amino acids such as an alanine residue. These substitutional variants are identified, for example, as "D203A"; "D218A" and "D269A." This nomenclature is used to identify Apo-2 ligand variants wherein the aspartic acid residues at positions 203, 218, and/or 269 (using the numbering shown in Figure 1) are substituted by alanine residues. Optionally, the Apo-2L variants of the present invention may comprise one or more of the amino acid substitutions. Optionally, such Apo-2L variants will be DR4 or DR5 receptor selective variants.

The description below relates to methods of producing Apo-2 ligand, including Apo-2 ligand variants, by culturing host cells transformed or transfected with a vector containing Apo-2 ligand encoding nucleic acid and recovering the polypeptide from the cell culture.

The DNA encoding Apo-2 ligand may be obtained from any cDNA library prepared from tissue believed to possess the Apo-2 ligand mRNA and to express it at a detectable level. Accordingly, human Apo-2 ligand DNA can be conveniently obtained from a cDNA library prepared from human tissues, such as the bacteriophage library of human placental cDNA as described in WO97/25428. The Apo-2 ligand-encoding gene may also be obtained from a genomic library or by oligonucleotide synthesis.

Libraries can be screened with probes (such as antibodies to the Apo-2 ligand or oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as described in Sambrook et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding Apo-2 ligand is to use PCR methodology [Sambrook et al., supra; Dieffenbach et al., PCR Primer:A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1995)].

Amino acid sequence fragments or variants of Apo-2 ligand can be prepared by introducing appropriate nucleotide changes into the Apo-2 ligand DNA, or by synthesis of the desired Apo-2 ligand polypeptide. Such fragments or variants represent insertions, substitutions, and/or deletions of residues within or at one or both of the ends of the intracellular region, the transmembrane region, or the extracellular region, or of the amino acid sequence shown for the full-length Apo-2 ligand in Figure 1. Any combination of insertion, substitution, and/or deletion can be made to arrive at the final construct, provided that the final construct possesses, for instance, a desired biological activity, such as apoptotic activity, as defined herein. In a preferred embodiment, the fragments or variants have

at least about 80% amino acid sequence identity, more preferably, at least about 90% sequence identity, and even more preferably, at least 95%, 96%, 97%, 98% or 99% sequence identity with the sequences identified herein for the intracellular, transmembrane, or extracellular domains of Apo-2 ligand, or the full-length sequence for Apo-2 ligand. The amino acid changes also may alter post-translational processes of the Apo-2 ligand, such as changing the number or position of glycosylation sites or altering the membrane anchoring characteristics.

Variations in the Apo-2 ligand sequence as described above can be made using any of the techniques and guidelines for conservative and non-conservative mutations set forth in U.S. Pat. No. 5,364,934. These include oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis.

Scanning amino acid analysis can be employed to identify one or more amino acids along a contiguous sequence. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine and cysteine. Alanine is typically a preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant. [Cunningham et al., Science, 244:1081 (1989)]. Alanine is also typically preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions [Creighton, The Proteins, (W.H. Freeman & Co., NY); Chothia, J. Mol. Biol., 150:1 (1976)].

Amino acids may be grouped according to similarities in the properties of their side chains (in A. L. Lehninger, in Biochemistry, second ed., pp. 73-75, Worth Publishers, New York (1975)):

(1) non-polar: Ala (A), Val (V), Leu (L), Ile (I), Pro (P), Phe (F), Trp (W), Met (M)

(2) uncharged polar: Gly (G), Ser (S), Thr (T), Cys (C), Tyr (Y), Asn (N), Gln (Q)

(3) acidic: Asp (D), Glu (E)

(4) basic: Lys (K), Arg (R), His(H)

Alternatively, naturally occurring residues may be divided into groups based on common side-chain properties:

(1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;

(2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

(5) residues that influence chain orientation: Gly, Pro;

(6) aromatic: Trp, Tyr, Phe.

Table 1

| Original Residue | Exemplary Substitutions | Preferred Substitutions |
|---|---|---|
| Ala (A) | Val; Leu; Ile | Val |
| Arg (R) | Lys; Gln; Asn | Lys |
| Asn (N) | Gln; His; Asp, Lys; Arg | Gln |
| Asp (D) | Glu; Asn | Glu |
| Cys (C) | Ser; Ala | Ser |
| Gln (Q) | Asn; Glu | Asn |
| Glu (E) | Asp; Gln | Asp |
| Gly (G) | Ala | Ala |
| His (H) | Asn; Gln; Lys; Arg | Arg |
| Ile (I) | Leu; Val; Met; Ala; Phe; Norleucine | Leu |
| Leu (L) | Norleucine; Ile; Val; Met; Ala; Phe | Ile |
| Lys (K) | Arg; Gln; Asn | Arg |
| Met (M) | Leu; Phe; Ile | Leu |
| Phe (F) | Trp; Leu; Val; Ile; Ala; Tyr | Tyr |
| Pro (P) | Ala | Ala |
| Ser (S) | Thr | Thr |
| Thr (T) | Val; Ser | Ser |
| Trp (W) | Tyr; Phe | Tyr |
| Tyr (Y) | Trp; Phe; Thr; Ser | Phe |
| Val (V) | Ile; Leu; Met; Phe; Ala; Norleucine | Leu |

Variations in the Apo-2 ligand sequence also included within the scope of the invention relate to amino-terminal derivatives or modified forms. Such Apo-2 ligand sequences include any of the Apo-2 ligand polypeptides described herein having a methionine or modified methionine (such as formyl methionyl or other blocked methionyl species) at the N-terminus of the polypeptide sequence.

The nucleic acid (e.g., cDNA or genomic DNA) encoding native or variant Apo-2 ligand may be inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Various vectors are publicly available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, each of which is described below. Optional signal sequences, origins of replication, marker genes, enhancer

elements and transcription terminator sequences that may be employed are known in the art and described in further detail in WO97/25428.

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the Apo-2 ligand nucleic acid sequence. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence, such as the Apo-2 ligand nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to Apo-2 ligand encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native Apo-2 ligand promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the Apo-2 ligand DNA.

Promoters suitable for use with prokaryotic and eukaryotic hosts are known in the art, and are described in further detail in WO97/25428.

A preferred method for the production of soluble Apo-2L in *E. coli* employs an inducible promoter for the regulation of product expression. The use of a controllable, inducible promoter allows for culture growth to the desirable cell density before induction of product expression and accumulation of significant amounts of product which may not be well tolerated by the host.

Several inducible promoter systems (T7 polymerase, trp and alkaline phosphatase (AP)) have been evaluated by Applicants for the expression of Apo-2L (form 114-281). The use of each of these three promoters resulted in significant amounts of soluble, biologically active Apo-2L trimer being recovered from the harvested cell paste. The AP promoter is preferred among these three inducible promoter systems tested because of tighter promoter control and the higher cell density and titers reached in harvested cell paste.

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures can be used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are

prepared, analyzed by restriction endonuclease digestion, and/or sequenced using standard techniques known in the art. [See, e.g., Messing et al., Nucleic Acids Res., 9:309 (1981); Maxam et al., Methods in Enzymology, 65:499 (1980)].

Expression vectors that provide for the transient expression in mammalian cells of DNA encoding Apo-2 ligand may be employed. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector [Sambrook et al., supra]. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of Apo-2 ligand that are biologically active Apo-2 ligand.

Other methods, vectors, and host cells suitable for adaptation to the synthesis of Apo-2 ligand in recombinant vertebrate cell culture are described in Gething et al., Nature, 293:620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117,058.

Suitable host cells for cloning or expressing the DNA in the vectors herein include prokaryote, yeast, or higher eukaryote cells. Suitable prokaryotes for this purpose include but are not limited to eubacteria, such as Gram-negative or Gram-positive organisms, for example, *Enterobacteriaceae* such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for Apo-2 ligand-encoding vectors. Suitable host cells for the expression of glycosylated Apo-2 ligand are derived from multicellular organisms. Examples of all such host cells, including CHO cells, are described further in WO97/25428.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors for Apo-2 ligand production and cultured in nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed.

Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with Agrobacterium tumefaciens is used for transformation of certain plant cells, as described by Shaw et al., Gene, 23:315 (1983) and WO 89/05859 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published 10 January 1991.

For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology, 52:456-457 (1978) may be employed. General aspects of mammalian cell host system transformations have been described in U.S. Pat. No. 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen et al., J. Bact., 130:946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g., polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown et al., Methods in Enzymology, 185:527-537 (1990) and Mansour et al., Nature, 336:348-352 (1988).

Prokaryotic cells used to produce Apo-2 ligand may be cultured in suitable culture media as described generally in Sambrook et al., supra. Particular forms of culture media that may be employed for culturing *E. coli* are described further in the Examples below. Mammalian host cells used to produce Apo-2 ligand may be cultured in a variety of culture media.

Examples of commercially available culture media include Ham's F10 (Sigma), Minimal Essential Medium ("MEM", Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ("DMEM", Sigma). Any such media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled

in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: A Practical Approach, M. Butler, ed. (IRL Press, 1991).

In accordance with one aspect of the present invention, one or more divalent metal ions will typically be added to or included in the culture media for culturing or fermenting the host cells. The divalent metal ions are preferably present in or added to the culture media at a concentration level sufficient to enhance storage stability, enhance solubility, or assist in forming stable Apo-2L trimers coordinated by one or more zinc ions. The amount of divalent metal ions which may be added will be dependent, in part, on the host cell density in the culture or potential host cell sensitivity to such divalent metal ions. At higher host cell densities in the culture, it may be beneficial to increase the concentration of divalent metal ions. If the divalent metal ions are added during or after product expression by the host cells, it may be desirable to adjust or increase the divalent metal ion concentration as product expression by the host cells increases. It is generally believed that trace levels of divalent metal ions which may be present in typical commonly available cell culture media may not be sufficient for stable trimer formation. Thus, addition of further quantities of divalent metal ions, as described herein, is preferred.

The divalent metal ions are preferably added to the culture media at a concentration which does not adversely or negatively affect host cell growth, if the divalent metal ions are being added during the growth phase of the host cells in the culture. In shake flask cultures, it was observed that $ZnSO_4$ added at concentrations of greater than 1 mM can result in lower host cell density. Those skilled in the art appreciate that bacterial cells can sequester metal ions effectively by forming metal ion complexes with cellular matrices. Thus, in the cell cultures, it is preferable to add the selected divalent metal ions to the culture media after the growth phase (after the desired host cell density is achieved) or just prior to product expression by the host cells. To ensure that sufficient amounts of divalent metal ions are present, additional divalent metal ions may be added or fed to the cell culture media during the product expression phase.

The divalent metal ion concentration in the culture media should not exceed the concentration which may be detrimental or toxic to the host cells. In the methods of the invention employing the host cell, *E. coli*, it is preferred that the concentration of the divalent metal ion concentration in the culture media does not exceed about 1mM (preferably, $\leq$ 1mM). Even more preferably, the divalent metal ion concentration in the culture media is about 50 micromolar to about 250 micromolar. Most

preferably, the divalent metal ion used in such methods is zinc sulfate. It is desirable to add the divalent metal ions to the cell culture in an amount wherein the metal ions and Apo-2 ligand trimer can be present at a one to one molar ratio.

The divalent metal ions can be added to the cell culture in any acceptable form. For instance, a solution of the metal ion can be made using water, and the divalent metal ion solution can then be added or fed to the culture media.

Expression of the Apo-2L may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis), or in situ hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, and particularly $^{32}P$. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionucleotides, fluorescers or enzymes. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected. Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like.

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native Apo-2 ligand polypeptide or against a synthetic peptide based on the DNA sequences provided herein or against exogenous sequence fused to Apo-2 ligand DNA and encoding a specific antibody epitope.

Apo-2 ligand preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates when directly produced without a secretory signal. If the Apo-2 ligand is membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g. Triton-X 100) or its extracellular region may be released by enzymatic cleavage.

When Apo-2 ligand is produced in a recombinant cell other than one of human origin, the Apo-2 ligand is free of proteins or polypeptides of human origin. However, it is usually necessary to recover or purify Apo-2 ligand from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to Apo-2 ligand. As a first step, the culture medium or lysate may be centrifuged to remove particulate cell debris. Apo-2 ligand thereafter is purified from contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE or CM; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; diafiltration and protein A Sepharose columns to remove contaminants such as IgG.

In a preferred embodiment, the Apo-2 ligand can be isolated by affinity chromatography. Apo-2 ligand fragments or variants in which residues have been deleted, inserted, or substituted are recovered in the same fashion as native Apo-2 ligand, taking account of any substantial changes in properties occasioned by the variation. For example, preparation of an Apo-2 ligand fusion with another protein or polypeptide, e.g., a bacterial or viral antigen, facilitates purification; an immunoaffinity column containing antibody to the antigen can be used to adsorb the fusion polypeptide.

A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native Apo-2 ligand may require modification to account for changes in the character of Apo-2 ligand or its variants upon expression in recombinant cell culture.

During any such purification steps, it may be desirable to expose the recovered Apo-2L to a divalent metal ion-containing solution or to purification material (such as a chromatography medium or support) containing one or more divalent metal ions. In a preferred embodiment, the divalent metal ions and/or reducing agent is used during recovery or purification of the Apo-2L. Optionally, both divalent metal ions and reducing agent, such as DTT or BME, may be used during recovery or purification of the Apo-2L. It is believed that use of divalent metal ions during recovery or purification will provide for stability of Apo-2L trimer or preserve Apo-2L trimer formed during the cell culturing step.

The description below also relates to methods of producing Apo-2 ligand covalently attached (hereinafter "conjugated") to one or more chemical groups. Chemical groups suitable for use in an Apo-2L conjugate of the present invention are preferably not significantly toxic or immunogenic. The chemical group is optionally selected to produce an Apo-

2L conjugate that can be stored and used under conditions suitable for storage. A variety of exemplary chemical groups that can be conjugated to polypeptides are known in the art and include for example carbohydrates, such as those carbohydrates that occur naturally on glycoproteins, polyglutamate, and non-proteinaceous polymers, such as polyols (see, e.g., U.S. Patent No. 6,245,901).

A polyol, for example, can be conjugated to polypeptides such as an Apo-2L at one or more amino acid residues, including lysine residues, as is disclosed in WO 93/00109, supra. The polyol employed can be any water-soluble poly(alkylene oxide) polymer and can have a linear or branched chain. Suitable polyols include those substituted at one or more hydroxyl positions with a chemical group, such as an alkyl group having between one and four carbons. Typically, the polyol is a poly(alkylene glycol), such as poly(ethylene glycol) (PEG), and thus, for ease of description, the remainder of the discussion relates to an exemplary embodiment wherein the polyol employed is PEG and the process of conjugating the polyol to a polypeptide is termed "pegylation." However, those skilled in the art recognize that other polyols, such as, for example, poly(propylene glycol) and polyethylene-polypropylene glycol copolymers, can be employed using the techniques for conjugation described herein for PEG.

The average molecular weight of the PEG employed in the pegylation of the Apo-2L can vary, and typically may range from about 500 to about 30,000 daltons (D). Preferably, the average molecular weight of the PEG is from about 1,000 to about 25,000 D, and more preferably from about 1,000 to about 5,000 D. In one embodiment, pegylation is carried out with PEG having an average molecular weight of about 1,000 D. Optionally, the PEG homopolymer is unsubstituted, but it may also be substituted at one end with an alkyl group. Preferably, the alkyl group is a C1-C4 alkyl group, and most preferably a methyl group. PEG preparations are commercially available, and typically, those PEG preparations suitable for use in the present invention are nonhomogeneous preparations sold according to average molecular weight. For example, commercially available PEG(5000) preparations typically contain molecules that vary slightly in molecular weight, usually ± 500 D.

The Apo-2 ligand of the invention may be in various forms, such as in monomer form or trimer form (comprising three monomers). Optionally, an Apo-2L trimer will be pegylated in a manner such that a PEG molecule is linked or conjugated to one, two or each of the three monomers that make up the trimeric Apo-2L. In such an embodiment, it is preferred that the PEG employed have an average molecular weight of about 1,000 to about 5,000 D. It is also contemplated that the Apo-2L trimers may be "partially" pegylated, i.e., wherein only one or two of the three monomers that make up the trimer are linked or conjugated to PEG.

A variety of methods for pegylating proteins are known in the art. Specific methods of producing proteins conjugated to PEG include the methods described in U.S. Pat. No. 4,179,337, U.S. Pat. No. 4,935,465 and U.S. Patent No. 5,849,535. Typically the protein is covalently bonded via one or more of the amino acid residues of the protein to a terminal reactive group on the polymer, depending mainly on the reaction conditions, the molecular weight of the polymer, etc. The polymer with the reactive group(s) is designated herein as activated polymer. The reactive group selectively reacts with free amino or other reactive groups on the protein. The PEG polymer can be coupled to the amino or other reactive group on the protein in either a random or a site specific manner. It will be understood, however, that the type and amount of the reactive group chosen, as well as the type of polymer employed, to obtain optimum results, will depend on the particular protein or protein variant employed to avoid having the reactive group react with too many particularly active groups on the protein. As this may not be possible to avoid completely, it is recommended that generally from about 0.1 to 1000 moles, preferably 2 to 200 moles, of activated polymer per mole of protein, depending on protein concentration, is employed. The final amount of activated polymer per mole of protein is a balance to maintain optimum activity, while at the same time optimizing, if possible, the circulatory half-life of the protein.

It is further contemplated that the Apo2L described herein may be also be linked or fused to leucine zipper sequences using techniques known in the art.

**********

Methods for generating death receptor antibodies and CD20 antibodies are also described herein. The antigen to be used for production of, or screening for, antibody may be, *e.g.*, a soluble form of the antigen or a portion thereof, containing the desired epitope. Alternatively, or additionally, cells expressing the antigen at their cell surface can be used to generate, or screen for, antibody. Other forms of the antigen useful for generating antibody will be apparent to those skilled in the art.

*(i) Polyclonal antibodies*

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues),

glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N=C=NR$, where R and $R^1$ are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, *e.g.*, 100 µg or 5 µg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

*(ii) Monoclonal antibodies*

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al.*, *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those

derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Manassas, Virginia USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson *et al.*, *Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra *et al.*, *Curr. Opinion in Immunol.*, 5:256-262 (1993) and Plückthun, *Immunol. Revs.*, 130:151-188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty *et al.*, *Nature*, 348:552-554 (1990). Clackson *et*

*al.*, *Nature*, 352:624-628 (1991) and Marks *et al.*, *J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks *et al.*, *Bio/Technology*, 10:779-783 (1992)), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse *et al.*, *Nuc. Acids. Res.*, 21:2265-2266 (1993). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4,816,567; Morrison, *et al.*, *Proc. Natl Acad. Sci. USA*, 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

*(iii) Humanized antibodies*

Methods for humanizing non-human antibodies have been described in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al.*, *Nature*, 321:522-525 (1986); Riechmann *et al.*, *Nature*, 332:323-327 (1988); Verhoeyen *et al.*, *Science*, 239:1534-1536 (1988)), by substituting hypervariable region sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some hypervariable region residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which

is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims *et al.*, *J. Immunol.*, 151:2296 (1993); Chothia *et al.*, *J. Mol. Biol.*, 196:901 (1987)). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta *et al.*, *J. Immunol.*, 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the hypervariable region residues are directly and most substantially involved in influencing antigen binding.

(iv) Human antibodies

As an alternative to humanization, human antibodies can be generated. For example, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region ($J_H$) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551 (1993); Jakobovits et al., Nature, 362:255-258 (1993); Bruggermann et al., Year in Immuno., 7:33 (1993); and US Patent Nos. 5,591,669, 5,589,369 and 5,545,807.

Alternatively, phage display technology (McCafferty et al., Nature 348:552-553 (1990)) can be used to produce human antibodies and antibody fragments in vitro, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody

V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B cell. Phage display can be performed in a variety of formats; for their review see, e.g., Johnson, Kevin S. and Chiswell, David J., Current Opinion in Structural Biology 3:564-571 (1993). Several sources of V-gene segments can be used for phage display. Clackson et al., Nature, 352:624-628 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks et al., J. Mol. Biol. 222:581-597 (1991), or Griffith et al., EMBO J. 12:725-734 (1993). See, also, US Patent Nos. 5,565,332 and 5,573,905.

Human antibodies may also be generated by in vitro activated B cells (see US Patents 5,567,610 and 5,229,275).

(v) Antibody fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., Journal of Biochemical and Biophysical Methods 24:107-117 (1992) and Brennan et al., Science, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from E. coli and chemically coupled to form $F(ab')_2$ fragments (Carter et al., Bio/Technology 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185; US Patent No. 5,571,894; and US Patent No. 5,587,458. The antibody fragment may also be a "linear antibody", e.g., as described in US Patent 5,641,870 for example. Such linear antibody fragments may be monospecific or bispecific.

(vi) Bispecific antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the CD20, DR4 or DR5 receptors. Bispecific antibodies may also be used to localize cytotoxic agents to a B cell. These antibodies possess a B cell marker-binding arm and an arm which

binds the cytotoxic agent (e.g. saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., Nature, 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986). According to another approach described in US Patent No. 5,731,168, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers

which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the $C_H3$ domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (US Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., Science, 229: 81 (1985); Shalaby et al., J. Exp. Med., 175: 217-225 (1992).

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., J. Immunol., 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., Proc. Natl. Acad. Sci. USA, 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain ($V_H$) connected to a light-chain variable domain ($V_L$) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the $V_H$ and $V_L$ domains of one fragment are forced to pair with the complementary $V_L$ and $V_H$ domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., J. Immunol., 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. J. Immunol.

147: 60 (1991). Antibodies with three or more antigen binding sites are described in WO01/77342 (Miller and Presta), expressly incorporated herein by reference.

The antibody used in the methods or included in the articles of manufacture herein is optionally conjugated to a cytotoxic agent.

Chemotherapeutic agents useful in the generation of such antibody-cytotoxic agent conjugates have been described above.

Conjugates of an antibody and one or more small molecule toxins, such as a calicheamicin, a maytansine (US Patent No. 5,208,020), a trichothene, and CC1065 are also contemplated herein. In one embodiment of the invention, the antibody is conjugated to one or more maytansine molecules (e.g. about 1 to about 10 maytansine molecules per antibody molecule). Maytansine may, for example, be converted to May-SS-Me which may be reduced to May-SH3 and reacted with modified antibody (Chari et al. Cancer Research 52: 127-131 (1992)) to generate a maytansinoid-antibody conjugate.

Alternatively, the antibody is conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics is capable of producing double-stranded DNA breaks at sub-picomolar concentrations. Structural analogues of calicheamicin which may be used include, but are not limited to, $\gamma_1^I$, $\alpha_2^I$, $\alpha_3^I$, N-acetyl-$\gamma_1^I$, PSAG and $\theta^I_1$ (Hinman et al. Cancer Research 53: 3336-3342 (1993) and Lode et al. Cancer Research 58: 2925-2928 (1998)).

Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. See, for example, WO 93/21232 published October 28, 1993.

The present invention further contemplates antibody conjugated with a compound with nucleolytic activity (e.g. a ribonuclease or a DNA endonuclease such as a deoxyribonuclease; DNase).

A variety of radioactive isotopes are available for the production of radioconjugated antagonists or antibodies. Examples include $At^{211}$, $I^{131}$, $I^{125}$, $Y^{90}$, $Re^{186}$, $Re^{188}$, $Sm^{153}$, $Bi^{212}$, $P^{32}$ and radioactive isotopes of Lu.

Conjugates of the antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine

compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. Science 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antagonist or antibody. See WO94/11026. The linker may be a "cleavable linker" facilitating release of the cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, dimethyl linker or disulfide-containing linker (Chari et al. Cancer Research 52: 127-131 (1992)) may be used.

Alternatively, a fusion protein comprising the antibody and cytotoxic agent may be made, e.g. by recombinant techniques or peptide synthesis.

The antibodies of the present invention may also be conjugated with a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Patent No. 4,975,278.

The enzyme component of such conjugates includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, *e.g.*, Massey, *Nature* 328: 457-458 (1987)). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the antibody by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well

known in the art (see, *e.g.*, Neuberger *et al.*, *Nature*, 312: 604-608 (1984)).

Other modifications of the antibody are contemplated herein. For example, the antibody may be linked to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, polyoxyalkylenes, or copolymers of polyethylene glycol and polypropylene glycol.

To increase the serum half life of the antibody, one may incorporate a salvage receptor binding epitope into the antibody (especially an antibody fragment) as described in US Patent 5,739,277, for example. As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (*e.g.*, $IgG_1$, $IgG_2$, $IgG_3$, or $IgG_4$) that is responsible for increasing the *in vivo* serum half-life of the IgG molecule. Alternatively, or additionally, one may increase, or decrease, serum half-life by altering the amino acid sequence of the Fc region of an antibody to generate variants with altered FcRn binding. Antibodies with altered FcRn binding and/or serum half life are described in WO00/42072 (Presta, L.).

**********

Formulations comprising Apo2L/TRAIL, death receptor antibodies, and/or CD20 antibodies are also provided by the present invention. It is believed that such formulations will be particularly suitable for storage as well as for therapeutic administration. The formulations may be prepared by known techniques. For instance, the formulations may be prepared by buffer exchange on a gel filtration column.

Typically, an appropriate amount of an acceptable salt or carrier is used in the formulation to render the formulation isotonic. Examples of pharmaceutically-acceptable carriers include saline, Ringer's solution and dextrose solution. The pH of the formulation is preferably from about 6 to about 9, and more preferably from about 7 to about 7.5. It will be apparent to those persons skilled in the art that certain carriers may be more preferable depending upon, for instance, the route of administration and concentrations of Apo-2 ligand, death receptor antibodies, and/or CD20 antibodies.

Therapeutic compositions can be prepared by mixing the desired molecules having the appropriate degree of purity with optional carriers, excipients, or stabilizers (Remington's Pharmaceutical Sciences, 16th edition, Osol, A. ed. (1980)), in the form of lyophilized formulations, aqueous solutions or aqueous suspensions. Acceptable carriers, excipients, or stabilizers are preferably nontoxic to recipients at the dosages and concentrations employed, and include buffers such as Tris, HEPES, PIPES, phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as

octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

Additional examples of such carriers include ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts, or electrolytes such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, and cellulose-based substances. Carriers for topical or gel-based forms include polysaccharides such as sodium carboxymethylcellulose or methylcellulose, polyvinylpyrrolidone, polyacrylates, polyoxyethylene-polyoxypropylene-block polymers, polyethylene glycol, and wood wax alcohols. For all administrations, conventional depot forms are suitably used. Such forms include, for example, microcapsules, nano-capsules, liposomes, plasters, inhalation forms, nose sprays, sublingual tablets, and sustained-release preparations.

Formulations to be used for *in vivo* administration should be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. The formulation may be stored in lyophilized form or in solution if administered systemically. If in lyophilized form, it is typically formulated in combination with other ingredients for reconstitution with an appropriate diluent at the time for use. An example of a liquid formulation is a sterile, clear, colorless unpreserved solution filled in a single-dose vial for subcutaneous injection.

Therapeutic formulations generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle. The formulations are preferably administered as repeated intravenous (i.v.), subcutaneous (s.c.), intramuscular (i.m.) injections or infusions, or as aerosol formulations suitable for intranasal or intrapulmonary delivery (for intrapulmonary delivery see, *e.g.*, EP 257,956).

Apo2L/TRAIL, death receptor antibodies, and CD20 antibodies can also be administered in the form of sustained-release preparations. Suitable

examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the protein, which matrices are in the form of shaped articles, *e.g.*, films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (*e.g.*, poly(2-hydroxyethyl-methacrylate) as described by Langer *et al.*, J. Biomed. Mater. Res., 15: 167-277 (1981) and Langer, Chem. Tech., 12: 98-105 (1982) or poly(vinylalcohol)), polylactides (U.S. Patent No. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman *et al.*, Biopolymers, 22: 547-556 (1983)), non-degradable ethylene-vinyl acetate (Langer *et al.*, *supra*), degradable lactic acid-glycolic acid copolymers such as the Lupron Depot (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid (EP 133,988).

The Apo2L/TRAIL, death receptor antibodies, and CD20 antibodies described herein can be employed in a variety of therapeutic applications. Among these applications are methods of treating various cancers and immune related diseases. Diagnosis in mammals of the various pathological conditions described herein can be made by the skilled practitioner. Diagnostic techniques are available in the art which allow, e.g., for the diagnosis or detection of cancer or immune related disease in a mammal. For instance, cancers may be identified through techniques, including but not limited to, palpation, blood analysis, x-ray, NMR and the like. Immune related diseases can also be readily identified. In systemic lupus erythematosus, the central mediator of disease is the production of auto-reactive antibodies to self proteins/tissues and the subsequent generation of immune-mediated inflammation. Multiple organs and systems are affected clinically including kidney, lung, musculoskeletal system, mucocutaneous, eye, central nervous system, cardiovascular system, gastrointestinal tract, bone marrow and blood. Rheumatoid arthritis (RA) is a chronic systemic autoimmune inflammatory disease that mainly involves the synovial membrane of multiple joints with resultant injury to the articular cartilage. The pathogenesis is T lymphocyte dependent and is associated with the production of rheumatoid factors, auto-antibodies directed against self IgG, with the resultant formation of immune complexes that attain high levels in joint fluid and blood. These complexes in the joint may induce the marked infiltrate of lymphocytes and monocytes into the synovium and subsequent marked synovial changes; the joint space/fluid if infiltrated by similar cells with the addition of numerous neutrophils. Tissues affected are primarily the joints, often in symmetrical pattern. However, extra-articular disease also occurs in two major forms. One form is the development of extra-articular lesions with ongoing progressive joint disease and typical lesions of pulmonary fibrosis, vasculitis, and cutaneous ulcers. The second form of extra-articular disease is the so called Felty's syndrome which occurs late in the RA disease course,

sometimes after joint disease has become quiescent, and involves the presence of neutropenia, thrombocytopenia and splenomegaly. This can be accompanied by vasculitis in multiple organs with formations of infarcts, skin ulcers and gangrene. Patients often also develop rheumatoid nodules in the subcutis tissue overlying affected joints; the nodules late stage have necrotic centers surrounded by a mixed inflammatory cell infiltrate. Other manifestations which can occur in RA include: pericarditis, pleuritis, coronary arteritis, interstitial pneumonitis with pulmonary fibrosis, keratoconjunctivitis sicca, and rheumatoid nodules.

The Apo2L/TRAIL, death receptor antibodies, and CD20 antibodies can be administered in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Optionally, administration may be performed through mini-pump infusion using various commercially available devices.

Effective dosages and schedules for administering Apo2L/TRAIL, death receptor antibodies, and CD20 antibodies may be determined empirically, and making such determinations is within the skill in the art. Single or multiple dosages may be employed. It is presently believed that an effective dosage or amount of Apo2L/TRAIL used alone may range from about 1 µg/kg to about 100 mg/kg of body weight or more per day. Interspecies scaling of dosages can be performed in a manner known in the art, e.g., as disclosed in Mordenti et al., Pharmaceut. Res., 8:1351 (1991).

When *in vivo* administration of an Apo2L/TRAIL is employed, normal dosage amounts may vary from about 10 ng/kg to up to 100 mg/kg of mammal body weight or more per day, preferably about 1 µg/kg/day to 10 mg/kg/day, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature; see, for example, U.S. Pat. Nos. 4,657,760; 5,206,344; or 5,225,212. It is anticipated that different formulations will be effective for different treatment compounds and different disorders, that administration targeting one organ or tissue, for example, may necessitate delivery in a manner different from that to another organ or tissue. Those skilled in the art will understand that the dosage of Apo2L/TRAIL that must be administered will vary depending on, for example, the mammal which will receive the Apo2L/TRAIL, the route of administration, and other drugs or therapies being administered to the mammal.

The CD20 antibody may be an antibody such as Rituximab or humanized 2H7, which is not conjugated to a cytotoxic agent. Suitable dosages for an unconjugated antibody are, for example, in the range from about 20 $mg/m^2$ to about 1000 $mg/m^2$. In one embodiment, the dosage of the antibody differs from that presently recommended for Rituximab. Exemplary dosage regimens

for the CD20 antibody include 375 mg/m2 weekly x 4 or 8; or 1000 mg x 2 (*e.g.* on days 1 and 15).

It is contemplated that yet additional therapies may be employed in the methods. The one or more other therapies may include but are not limited to, administration of radiation therapy, cytokine(s), growth inhibitory agent(s), chemotherapeutic agent(s), cytotoxic agent(s), tyrosine kinase inhibitors, ras farnesyl transferase inhibitors, angiogenesis inhibitors, and cyclin-dependent kinase inhibitors which are known in the art and defined further with particularity in Section I above.

Exemplary therapeutic antibodies include anti-HER2 antibodies including rhuMAb 4D5 (HERCEPTIN.) (Carter *et al., Proc. Natl. Acad. Sci. USA*, 89:4285-4289 (1992), U.S. Patent No. 5,725,856); anti-IL-8 (St John *et al., Chest*, 103:932 (1993), and International Publication No. WO 95/23865); anti-VEGF antibodies including humanized and/or affinity matured anti-VEGF antibodies such as the humanized anti-VEGF antibody huA4.6.1 AVASTIN. (Kim *et al., Growth Factors*, 7:53-64 (1992), International Publication No. WO 96/30046, and WO 98/45331, published October 15, 1998); anti-PSCA antibodies (WO01/40309); anti-CD40 antibodies, including S2C6 and humanized variants thereof (WO00/75348); anti-CD11a antibodies including Raptiva™ (US Patent No. 5,622,700, WO 98/23761, Steppe *et al., Transplant Intl.* 4:3-7 (1991), and Hourmant *et al., Transplantation* 58:377-380 (1994)); anti-IgE antibodies (Presta *et al., J. Immunol.* 151:2623-2632 (1993), and International Publication No. WO 95/19181;US Patent No. 5,714,338, issued February 3, 1998 or US Patent No. 5,091,313, issued February 25, 1992, WO 93/04173 published March 4, 1993, or International Application No. PCT/US98/13410 filed June 30, 1998, US Patent No. 5,714,338); anti-CD18 antibodies (US Patent No. 5,622,700, issued April 22, 1997, or as in WO 97/26912, published July 31, 1997); anti-Apo-2 receptor antibody antibodies (WO 98/51793 published November 19, 1998); anti-TNF-alpha antibodies including cA2 (REMICADE.), CDP571 and MAK-195 (See, US Patent No. 5,672,347 issued September 30, 1997, Lorenz *et al. J. Immunol.* 156(4):1646-1653 (1996), and Dhainaut *et al. Crit. Care Med.* 23(9):1461-1469 (1995)); anti-Tissue Factor (TF) antibodies (European Patent No. 0 420 937 B1 granted November 9, 1994); anti-human $\alpha_4$-$\beta_7$ integrin antibodies (WO 98/06248 published February 19, 1998); anti-EGFR antibodies (chimerized or humanized 225 antibody as in WO 96/40210 published December 19, 1996); anti-CD3 antibodies such as OKT3 (US Patent No. 4,515,893 issued May 7, 1985); anti-CD25 or anti-Tac antibodies such as CHI-621 (SIMULECT.) and ZENAPAX. (See US Patent No. 5,693,762 issued December 2, 1997); anti-CD4 antibodies such as the cM-7412 antibody (Choy *et al. Arthritis Rheum* 39(1):52-56 (1996)); anti-CD52 antibodies such as CAMPATH-1H (Riechmann *et al. Nature* 332:323-337 (1988); anti-Fc receptor antibodies such as the M22 antibody directed against Fc.RI as in Graziano *et al. J. Immunol.* 155(10):4996-5002 (1995); anti-carcinoembryonic antigen (CEA) antibodies such as hMN-14 (Sharkey *et*

*al. Cancer Res.* 55(23Suppl): 5935s-5945s (1995); antibodies directed against breast epithelial cells including huBrE-3, hu-Mc 3 and CHL6 (Ceriani *et al. Cancer Res.* 55(23): 5852s-5856s (1995); and Richman *et al. Cancer Res.* 55(23 Supp): 5916s-5920s (1995)); antibodies that bind to colon carcinoma cells such as C242 (Litton *et al. Eur J. Immunol.* 26(1):1-9 (1996)); anti-CD38 antibodies, *e.g.* AT 13/5 (Ellis *et al. J. Immunol.* 155(2):925-937 (1995)); anti-CD33 antibodies such as Hu M195 (Jurcic *et al. Cancer Res* 55(23 Suppl):5908s-5910s (1995) and CMA-676 or CDP771; anti-CD22 antibodies such as LL2 or LymphoCide (Juweid *et al. Cancer Res* 55(23 Suppl):5899s-5907s (1995); anti-EpCAM antibodies such as 17-1A (PANOREX®); anti-GpIIb/IIIa antibodies such as abciximab or c7E3 Fab (REOPRO®); anti-RSV antibodies such as MEDI-493 (SYNAGIS®); anti-CMV antibodies such as PROTOVIR®; anti-HIV antibodies such as PRO542; anti-hepatitis antibodies such as the anti-Hep B antibody OSTAVIR®; anti-CA 125 antibody OvaRex; anti-idiotypic GD3 epitope antibody BEC2; anti-αvβ3 antibody VITAXIN®; anti-human renal cell carcinoma antibody such as ch-G250; ING-1; anti-human 17-1A antibody (3622W94); anti-human colorectal tumor antibody (A33); anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma (SF-25); and anti-human leukocyte antigen (HLA) antibodies such as Smart ID10 and the anti-HLA DR antibody Oncolym (Lym-1).

Preparation and dosing schedules for chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in *Chemotherapy Service* Ed., M.C. Perry, Williams & Wilkins, Baltimore, MD (1992). The chemotherapeutic agent may precede, or follow administration of the Apo2L/TRAIL, death receptor antibody, and/or CD20 antibody, or may be given simultaneously therewith.

Sometimes, it may be beneficial to also administer one or more cytokines or growth inhibitory agent.

The Apo2L/TRAIL, death receptor antibodies, and CD20 antibodies (and one or more other therapies) may be administered concurrently or sequentially. Following administration, treated cells *in vitro* can be analyzed. Where there has been *in vivo* treatment, a treated mammal can be monitored in various ways well known to the skilled practitioner. For instance, cancer cells can be examined pathologically to assay for necrosis or serum can be analyzed for immune system responses.

For RA, and other autoimmune diseases, the Apo2L/TRAIL, death receptor antibody, and/or CD20 antibody may be combined with any one or more of the immunosuppressive agents, chemotherapeutic agents and/or cytokines listed in the definitions section above; any one or more disease-modifying antirheumatic drugs (DMARDs), such as hydroxycloroquine, sulfasalazine, methotrexate, leflunomide, azathioprine, D-penicillamine, Gold (oral), Gold (intramuscular), minocycline, cyclosporine, Staphylococcal protein A immunoadsorption; intravenous immunoglobulin

(IVIG); nonsteroidal antiinflammatory drugs (NSAIDs); glucocorticoid (*e.g.* via joint injection); corticosteroid (*e.g.* methylprednisolone and/or prednisone); folate; an anti-tumor necrosis factor (TNF) antibody, *e.g.* etanercept/ENBREL™, infliximab/REMICADE™, D2E7 (Knoll) or CDP-870 (Celltech); IL-1R antagonist (*e.g.* Kineret); IL-10 antagonist (*e.g.* Ilodecakin); a blood clotting modulator (*e.g.* WinRho); an IL-6 antagonist/anti-TNF (CBP 1011); CD40 antagonist (*e.g.* IDEC 131); Ig-Fc receptor antagonist (MDX33); immunomodulator (*e.g.* thalidomide or ImmuDyn); anti-CD5 antibody (*e.g.* H5g1.1); macrophage inhibitor (*e.g.* MDX 33); costimulatory blocker (*e.g.* BMS 188667 or Tolerimab); complement inhibitor (*e.g.* h5G1.1, 3E10 or an anti-decay accelerating factor (DAF) antibody); or IL-2 antagonist (zxSMART).

For B cell malignancies, e.g., the Apo2L/TRAIL, death receptor antibody, and/or CD20 antibody may be combined with a chemotherapeutic agent; cytokine, *e.g.* a lymphokine such as IL-2, IL-12, or an interferon, such as interferon alpha-2a; other antibody, *e.g.*, a radiolabeled antibody such as ibritumomab tiuxetan (ZEVALIN®), iodine $I^{131}$ tositumomab (BEXXAR™), $^{131}I$ Lym-1 (ONCOLYM™), $^{90}Y$-LYMPHOCIDE™; anti-CD52 antibody, such as alemtuzumab (CAMPATH-1H™), anti-HLA-DR-β antibody, such as apolizumab, anti-CD80 antibody (*e.g.* IDEC-114), epratuzumab, Hu1D10 (SMART 1D10™), CD19 antibody, CD40 antibody or CD22 antibody; an immunomodulator (*e.g.* thalidomide or ImmuDyn); an inhibitor of angiogenesis (*e.g.* an anti-vascular endothelial growth factor (VEGF) antibody such as AVASTIN™ or thalidomide); idiotype vaccine (EPOCH); ONCO-TCS™; HSPPC-96 (ONCOPHAGE™); liposomal therapy (*e.g.* daunorubicin citrate liposome), etc.

In another embodiment of the invention, articles of manufacture containing materials useful for the treatment of cancer or immune related disease, described above, are provided. In one aspect, the article of manufacture comprises (a) a container comprising CD20 antibody (preferably the container comprises the antibody and a pharmaceutically acceptable carrier or diluent within the container); (b) a container comprising Apo2L/TRAIL or death receptor antibody (preferably the container comprises the Apo2L/TRAIL or death receptor antibody and a pharmaceutically acceptable carrier or diluent within the container); and (c) a package insert with instructions for treating cancer or immune related disease in a patient, wherein the instructions indicate that amounts of the CD20 antibody and the Apo2L/TRAIL or death receptor antibody are administered to the patient that are effective to provide synergistic activity in treating the disease.

In all of these aspects, the package insert is on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, *etc.* The containers may be formed from a variety of materials such as glass or plastic. The container holds or contains a composition that is effective for treating the cancer or immune related disease and may

have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is the CD20 antibody, Apo2L/TRAIL or death receptor antibody. The label or package insert indicates that the composition is used for treating cancer or immune related disease in a patient or subject eligible for treatment with specific guidance regarding dosing amounts and intervals of antibody and any other medicament being provided. The article of manufacture may further comprise an additional container comprising a pharmaceutically acceptable diluent buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution, and/or dextrose solution. The article of manufacture may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

**EXAMPLES**

Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by way of reference to the ATCC is the American Type Culture Collection, Manassas, Virginia.

Example 1

Analysis of Apo2L/TRAIL receptor expression in B lymphoma cell lines

To examine the cell-surface expression of Apo2L/TRAIL receptors (DR4, DR5, DcR1, and DcR2) in human lymphoma cell lines, the B lymphoma cell lines Ramos, Daudi, Raji, and BJAB (ATCC) were analyzed by FACS using monoclonal antibodies specific for DR4 (mAb 4H6.17.8; ATCC HB-12455), DR5 (mAb 3H3.14.5; HB-12534), DcR1 (mAb 6G9; Genentech, Inc.), or DcR2 (mAb 1G9, Genentech, Inc.) For Ramos cells, the analysis was carried out twice to ensure reproducibility (RAMOS A and B).

As illustrated in Figure 4, DR4 and DR5 were expressed at significant levels (mean fluorescence shift of approximately 0.5 - 1.7 units) in all four of the cell lines, while DcR1 and DcR2 were expressed at lower or minimal levels (mean fluorescence shift of approximately 0 - 0.3 units).

Example 2

Analysis of CD20 expression in B lymphoma cell lines

To examine the cell-surface expression of CD20 in human lymphoma cell lines, the B lymphoma cell lines Ramos, Daudi, Raji, and BJAB (ATCC) were

analyzed by FACS using a monoclonal antibody specific for CD20 (RITUXAN®, Genentech, Inc.). For Ramos cells, the analysis was carried out twice to ensure reproducibility (RAMOS A and B).

As illustrated in Figure 5, all four cell lines expressed high levels of CD20, indicated by a mean fluorescence shift of approximately 5 - 15 units.

Example 3

Effect of Apo2L/TRAIL, RITUXAN®, or combination treatment on the growth of pre-established subcutaneous BJAB lymphoma tumor xenografts in SCID mice

SCID mice were injected subcutaneously with human B-cell non˜Hodgkin's BJAB lymphoma cells (ATCC) (20 million cells per mouse) and tumors were allowed to grow to ~200 $mm^3$. The mice were then divided into 4 study groups (8 mice per group) and treated with five intraperitoneal (IP) doses per week over 2 weeks (i.e., days 0-4 and 7-11) of vehicle (0.5M Arg-Succinate/20mM Tris/0.02% Tween 20 pH =7.2), Apo2L/TRAIL (amino acids 114-281 of Figure 1) (60 mg/kg), or with 1 IP dose per week over 2 weeks (i.e., days 0 and 7) of RITUXAN® (4 mg/kg, Genentech, Inc.), or the combination of these latter Apo2L/TRAIL and RITUXAN® regimens (Figure 6).

Tumors in vehicle-treated mice grew rapidly, while single-agent Apo2L/TRAIL or RITUXAN® treatment markedly delayed tumor growth. One mouse in the Apo2L/TRAIL group showed complete tumor ablation, leaving a tumor incidence (TI) of 7/8. RITUXAN® treatment did not ablate any tumors but showed a more prolonged effect. Importantly, combined treatment with Apo2L/TRAIL and RITUXAN® caused a dramatic reduction in tumor volume in all mice, with 5 out of 8 mice showing complete tumor ablation and 3/8 showing minimal tumor growth for at least 28 days. These results indicate that Apo2L/TRAIL and RITUXAN® can exert synergistic anti-tumor activity against lymphoma xenografts.

Example 4

Effect of Apo2L/TRAIL, RITUXAN®, or combination treatment on the growth of pre-established subcutaneous BJAB lymphoma tumor xenografts grown in SCID mice

A similar study to the one described in Example 3 was conducted. SCID mice were injected subcutaneously with human B-cell non˜Hodgkin's BJAB lymphoma cells (ATCC) (20 million cells per mouse) and tumors were allowed to grow to ~200 $mm^3$. The mice were then divided into 4 study groups (8 mice per group) and treated with five intraperitoneal (IP) doses per week over 2 weeks (i.e., days 0-4 and 7-11) of vehicle (0.5M Arg-Succinate/20mM Tris/0.02% Tween 20 pH =7.2), Apo2L/TRAIL ("Apo2L.0"; amino acids 114-281 of Fig.1) (60 mg/kg), or with 1 IP dose per week over 2 weeks (i.e., days 0 and 7) of RITUXAN® (4 mg/kg), or the combination of these latter Apo2L/TRAIL and RITUXAN® regimens.

The results are shown in Fig. 7. Tumors in vehicle-treated mice grew rapidly, while single-agent Apo2L/TRAIL or RITUXAN® treatment markedly delayed tumor growth. Neither Apo2L/TRAIL nor RITUXAN® alone caused any complete regressions, while RITUXAN® showed a more prolonged effect. As in the study described in Example 3, combined treatment with Apo2L/TRAIL and RITUXAN® caused a remarkable reduction in tumor volume in all mice, with 6 out of 7 mice showing complete tumor ablation. These results indicate that Apo2L/TRAIL and RITUXAN® can exert synergistic anti-tumor activity against lymphoma xenografts.

Example 5

Effect of Apo2L/TRAIL, RITUXAN®, or combination treatment on caspase processing in pre-established subcutaneous BJAB lymphoma tumor xenografts grown in SCID mice

To examine the processing of apoptosis-mediating caspases in treated tumors (indicated by proteolytic caspase processing), SCID mice were injected subcutaneously with human B-cell non Hodgkin's BJAB lymphoma cells (ATCC) (20 million cells per mouse) and tumors were allowed to grow to ~200 $mm^3$. The mice were then treated with vehicle (0.5M Arg-Succinate/20mM Tris/0.02% Tween 20 pH =7.2) (n=1), 1 IP dose of Apo2L/TRAIL (60 mg/kg) (n=1), or 1 IP dose of RITUXAN® (4 mg/kg, Genentech, Inc.)(n=2), or the combination of these latter Apo2L/TRAIL and RITUXAN® doses (n=2). Two days after treatment, the tumors were harvested, lysed in lysis buffer, and subjected to immunoblot with specific antibodies against Caspase 8, 3, 9, and 7 (with anti-beta actin antibody as a loading control) to visualize caspase processing (Figure 8).

Apo2L/TRAIL treatment (A) induced increased processing of caspase 8, 3, 9, and 7 as compared to the vehicle control (V), while RITUXAN® (R) did not induce caspase processing. Notably, combination treatment with Apo2L/TRAIL and RITUXAN® (AR) did not further increase caspase processing as compared to Apo2L/TRAIL alone. These results suggest that the synergistic anti-tumor activity between Apo2L/TRAIL and RITUXAN® is not necessarily mediated by enhancement of apoptosis, suggesting that combination of apoptosis activation mediated by Apo2L/TRAIL and complement-dependent lysis together with ADCC mediated by RITUXAN® may underlie the observed anti-tumor synergy.

Example 6

Effect of agonistic DR5 antibody, RITUXAN®, or combination treatment on the growth of pre-established subcutaneous BJAB lymphoma tumor xenografts in SCID mice

SCID mice were injected subcutaneously with human B-cell non-Hodgkin's BJAB lymphoma cells (ATCC) (20 million cells per mouse) and tumors were allowed to grow to ~200 $mm^3$. The mice were then divided into 4

study groups (7 mice per group) and treated with one intraperitoneal (IP) injection per week over 2 weeks (i.e., days 0 and 7) of vehicle (0.5M Arg-Succinate/20mM Tris/0.02% Tween 20 pH =7.2), agonist DR5 monoclonal antibody ("Apomab")(10 mg/kg), or RITUXAN® (4 mg/kg), or the combination of these latter DR5 antibody and RITUXAN® regimens (Figure 9). Tumors in vehicle-treated mice grew rapidly, while single-agent DR5 antibody or RITUXAN® treatment markedly delayed tumor growth. Importantly, combined treatment with DR5 antibody and RITUXAN® caused a dramatic reduction in tumor volume in all mice, with 5 out of 7 mice showing complete tumor ablation and 2/7 showing minimal tumor growth for at least 35 days. These results indicate that agonist DR5 antibody and RITUXAN® can exert synergistic anti-tumor activity against lymphoma xenografts.

Example 7

Effect of agonistic DR5 antibody, RITUXAN®, or combination treatment on caspase processing in pre-established subcutaneous BJAB lymphoma tumor xenografts grown in SCID mice

To examine the processing of apoptosis-mediating caspases in treated tumors (indicated by proteolytic caspase processing), SCID mice were injected subcutaneously with human B-cell non-Hodgkin's BJAB lymphoma cells (ATCC) (20 million cells per mouse) and tumors were allowed to grow to ~200 $mm^3$. The mice were then treated with vehicle (0.5M Arg-Succinate/20mM Tris/0.02% Tween 20 pH =7.2) (n=1), or 1 IP dose of RITUXAN® (4 mg/kg)(n=2), or 1 IP dose of agonist DR5 antibody (10 mg/kg) (n=2), or the combination of these latter DR5 antibody and RITUXAN® doses (n=2). Two days after treatment, the tumors were harvested, lysed in lysis buffer, and subjected to immunoblot with specific antibodies against Caspase 8, 3, 9, and 7 (with anti-beta actin antibody as a loading control) to visualize caspase processing (Figure 10).

Agonist DR5 antibody treatment (A) induced increased processing of caspase 8, 3, 9, and 7 as compared to the vehicle control (V), while RITUXAN® (R) did not induce caspase processing. Notably, combination treatment with DR5 antibody and RITUXAN® (AR) did not further increase caspase processing as compared to DR5 antibody alone. These results suggest that the synergistic anti-tumor activity between DR5 antibody and RITUXAN® is not necessarily mediated by enhancement of apoptosis, but rather that the combination of apoptosis activation mediated by agonist DR5 antibody and complement-dependent lysis together with ADCC mediated by RITUXAN® may underlie the observed anti-tumor synergy.

Further data illustrating expression of CD20 and Apo2L/TRAIL receptors in NHL cell lines and the effects of Rituximab, Apo2L/TRAIL and combinations thereof on cancer cells are provided in Figures 11-16.

WHAT IS CLAIMED IS:

1. A use of a synergistic effective amount of Apo2L/TRAIL polypeptide and CD20 antibody for the manufacture of medicament for the treatment of mammalian cancer cells.

2. The use of claim 1 wherein said Apo2L/TRAIL polypeptide comprises amino acids 1-281 of Fig. 1 (SEQ ID NO:1) or a fragment or variant thereof.

3. The use of claim 1 wherein said Apo2L/TRAIL polypeptide comprises amino acids 114-281 of Fig. 1 (SEQ ID NO:1).

4. The use of claim 1 wherein said cancer cells are exposed to said synergistic effective amount of Apo2L/TRAIL polypeptide and CD20 antibody *in vivo*.

5. The use of claim 1 wherein said cancer cells are lymphoma cells.

6. The use of claim 1 further comprising exposing the cancer cells to one or more growth inhibitory agents.

7. The use of claim 1 further comprising exposing the cells to radiation.

8. The use of claim 1 wherein said Apo2L/TRAIL polypeptide is expressed in a recombinant host cell selected from the group consisting of a CHO cell, yeast cell and *E. coli*.

9. The use of claim 1 wherein said Apo2L/TRAIL polypeptide is linked to a polyethylene glycol molecule.

10. The use of claim 1 wherein said CD20 antibody is a monoclonal antibody.

11. The use of claim 10 wherein said CD20 antibody is the antibody Rituximab.

12. A use of a synergistic effective amount of Apo2L/TRAIL polypeptide and CD20 antibody for the manufacture of medicament for the treatment of an immune related disease.

13. The use of claim 12 wherein said Apo2L/TRAIL polypeptide comprises amino acids 1-281 of Fig. 1 (SEQ ID NO:1) or a fragment or variant thereof.

14. The use of claim 12 wherein said Apo2L/TRAIL polypeptide comprises amino acids 114-281 of Fig. 1 (SEQ ID NO:1).

15. The use of claim 12 wherein said Apo2L/TRAIL polypeptide is expressed in a recombinant host cell selected from the group consisting of a CHO cell, yeast cell and *E. coli*.

16. The use of claim 12 wherein said Apo2L/TRAIL polypeptide is linked to a polyethylene glycol molecule.

17. The use of claim 12 wherein said immune related disease is rheumatoid arthritis or multiple sclerosis.

18. The use of claim 12 wherein said CD20 antibody is a monoclonal antibody.

19. The use of claim 18 wherein said CD20 antibody is the antibody Rituximab.

20. The use of claim 1 or 12 wherein said Apo2L/TRAIL polypeptide and CD20 antibody are administered sequentially.

21. The use of claim 1 or 12 wherein said Apo2L/TRAIL polypeptide and CD20 antibody are administered concurrently.

22. A use of synergistic effective amounts of Apo2L/TRAIL polypeptide and CD20 antibody in the preparation of a formulation or a kit or an article of manufacture for treating mammalian cancer cells.

23. The use of claim 22 wherein said cancer cells are exposed to said synergistic effective amount of Apo2L/TRAIL polypeptide and CD20 antibody *in vivo*.

24. The use of claim 22 wherein said cancer cells are lymphoma cells.

25. The use of claim 22 further comprising exposing the cancer cells to one or more growth inhibitory agents.

26. The use of claim 22 further comprising exposing the cells to radiation.

27. The use of claim 22 wherein said CD20 antibody is a monoclonal antibody.

28. The use of claim 27 wherein said CD20 antibody is the antibody Rituximab.

29. The use of claim 22 wherein said Apo2L/TRAIL polypeptide comprises amino acids 114-281 of Figure 1.

30. The use of claim 24 wherein said lymphoma cells are non-Hodgkin's lymphoma cells.

31. A use of a synergistic effective amount of Apo2L/TRAIL polypeptide and CD20 antibody in the preparation of a formulation or a kit or an article of manufacture for treating an immune related disease in a mammal.

32. The use of claim 31 wherein said immune related disease is rheumatoid arthritis or multiple sclerosis.

33. The use of claim 31 wherein said CD20 antibody is a monoclonal antibody.

34. The use of claim 33 wherein said CD20 antibody is the antibody Rituximab.

35. The use of claim 22 or 31 wherein said Apo2L/TRAIL polypeptide and CD20 antibody are administered sequentially.

36. The use of claim 22 or 31 wherein said Apo2L/TRAIL polypeptide and CD20 antibody are administered concurrently.

37. An article of manufacture for the treatment of cancer or immune related disease, comprising (a) a container comprising CD20 antibody; (b) a container comprising Apo2L/TRAIL polypeptide; and (c) a package insert with instructions for treating cancer or immune related disease in a patient, wherein the instructions indicate that amounts of the CD20 antibody and Apo2L/TRAIL polypeptide are administered to the patient that are effective to provide synergistic activity in treating the disease.

38. The article of manufacture of claim 37 wherein the CD20 antibody is Rituximab or humanized 2H7.

39. A use of Apo2L/TRAIL polypeptide and Rituximab in the preparation of a formulation or an article of manufacture for the treatment of lymphoma.

40. The use of claim 39 wherein the lymphoma is human B-cell non-Hodgkin's lymphoma.

41. The use of claim 39 wherein the Apo2L/TRAIL polypeptide comprises amino acids 114-281 of Figure 1.

Sequence Listing

<110> Genentech, Inc.

<120> METHODS OF USING DEATH RECEPTOR LIGANDS AND CD20 ANTIBODIES

<130> P2031R1A PCT

<140> PCT/US2005/031907
<141> 2005-09-07

<150> US 60/607,909
<151> 2004-09-08

<150> US 60/666,553
<151> 2005-03-30

<160> 11

<210> 1
<211> 281
<212> PRT
<213> Homo sapiens

<400> 1

Met Ala Met Met Glu Val Gln Gly Gly Pro Ser Leu Gly Gln Thr
1 5 10 15

Cys Val Leu Ile Val Ile Phe Thr Val Leu Leu Gln Ser Leu Cys
20 25 30

Val Ala Val Thr Tyr Val Tyr Phe Thr Asn Glu Leu Lys Gln Met
35 40 45

Gln Asp Lys Tyr Ser Lys Ser Gly Ile Ala Cys Phe Leu Lys Glu
50 55 60

Asp Asp Ser Tyr Trp Asp Pro Asn Asp Glu Glu Ser Met Asn Ser
65 70 75

Pro Cys Trp Gln Val Lys Trp Gln Leu Arg Gln Leu Val Arg Lys
80 85 90

Met Ile Leu Arg Thr Ser Glu Glu Thr Ile Ser Thr Val Gln Glu
95 100 105

Lys Gln Gln Asn Ile Ser Pro Leu Val Arg Glu Arg Gly Pro Gln
110 115 120

Arg Val Ala Ala His Ile Thr Gly Thr Arg Gly Arg Ser Asn Thr
125 130 135

Leu Ser Ser Pro Asn Ser Lys Asn Glu Lys Ala Leu Gly Arg Lys
140 145 150

Ile Asn Ser Trp Glu Ser Ser Arg Ser Gly His Ser Phe Leu Ser
155 160 165

Asn Leu His Leu Arg Asn Gly Glu Leu Val Ile His Glu Lys Gly
170 175 180

Phe Tyr Tyr Ile Tyr Ser Gln Thr Tyr Phe Arg Phe Gln Glu Glu
185 190 195

Ile Lys Glu Asn Thr Lys Asn Asp Lys Gln Met Val Gln Tyr Ile
200 205 210

Tyr Lys Tyr Thr Ser Tyr Pro Asp Pro Ile Leu Leu Met Lys Ser

215 220 225

Ala Arg Asn Ser Cys Trp Ser Lys Asp Ala Glu Tyr Gly Leu Tyr
230 235 240

Ser Ile Tyr Gln Gly Gly Ile Phe Glu Leu Lys Glu Asn Asp Arg
245 250 255

Ile Phe Val Ser Val Thr Asn Glu His Leu Ile Asp Met Asp His
260 265 270

Glu Ala Ser Phe Phe Gly Ala Phe Leu Val Gly
275 280

<210> 2
<211> 1042
<212> DNA
<213> Homo sapiens

<220>
<221> N
<222> 447
<223> may be T or G

<400> 2
tttcctcact gactataaaa gaatagagaa ggaagggctt cagtgaccgg 50

ctgcctggct gacttacagc agtcagactc tgacaggatc atggctatga 100

tggaggtcca ggggggaccc agcctgggac agacctgcgt gctgatcgtg 150

atcttcacag tgctcctgca gtctctctgt gtggctgtaa cttacgtgta 200

ctttaccaac gagctgaagc agatgcagga caagtactcc aaaagtggca 250

ttgcttgttt cttaaaagaa gatgacagtt attgggaccc caatgacgaa 300

gagagtatga acagcccctg ctggcaagtc aagtggcaac tccgtcagct 350

cgttagaaag atgattttga gaacctctga ggaaaccatt tctacagttc 400

aagaaaagca acaaaatatt tctcccctag tgagagaaag aggtccncag 450

agagtagcag ctcacataac tgggaccaga ggaagaagca acacattgtc 500

ttctccaaac tccaagaatg aaaaggctct gggccgcaaa ataaactcct 550

gggaatcatc aaggagtggg cattcattcc tgagcaactt gcacttgagg 600

aatggtgaac tggtcatcca tgaaaaaggg ttttactaca tctattccca 650

aacatacttt cgatttcagg aggaaataaa agaaaacaca aagaacgaca 700

aacaaatggt ccaatatatt tacaaataca caagttatcc tgaccctata 750

ttgttgatga aaagtgctag aaatagttgt tggtctaaag atgcagaata 800

tggactctat tccatctatc aaggggggaat atttgagctt aaggaaaatg 850

acagaatttt tgtttctgta acaaatgagc acttgataga catggaccat 900

gaagccagtt ttttcggggc ctttttagtt ggctaactga cctggaaaga 950

aaaagcaata acctcaaagt gactattcag ttttcaggat gatacactat 1000

gaagatgttt caaaaaatct gaccaaaaca aacaaacaga aa 1042

<210> 3

<211> 468
<212> PRT
<213> Homo sapiens

<400> 3

```
Met Ala Pro Pro Pro Ala Arg Val His Leu Gly Ala Phe Leu Ala
  1               5                  10                  15

Val Thr Pro Asn Pro Gly Ser Ala Ala Ser Gly Thr Glu Ala Ala
             20                  25                  30

Ala Ala Thr Pro Ser Lys Val Trp Gly Ser Ser Ala Gly Arg Ile
         35                  40                  45

Glu Pro Arg Gly Gly Gly Arg Gly Ala Leu Pro Thr Ser Met Gly
     50                  55                  60

Gln His Gly Pro Ser Ala Arg Ala Arg Ala Gly Arg Ala Pro Gly
 65                  70                  75

Pro Arg Pro Ala Arg Glu Ala Ser Pro Arg Leu Arg Val His Lys
             80                  85                  90

Thr Phe Lys Phe Val Val Val Gly Val Leu Leu Gln Val Val Pro
         95                 100                 105

Ser Ser Ala Ala Thr Ile Lys Leu His Asp Gln Ser Ile Gly Thr
    110                 115                 120

Gln Gln Trp Glu His Ser Pro Leu Gly Glu Leu Cys Pro Pro Gly
125                 130                 135

Ser His Arg Ser Glu Arg Pro Gly Ala Cys Asn Arg Cys Thr Glu
            140                 145                 150

Gly Val Gly Tyr Thr Asn Ala Ser Asn Asn Leu Phe Ala Cys Leu
        155                 160                 165

Pro Cys Thr Ala Cys Lys Ser Asp Glu Glu Glu Arg Ser Pro Cys
    170                 175                 180

Thr Thr Thr Arg Asn Thr Ala Cys Gln Cys Lys Pro Gly Thr Phe
185                 190                 195

Arg Asn Asp Asn Ser Ala Glu Met Cys Arg Lys Cys Ser Thr Gly
            200                 205                 210

Cys Pro Arg Gly Met Val Lys Val Lys Asp Cys Thr Pro Trp Ser
        215                 220                 225

Asp Ile Glu Cys Val His Lys Glu Ser Gly Asn Gly His Asn Ile
    230                 235                 240

Trp Val Ile Leu Val Val Thr Leu Val Val Pro Leu Leu Leu Val
245                 250                 255

Ala Val Leu Ile Val Cys Cys Cys Ile Gly Ser Gly Cys Gly Gly
            260                 265                 270

Asp Pro Lys Cys Met Asp Arg Val Cys Phe Trp Arg Leu Gly Leu
        275                 280                 285

Leu Arg Gly Pro Gly Ala Glu Asp Asn Ala His Asn Glu Ile Leu
    290                 295                 300

Ser Asn Ala Asp Ser Leu Ser Thr Phe Val Ser Glu Gln Gln Met
305                 310                 315
```

Glu Ser Gln Glu Pro Ala Asp Leu Thr Gly Val Thr Val Gln Ser
320 325 330

Pro Gly Glu Ala Gln Cys Leu Leu Gly Pro Ala Glu Ala Glu Gly
335 340 345

Ser Gln Arg Arg Arg Leu Leu Val Pro Ala Asn Gly Ala Asp Pro
350 355 360

Thr Glu Thr Leu Met Leu Phe Phe Asp Lys Phe Ala Asn Ile Val
365 370 375

Pro Phe Asp Ser Trp Asp Gln Leu Met Arg Gln Leu Asp Leu Thr
380 385 390

Lys Asn Glu Ile Asp Val Val Arg Ala Gly Thr Ala Gly Pro Gly
395 400 405

Asp Ala Leu Tyr Ala Met Leu Met Lys Trp Val Asn Lys Thr Gly
410 415 420

Arg Asn Ala Ser Ile His Thr Leu Leu Asp Ala Leu Glu Arg Met
425 430 435

Glu Glu Arg His Ala Lys Glu Lys Ile Gln Asp Leu Leu Val Asp
440 445 450

Ser Gly Lys Phe Ile Tyr Leu Glu Asp Gly Thr Gly Ser Ala Val
455 460 465

Ser Leu Glu

<210> 4
<211> 1407
<212> DNA
<213> Homo sapiens

<400> 4
atggcgccac caccagctag agtacatcta ggtgcgttcc tggcagtgac 50

tccgaatccc gggagcgcag cgagtgggac agaggcagcc gcggccacac 100

ccagcaaagt gtggggctct tccgcgggga ggattgaacc acgaggcggg 150

ggccgaggag cgctccctac ctccatggga cagcacggac ccagtgcccg 200

ggcccgggca gggcgcgccc caggacccag gccggcgcgg gaagccagcc 250

ctcggctccg ggtccacaag accttcaagt ttgtcgtcgt cggggtcctg 300

ctgcaggtcg tacctagctc agctgcaacc atgatcaatc aattggcaca 350

aattggcaca cagcaatggg aacatagccc tttgggagag ttgtgtccac 400

caggatctca tagatcagaa cgtcctggag cctgtaaccg gtgcacagag 450

ggtgtgggtt acaccaatgc ttccaacaat ttgtttgctt gcctcccatg 500

tacagcttgt aaatcagatg aagaagagag aagtccctgc accacgacca 550

ggaacacagc atgtcagtgc aaaccaggaa ctttccggaa tgacaattct 600

gctgagatgt gccggaagtg cagcacaggg tgccccagag ggatggtcaa 650

ggtcaaggat tgtacgccct ggagtgacat cgagtgtgtc cacaaagaat 700

caggcaatgg acataatata tgggtgattt tggttgtgac tttggttgtt 750

ccgttgctgt tggtggctgt gctgattgtc tgttgttgca tcggctcagg 800

ttgtggaggg gaccccaagt gcatggacag ggtgtgtttc tggcgcttgg 850

gtctcctacg agggcctggg gctgaggaca atgctcacaa cgagattctg 900

agcaacgcag actcgctgtc cactttcgtc tctgagcagc aaatggaaag 950

ccaggagccg gcagatttga caggtgtcac tgtacagtcc ccaggggagg 1000

cacagtgtct gctgggaccg gcagaagctg aagggtctca gaggaggagg 1050

ctgctggttc cagcaaatgg tgctgacccc actgagactc tgatgctgtt 1100

ctttgacaag tttgcaaaca tcgtgccctt tgactcctgg gaccagctca 1150

tgaggcagct ggacctcacg aaaaatgaga tcgatgtggt cagagctggt 1200

acagcaggcc caggggatgc cttgtatgca atgctgatga aatgggtcaa 1250

caaaactgga cggaacgcct cgatccacac cctgctggat gccttggaga 1300

ggatggaaga gagacatgca aaagagaaga ttcaggacct cttggtggac 1350

tctggaaagt tcatctactt agaagatggc acaggctctg ccgtgtcctt 1400

ggagtga 1407

<210> 5
<211> 411
<212> PRT
<213> Homo sapiens

<400> 5

```
Met Glu Gln Arg Gly Gln Asn Ala Pro Ala Ala Ser Gly Ala Arg
  1               5                  10                  15

Lys Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala Arg Pro
             20                  25                  30

Gly Leu Arg Val Pro Lys Thr Leu Val Leu Val Val Ala Ala Val
         35                  40                  45

Leu Leu Leu Val Ser Ala Glu Ser Ala Leu Ile Thr Gln Gln Asp
     50                  55                  60

Leu Ala Pro Gln Gln Arg Ala Ala Pro Gln Gln Lys Arg Ser Ser
 65                  70                  75

Pro Ser Glu Gly Leu Cys Pro Pro Gly His His Ile Ser Glu Asp
             80                  85                  90

Gly Arg Asp Cys Ile Ser Cys Lys Tyr Gly Gln Asp Tyr Ser Thr
         95                 100                 105

His Trp Asn Asp Leu Leu Phe Cys Leu Arg Cys Thr Arg Cys Asp
    110                 115                 120

Ser Gly Glu Val Glu Leu Ser Pro Cys Thr Thr Thr Arg Asn Thr
125                 130                 135

Val Cys Gln Cys Glu Glu Gly Thr Phe Arg Glu Glu Asp Ser Pro
            140                 145                 150

Glu Met Cys Arg Lys Cys Arg Thr Gly Cys Pro Arg Gly Met Val
        155                 160                 165
```

Lys Val Gly Asp Cys Thr Pro Trp Ser Asp Ile Glu Cys Val His
170 175 180

Lys Glu Ser Gly Ile Ile Ile Gly Val Thr Val Ala Ala Val Val
185 190 195

Leu Ile Val Ala Val Phe Val Cys Lys Ser Leu Leu Trp Lys Lys
200 205 210

Val Leu Pro Tyr Leu Lys Gly Ile Cys Ser Gly Gly Gly Gly Asp
215 220 225

Pro Glu Arg Val Asp Arg Ser Ser Gln Arg Pro Gly Ala Glu Asp
230 235 240

Asn Val Leu Asn Glu Ile Val Ser Ile Leu Gln Pro Thr Gln Val
245 250 255

Pro Glu Gln Glu Met Glu Val Gln Glu Pro Ala Glu Pro Thr Gly
260 265 270

Val Asn Met Leu Ser Pro Gly Glu Ser Glu His Leu Leu Glu Pro
275 280 285

Ala Glu Ala Glu Arg Ser Gln Arg Arg Arg Leu Leu Val Pro Ala
290 295 300

Asn Glu Gly Asp Pro Thr Glu Thr Leu Arg Gln Cys Phe Asp Asp
305 310 315

Phe Ala Asp Leu Val Pro Phe Asp Ser Trp Glu Pro Leu Met Arg
320 325 330

Lys Leu Gly Leu Met Asp Asn Glu Ile Lys Val Ala Lys Ala Glu
335 340 345

Ala Ala Gly His Arg Asp Thr Leu Tyr Thr Met Leu Ile Lys Trp
350 355 360

Val Asn Lys Thr Gly Arg Asp Ala Ser Val His Thr Leu Leu Asp
365 370 375

Ala Leu Glu Thr Leu Gly Glu Arg Leu Ala Lys Gln Lys Ile Glu
380 385 390

Asp His Leu Leu Ser Ser Gly Lys Phe Met Tyr Leu Glu Gly Asn
395 400 405

Ala Asp Ser Ala Leu Ser
410

<210> 6
<211> 440
<212> PRT
<213> Homo sapiens

<400> 6
Met Glu Gln Arg Gly Gln Asn Ala Pro Ala Ala Ser Gly Ala Arg
1 5 10 15

Lys Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala Arg Pro
20 25 30

Gly Pro Arg Val Pro Lys Thr Leu Val Leu Val Val Ala Ala Val
35 40 45

Leu Leu Leu Val Ser Ala Glu Ser Ala Leu Ile Thr Gln Gln Asp
50 55 60

Leu Ala Pro Gln Gln Arg Ala Ala Pro Gln Gln Lys Arg Ser Ser
65 70 75

Pro Ser Glu Gly Leu Cys Pro Pro Gly His His Ile Ser Glu Asp
80 85 90

Gly Arg Asp Cys Ile Ser Cys Lys Tyr Gly Gln Asp Tyr Ser Thr
95 100 105

His Trp Asn Asp Leu Leu Phe Cys Leu Arg Cys Thr Arg Cys Asp
110 115 120

Ser Gly Glu Val Glu Leu Ser Pro Cys Thr Thr Thr Arg Asn Thr
125 130 135

Val Cys Gln Cys Glu Glu Gly Thr Phe Arg Glu Glu Asp Ser Pro
140 145 150

Glu Met Cys Arg Lys Cys Arg Thr Gly Cys Pro Arg Gly Met Val
155 160 165

Lys Val Gly Asp Cys Thr Pro Trp Ser Asp Ile Glu Cys Val His
170 175 180

Lys Glu Ser Gly Thr Lys His Ser Gly Glu Ala Pro Ala Val Glu
185 190 195

Glu Thr Val Thr Ser Ser Pro Gly Thr Pro Ala Ser Pro Cys Ser
200 205 210

Leu Ser Gly Ile Ile Ile Gly Val Thr Val Ala Ala Val Val Leu
215 220 225

Ile Val Ala Val Phe Val Cys Lys Ser Leu Leu Trp Lys Lys Val
230 235 240

Leu Pro Tyr Leu Lys Gly Ile Cys Ser Gly Gly Gly Gly Asp Pro
245 250 255

Glu Arg Val Asp Arg Ser Ser Gln Arg Pro Gly Ala Glu Asp Asn
260 265 270

Val Leu Asn Glu Ile Val Ser Ile Leu Gln Pro Thr Gln Val Pro
275 280 285

Glu Gln Glu Met Glu Val Gln Glu Pro Ala Glu Pro Thr Gly Val
290 295 300

Asn Met Leu Ser Pro Gly Glu Ser Glu His Leu Leu Glu Pro Ala
305 310 315

Glu Ala Glu Arg Ser Gln Arg Arg Arg Leu Leu Val Pro Ala Asn
320 325 330

Glu Gly Asp Pro Thr Glu Thr Leu Arg Gln Cys Phe Asp Asp Phe
335 340 345

Ala Asp Leu Val Pro Phe Asp Ser Trp Glu Pro Leu Met Arg Lys
350 355 360

Leu Gly Leu Met Asp Asn Glu Ile Lys Val Ala Lys Ala Glu Ala
365 370 375

Ala Gly His Arg Asp Thr Leu Tyr Thr Met Leu Ile Lys Trp Val
380 385 390

Asn Lys Thr Gly Arg Asp Ala Ser Val His Thr Leu Leu Asp Ala

395 400 405

Leu Glu Thr Leu Gly Glu Arg Leu Ala Lys Gln Lys Ile Glu Asp
410 415 420

His Leu Leu Ser Ser Gly Lys Phe Met Tyr Leu Glu Gly Asn Ala
425 430 435

Asp Ser Ala Met Ser
440

<210> 7
<211> 107
<212> PRT
<213> Artificial sequence

<220>
<223> synthetic

<400> 7
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
1 5 10 15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Ser Ser Val Ser
20 25 30

Tyr Met His Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Pro
35 40 45

Leu Ile Tyr Ala Pro Ser Asn Leu Ala Ser Gly Val Pro Ser Arg
50 55 60

Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
65 70 75

Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Trp
80 85 90

Ser Phe Asn Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile
95 100 105

Lys Arg

<210> 8
<211> 122
<212> PRT
<213> Artificial sequence

<220>
<223> synthetic

<400> 8
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
1 5 10 15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr
20 25 30

Ser Tyr Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
35 40 45

Glu Trp Val Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr
50 55 60

Asn Gln Lys Phe Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser
65 70 75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ala Arg Val Val Tyr Tyr Ser Asn Ser
95 100 105

Tyr Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val
110 115 120

Ser Ser

<210> 9
<211> 213
<212> PRT
<213> Artificial sequence

<220>
<223> synthetic

<400> 9
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
1 5 10 15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Ser Ser Val Ser
20 25 30

Tyr Met His Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Pro
35 40 45

Leu Ile Tyr Ala Pro Ser Asn Leu Ala Ser Gly Val Pro Ser Arg
50 55 60

Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
65 70 75

Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Trp
80 85 90

Ser Phe Asn Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile
95 100 105

Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser
110 115 120

Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu
125 130 135

Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp
140 145 150

Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln
155 160 165

Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu
170 175 180

Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu Val
185 190 195

Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn Arg
200 205 210

Gly Glu Cys

<210> 10
<211> 452

<212> PRT
<213> Artificial sequence

<220>
<223> synthetic

<400> 10

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr
             20                  25                  30

Ser Tyr Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
             35                  40                  45

Glu Trp Val Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr
             50                  55                  60

Asn Gln Lys Phe Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser
             65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
             80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Val Val Tyr Tyr Ser Asn Ser
             95                 100                 105

Tyr Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val
            110                 115                 120

Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro
            125                 130                 135

Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu
            140                 145                 150

Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser
            155                 160                 165

Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln
            170                 175                 180

Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser
            185                 190                 195

Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys
            200                 205                 210

Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys
            215                 220                 225

Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
            230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr
            245                 250                 255

Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp
            260                 265                 270

Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp
            275                 280                 285

Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln
            290                 295                 300

Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His
```

305 310 315

Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn
320 325 330

Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys
335 340 345

Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg
350 355 360

Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys
365 370 375

Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly
380 385 390

Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser
395 400 405

Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser
410 415 420

Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu
425 430 435

Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro
440 445 450

Gly Lys

<210> 11
<211> 452
<212> PRT
<213> Artificial sequence

<220>
<223> synthetic

<400> 11
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
1 5 10 15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr
20 25 30

Ser Tyr Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
35 40 45

Glu Trp Val Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr
50 55 60

Asn Gln Lys Phe Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser
65 70 75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ala Arg Val Val Tyr Tyr Ser Asn Ser
95 100 105

Tyr Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val
110 115 120

Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro
125 130 135

```
Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu
                140                 145                 150

Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser
                155                 160                 165

Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln
                170                 175                 180

Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser
                185                 190                 195

Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys
                200                 205                 210

Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys
                215                 220                 225

Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
                230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr
                245                 250                 255

Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp
                260                 265                 270

Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp
                275                 280                 285

Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln
                290                 295                 300

Tyr Asn Ala Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His
                305                 310                 315

Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn
                320                 325                 330

Lys Ala Leu Pro Ala Pro Ile Ala Ala Thr Ile Ser Lys Ala Lys
                335                 340                 345

Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg
                350                 355                 360

Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys
                365                 370                 375

Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly
                380                 385                 390

Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser
                395                 400                 405

Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser
                410                 415                 420

Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu
                425                 430                 435

Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro
                440                 445                 450

Gly Lys
```