USE OF APO2L RECEPTOR AGONISTS AND NK CELLS OR NK CELL ACTIVATORS

RELATED APPLICATIONS

This application claims priority under Section 119(e) to U.S. provisional application number 60/581,129 filed June 18, 2004, the contents of which are hereby incorporated by reference.

FIELD OF THE INVENTION

This invention relates generally to methods of enhancing induction of apoptosis or cytolytic activity in mammalian cells. In particular, it pertains to the use of Apo-2L receptor agonists and NK cells, or activating agents thereof, to induce apoptosis or cytolytic activity in mammalian cells. Various Apo-2L receptor agonists contemplated by the invention include the ligand known as Apo-2 ligand or TRAIL, as well as agonist antibodies directed to one or more Apo-2L receptors. Various NK cell activating agents contemplated by the invention include but are not limited to Toll receptor activating agents, IL-2, IL-12, IL-15, IFN-alpha, IFN-beta, and agonist antibodies to activating receptors such as NKp30, NKp44, NKG2D.

BACKGROUND OF THE INVENTION

Control of cell numbers in mammals is believed to be determined, in part, by a balance between cell proliferation and cell death. One form of cell death, sometimes referred to as necrotic cell death, is typically characterized as a pathologic form of cell death resulting from some trauma or cellular injury. In contrast, there is another, "physiologic" form of cell death which usually proceeds in an orderly or controlled manner. This orderly or controlled form of cell death is often referred to as "apoptosis" [see, *e.g.*, Barr et al., Bio/Technology, 12:487-493 (1994); Steller et al., Science, 267:1445-1449 (1995)]. Apoptotic cell death naturally occurs in many physiological processes, including embryonic development and clonal selection in the immune system [Itoh et al., Cell, 66:233-243 (1991)].

Various molecules, such as 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, 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) have been identified as members of the tumor necrosis factor ("TNF") family of cytokines [See, e.g., 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; WO98/18921 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)]. Among these molecules, TNF-alpha, TNF-beta, CD30 ligand, 4-1BB ligand, Apo-1 ligand, Apo-2 ligand (Apo2L/TRAIL) and Apo-3 ligand (TWEAK) have been reported to be involved in apoptotic cell death.

Apo2L/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); 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); 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 *in vitro*, 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; 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)]. 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)].

Induction of various cellular responses mediated by the TNF family cytokines is believed to be initiated by their binding to specific cell receptors. Two distinct TNF receptors of approximately 55-kDa (TNFR1) and 75-kDa (TNFR2) have been identified [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] and human and mouse cDNAs corresponding to both receptor types have been isolated and characterized [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)]. Extensive polymorphisms have been associated with both TNF receptor genes [see, e.g., Takao et al., Immunogenetics, 37:199-203 (1993)]. Both TNFRs share the typical structure of cell surface receptors including extracellular, transmembrane and intracellular regions. The extracellular portions of both receptors are found naturally also as soluble TNF-binding proteins [Nophar, Y. et al., EMBO J., 9:3269 (1990); and Kohno, T. et al., Proc. Natl. Acad. Sci. U.S.A., 87:8331 (1990)]. The cloning of recombinant soluble TNF receptors was reported by Hale et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 113 (P424)].

The extracellular portion of type 1 and type 2 TNFRs (TNFR1 and TNFR2) contains a repetitive amino acid sequence pattern of four cysteine-rich domains (CRDs) designated 1 through 4, starting from the NH_2-terminus. Each CRD is about 40 amino acids long and contains 4 to 6 cysteine residues at positions which are well conserved [Schall et al., supra; Loetscher et al., supra; Smith et al., supra; Nophar et al., supra; Kohno et al., supra]. In TNFR1, the approximate boundaries of the four CRDs are as follows: CRD1- amino acids 14 to about 53; CRD2- amino acids from about 54 to about 97; CRD3- amino acids from about 98 to about 138; CRD4- amino acids from about 139 to about 167. In TNFR2, CRD1 includes amino acids 17 to about 54; CRD2- amino acids from about 55 to about 97; CRD3- amino acids from about 98 to about 140; and CRD4- amino acids from about 141 to about 179 [Banner et al., Cell, 73:431-435 (1993)]. The potential role of the CRDs in ligand binding is also described by Banner et al., supra.

A similar repetitive pattern of CRDs exists in several other cell-surface proteins, including the p75 nerve growth factor receptor (NGFR) [Johnson et al., Cell, 47:545 (1986); Radeke et al., Nature, 325:593 (1987)], the B cell antigen CD40 [Stamenkovic et al., EMBO J., 8:1403 (1989)], the T cell antigen OX40 [Mallet et al., EMBO J., 9:1063 (1990)] and the Fas antigen [Yonehara et al., J. Exp. Med., 169:1747-1756 (1989) and Itoh et al., Cell, 66:233-243 (1991)]. CRDs are also found in the soluble TNFR (sTNFR)-like T2 proteins of the Shope and myxoma poxviruses [Upton et al., Virology, 160:20-29 (1987); Smith et al., Biochem. Biophys. Res. Commun., 176:335 (1991); Upton et al., Virology, 184:370 (1991)]. Optimal alignment of these sequences indicates that the positions of the cysteine residues are well conserved. These receptors are sometimes collectively referred to as members of the TNF/NGF receptor superfamily. Recent studies on p75NGFR showed that the deletion of CRD1 [Welcher, A.A. et al., Proc. Natl. Acad. Sci. USA, 88:159-163 (1991)] or a 5-amino acid insertion in this domain [Yan, H. and Chao, M.V., J. Biol. Chem., 266:12099-12104 (1991)] had little or no effect on NGF binding [Yan, H. and Chao, M.V., supra]. p75 NGFR contains a proline-rich stretch of about 60 amino acids, between its CRD4 and transmembrane region, which is not involved in NGF binding [Peetre, C. et al., Eur. J. Hematol., 41:414-419 (1988); Seckinger, P. et al., J. Biol. Chem., 264:11966-11973 (1989); Yan, H. and Chao, M.V., supra]. A similar proline-rich region is found in TNFR2 but not in TNFR1.

The TNF family ligands identified to date, with the exception of lymphotoxin-α, are type II transmembrane proteins, whose C-terminus is extracellular. In contrast, most receptors in the TNF receptor (TNFR) family identified to date are type I transmembrane proteins. In both the TNF ligand and receptor families, however, homology identified between family members has been found mainly in the extracellular domain ("ECD").

Several of the TNF family cytokines, including TNF-α, Apo-1 ligand and CD40 ligand, are cleaved proteolytically at the cell surface; the resulting protein in each case typically forms a homotrimeric molecule that functions as a soluble cytokine. TNF receptor family proteins are also usually cleaved proteolytically to release soluble receptor ECDs that can function as inhibitors of the cognate cytokines.

Recently, other members of the TNFR family have been identified. Such newly identified members of the TNFR family include CAR1, HVEM and osteoprotegerin (OPG) [Brojatsch et al., Cell, 87:845-855 (1996); Montgomery et al., Cell, 87:427-436 (1996); Marsters et al., J. Biol. Chem., 272:14029-14032 (1997); Simonet et al., Cell, 89:309-319 (1997)]. Unlike other known TNFR-like molecules, Simonet et al., supra, report that OPG contains no hydrophobic transmembrane-spanning sequence. OPG is believed to act as a decoy receptor, as discussed below.

Pan et al. have disclosed another TNF receptor family member referred to as "DR4" [Pan et al., Science, 276:111-113 (1997)]. The DR4 was reported to contain a cytoplasmic death domain capable of engaging the cell suicide apparatus. Pan et al. disclose that DR4 is believed to be a receptor for the ligand known as Apo-2 ligand or TRAIL.

In Sheridan et al., Science, 277:818-821 (1997) and Pan et al., Science, 277:815-818 (1997), another molecule believed to be a receptor for Apo2L/TRAIL is described [see also, WO98/51793 published November 19, 1998; WO98/41629 published September 24, 1998]. That molecule is referred to as DR5 (it 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]. Like DR4, DR5 is reported to contain a cytoplasmic death domain and be capable of signaling apoptosis. 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).

A further group of recently identified TNFR family members are referred to as "decoy receptors," which are believed to function as inhibitors, rather than transducers of signaling. This group includes 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)] and 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)], both cell surface molecules, as well as OPG [Simonet et al., supra] and DCR3 [Pitti et al., Nature, 396:699-703 (1998)], both of which are secreted, soluble proteins. Apo2L/TRAIL has been reported to bind those receptors referred to as DcR1, DcR2 and OPG.

Apo2L/TRAIL is believed to act through the cell surface "death receptors" DR4 and DR5 to activate caspases, or enzymes that carry out the intracellular cell death program. [See, e.g., Salvesen et al., Cell, 91:443-446 (1997)]. 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)]. In contrast to DR4 and DR5, the DcR1 and DcR2 receptors do not signal apoptosis.

For a review of the TNF family of cytokines and their receptors, see 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); Gruss and Dower, supra, and Nagata, Cell, 88:355-365 (1997); Locksley et al., Cell, 104:487-501 (2001); Wallach, "TNF Ligand and TNF/NGF Receptor Families", Cytokine Research, Academic Press, pages 377-411 (2000).

A number of molecules in the Toll receptor (TLR) family have been identified in humans, of which TLRs 2, 4, 5 and 9 are believed to be activated by highly conserved microbial products such as lipoproteins, LPS, flagellin and unmethylated CpG DNA, respectively. TLR3 can be activated by double stranded RNA, often produced during replication of viruses, while TLR7 can be activated by small molecule compounds such as the antiviral imidazoquinolines: imiquimod and R-848. Human TLR8 can also be activated by R-848, and recent reports demonstrate that single-stranded RNA represents a physiological ligand for TLR8.

TLRs are widely expressed on cells that are important for innate responses to pathogens. On antigen presenting cells ("APCs"), activation of different TLRs results in a variety of responses including the production of cytokines and co-stimulatory molecules that initiate and shape the adaptive response to particular pathogens. NK cells also express members of the TLR family. Purified NK cells express TLR3 and their cytolytic activity towards certain tumor cells can be activated by poly(I:C).

NK cells employ a series of activating and inhibitory receptors to identify and eliminate target cells. One class of activating receptors that trigger NK cytotoxicity are referred to as NCRs (natural cytotoxicity receptors), which include the immunoglobulin family members NKp46, NKp30 and NKp44 and the C-type lectin, NKG2D. The activity of the

NCRs is opposed by signaling from inhibitory receptors specific for classical MHC class I molecules, which are constitutively expressed by normal cells. The perforin-dependent cytotoxic granule exocytosis pathway is a well-characterized mechanism by which NK cells kill target cells. Cytotoxic granules are specialized secretory lysosomes that contain the pore-forming protein perforin and a family of serine proteases known as granzymes, which trigger rapid apoptosis in target cells. NK cells also use cell surface "perforin-independent" mechanisms to induce cytotoxicity in target cells.

SUMMARY OF THE INVENTION

Applicants have found that Apo-2 ligand or other Apo-2L receptor agonists and NK cells, or activating agents thereof, can be effectively used in combination to induce apoptosis or cytolytic activity in mammalian cells, particularly in diseased mammalian cells.

The invention provides various methods for the use of Apo-2 ligand and NK cells or NK cell activating agent(s) to enhance apoptosis or cytolytic activity in mammalian cells. For example, the invention provides methods for inducing apoptosis comprising exposing a mammalian cell, such as a cancer cell or virally- or bacteria- infected cell, to NK cells or NK cell activating agent(s) and one or more Apo-2 ligand receptor agonists.

The cells may be in cell culture or in a mammal, *e.g.* a mammal suffering from cancer or a condition in which induction of apoptosis in the cells is desirable. Thus, the invention includes methods for treating a mammal suffering from a disorder such as cancer or viral infection, comprising administering an effective amount of Apo-2 ligand and NK cells or NK cell activating agent(s), as disclosed herein.

Optionally, the methods may employ agonistic anti-Apo-2 ligand receptor antibody(s) which mimics the apoptotic activity of Apo-2 ligand. Thus, the invention provides various methods for the use of Apo-2 ligand receptor agonist antibody(s) and NK cells or NK cell activating agent(s) to induce apoptosis in mammalian cells. In a preferred embodiment, the agonist antibody will comprise an antibody against the DR4 or DR5 receptor.

In optional embodiments, there are provided methods of enhancing apoptosis in mammalian cancer cells, comprising exposing mammalian cancer cells to an effective amount of NK cells or NK cell activating agent(s) and Apo-2 ligand receptor agonist, wherein said mammalian cancer cells are exposed to the NK cells or NK cell activating agent(s) prior to exposure to said Apo-2 ligand receptor agonist. The Apo-2 ligand receptor agonist optionally comprises Apo2L polypeptide or anti-DR4 receptor antibody or anti-DR5 receptor antibody.

The invention also provides compositions which comprise Apo-2 ligand or Apo-2L receptor agonist antibody and/or NK cells or NK cell activating agent(s). Optionally, the compositions of the invention will include pharmaceutically acceptable carriers or diluents. Preferably, the compositions will include Apo-2 ligand or agonist antibody and/or NK cells or NK cell activating agent(s) in an amount which is effective to synergistically induce apoptosis in mammalian cells.

The invention also provides articles of manufacture and kits which include Apo-2 ligand or Apo-2L receptor agonist antibody and/or NK cells or NK cell activating agent(s).

Further optional embodiments are illustrated by the following methods:

1. A method of enhancing apoptosis or cytotoxicity in mammalian cells comprising exposing mammalian cells to an effective amount of Apo-2 ligand receptor agonist and NK cells or NK cell activating agent(s).

2. The method of claim 1 wherein said Apo-2 ligand receptor agonist comprises Apo-2 ligand polypeptide.

3. The method of claim 1 wherein said mammalian cells are cancer cells.

4. The method of claim 1 wherein the mammalian cells are virally-infected or bacteria-infected cells.

5. The method of claim 2 wherein said Apo-2 ligand polypeptide comprises amino acids 39 to 281 of Figure 4 or a biologically active fragment thereof.

6. The method of claim 5 wherein said Apo-2 ligand polypeptide comprises amino acids 114 to 281 of Figure 4.

7. The method of claim 5 wherein said Apo-2 ligand polypeptide is linked to one or more polyethylene glycol (PEG) molecules.

8. The method of claim 1 wherein said Apo-2 ligand receptor agonist is an agonistic anti-Apo-2 ligand receptor antibody.

9. The method of claim 8 wherein said agonistic antibody comprises an anti-DR4 antibody.

10. The method of claim 8 wherein said agonistic antibody comprises an anti-DR5 antibody.

11. The method of claim 9 wherein said anti-DR4 antibody is a chimeric, humanized or human antibody.

12. The method of claim 10 wherein said anti-DR5 antibody is a chimeric, humanized or human antibody.

13. The method of claim 1 wherein said NK cells are purified NK cells from a donor mammal.

14. The method of claim 1 wherein said NK cell activating agent is selected from the group consisting of Toll receptor activating agents, IL-2, IL-12, IL-15, IFN-alpha, and IFN-beta.

15. The method of claim 1 wherein said NK cell activating agent is selected from the group consisting of agonist antibodies to activating receptors such as NKp30, NKp44, NKG2D.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the effects of various TLR activators on the activity of human NK cells, as measured in a ^{51}Cr release assay.

Figures 2A-C illustrate induction of Apo2L/TRAIL on human NK cells. (A) NK cells were treated with poly(I:C), R-848 or human interferon-alpha in the presence or absence of cyclohexamide and Apo2L/TRAIL message was measured in the extracted RNA. (B) Resting and poly(I:C) stimulated NK cell lysates or culture supernatants were assessed for presence of Apo2L/TRAIL protein by quantitative ELISA. (C) Staining for cell surface Apo2L/TRAIL on purified NK cells treated with the agent referred to in respective panel. The solid line corresponds to isotype control and the lighter line to the Apo2L/TRAIL staining. Results shown are a representative donor (out of three or more donors) in all three panels.

Figures 3A-C illustrate assay results showing role(s) of Apo2L/TRAIL in activated NK cell activity. (A) % lysis obtained at E:T ratio of 50:1 (B) purified NK cells were treated with poly(I:C) or R-848 and incubated with B16BL10 cells at the indicated ratios. (C) Purified NK cells stimulated with R-848 were incubated with HCT116 target cells at the indicated ratios. The role of Apo2L/TRAIL in lysis assays in panels B and C was determined by pre-incubation with neutralizing and non-neutralizing anti-Apo2L/TRAIL antibodies. The data has been confirmed with NK cells from at least 3 donors and less than 5% SD was observed.

Figure 4 shows the nucleotide sequence of human Apo-2 ligand cDNA (SEQ ID NO:1) and its derived amino acid sequence (SEQ ID NO:2). The "N" at nucleotide position 447 is used to indicate the nucleotide base may be a "T" or "G".

Figures 5A and 5B show the nucleotide sequence of a cDNA (SEQ ID NO:3) for full length human DR4 and its derived amino acid sequence (SEQ ID NO:4). The respective nucleotide and amino acid sequences for human DR4 are also reported in Pan et al., Science, 276:111 (1997).

Figure 6 shows the 411 amino acid sequence of human DR5 (SEQ ID NO:5) as published in WO 98/51793 on November 19, 1998.

Figure 7 shows a transcriptional splice variant of human DR5. This DR5 splice variant encodes the 440 amino acid sequence of human DR5 (SEQ ID NO:6) as published in WO 98/35986 on August 20, 1998.

Figure 8A illustrates the effects of the recited agents on B16 melanoma cells and suggests that the cells were lysed by activated NK cells in an Apo2L/TRAIL dependent manner. Figure 8B shows the assay results where B16 cells were labeled with ^{51}Cr and cultured with purified resting or stimulated NK cells at the indicated ratios. The role of Apo2L/TRAIL was evaluated by preincubation of NK cells with either a neutralizing (5C2) or non-neutralizing (1D1) mAb. The % lysis was plotted, and the results are representative of 3 experiments (less than 5% SD was observed). As shown in Figure 8B, the indicated cell lines and primary monocyte derived dendritic cells (DC) were labeled with ^{51}Cr and incubated with varying soluble Apo2L/TRAIL protein concentrations (indicated as "sApo2L") for 4 hours. The supernatants were then assessed for Cr release. Similar cytolysis results were obtained in at least 3 experiments.

Figure 9A is a bar diagram of assay results suggesting that cytotoxic granules of activated NK cells may be essential for lysis of 4T1 cells. The cytolytic activity of resting and activated NK cells was evaluated against 4T1 target cells. The activated NK cells were treated with inhibitors of Perforin maturation (concanamycin A), GraB (Z-AAD-FMK), PI3K (wortmannin), MEK1 kinase (PD98059), S6 kinase (rapamycin) and JNK (SP) to assess the role of cytotoxic granules. The E/T ratio was 25 to 1, and results shown are from one representative donor (out of three donors). Figure 9B illustrates assay results suggesting activated NK cells are capable of inducing activation of caspase-3 in 4T1 cells. 4T1 target cells were treated with Apo2L/TRAIL protein (100ng/ml) or with activated NK cells ("Act NK") for 30 minutes, 1 hour or 4 hours. The E/T ratio was 10:1. Extracts of the 4T1 cells were analysed by SDS-PAGE and immunoblot analysis for pro-caspase-3, cleaved caspase-3 and cleaved PARP.

Figure 10 shows the results of a PARP cleavage assay. 4T1 cells were treated with Apo2L/TRAIL, resting NK cells ("NK") or activated NK cells ("Act NK") separately or in combination. The lysates were analyzed for cleavage of PARP, an indicator of caspase-3 activation. NK cells from multiple donors were utilized and results from one representative donor are shown.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

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 techniques known in the art, for instance, by cell viability assays, FACS analysis or DNA electrophoresis, and more specifically by binding of annexin V, fragmentation of DNA, PARP cleavage, cell shrinkage, dilation of endoplasmatic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). These techniques and assays are described in the art, for example, in WO97/25428 and WO97/01633.

As used herein, the term "synergy" or "synergism" or "synergistically" refers to the interaction of two or more agents so that their combined effect is greater than the sum of the effects that result from the same treatment using the respective agents separately.

The terms "Apo-2 ligand", "Apo-2L", or "TRAIL" are used herein to refer to a polypeptide which includes amino acid residues 95-281, inclusive, 114-281, inclusive, residues 91-281, inclusive, residues 92-281, inclusive, residues 41-281, inclusive, residues 15-281, inclusive, or residues 1-281, inclusive, of the amino acid sequence shown in Figure 1A of Pitti et al., J. Biol. Chem., 271:12687-12690 (1996) (provided herein in Fig. 4), as well as biologically active (e.g., having apoptotic activity) fragments, deletional, insertional, or substitutional variants of the above sequences. In one embodiment, the polypeptide sequence comprises residues 114-281 of Fig. 4. Optionally, the polypeptide sequence has at least residues 91-281 or residues 92-281. In another preferred embodiment, the biologically active fragments or variants have at least about 80% amino acid sequence identity, more preferably at least about 90% amino acid sequence identity, and even more preferably, at least about 95%, 96%, 97%, 98%, or 99% amino acid sequence identity with any one of the above sequences. The definition encompasses substitutional variants of the Apo-2 ligand comprising amino acids 91-281 of Figure 1A of Pitti et al., J. Biol. Chem., 271:12687-12690 (1996) (Fig. 4 herein) in which at least one of the amino acids at positions 203, 218 or 269 (using the numbering of the sequence provided in Fig. 4) are substituted by an alanine residue. The definition encompasses Apo-2 ligand isolated from an Apo-2 ligand source, such as from human tissue types, or from another source, or prepared by recombinant or synthetic methods. The Apo-2 ligand may for example, be a soluble polypeptide or expressed on the cell surface of mammalian cells. The term Apo-2 ligand also refers to the polypeptides

described in WO 97/25428, supra, and WO97/01633, supra. It is contemplated that the Apo-2 ligand polypeptide may be linked to one or more polymer molecules such as polyethylene glycol.

"Percent (%) amino acid sequence identity" with respect to the Apo-2L polypeptide 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 an Apo-2L 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, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, ALIGN-2 or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared. Optionally, % amino acid sequence identity values are obtained by using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc. and the source code has been filed with user documentation in the U.S. Copyright Office, Washington, D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available through Genentech, Inc., South San Francisco, California. The ALIGN-2 program should be compiled for use on a UNIX operating system, preferably digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary. However, % amino acid sequence identity may also be determined using the sequence comparison program NCBI-BLAST2 (Altschul et al., Nucleic Acids Res. 25:3389-3402 (1997)). The NCBI-BLAST2 sequence comparison program may be downloaded from http://www.ncbi.nlm.nih.gov. NCBI-BLAST2 uses several search parameters, wherein all of those search parameters are set to default values including, for example, unmask = yes, strand = all, expected occurrences = 10, minimum low complexity length = 15/5, multi-pass e-value = 0.01, constant for multi-pass = 25, dropoff for final gapped alignment = 25 and scoring matrix = BLOSUM62.

The term "antibody" when used in reference to an "agonistic anti-Apo-2 ligand receptor antibody" 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 bind one or more Apo-2 ligand receptors and/or are capable of activating the apoptosis signaling pathway of the mammalian cell expressing one or more of the Apo-2 ligand receptors or mimic (e.g., have comparable or at least

equal to) the apoptotic activity of Apo-2 ligand or have greater apoptotic activity than that of Apo-2 ligand.

"Apo-2 ligand receptor" includes the receptors referred to in the art as "DR4" and "DR5". 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]. The DR4 receptor was reported to contain a cytoplasmic death domain capable of engaging the cell suicide apparatus. Pan et al. disclose that DR4 is believed to be a receptor for the ligand known as Apo2L/TRAIL. The amino acid sequence of the full length DR4 receptor is provided herein as Fig. 5. 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 (corresponding to issued US Patent 6,072,047); 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]. Like DR4, DR5 is reported to contain a cytoplasmic death domain and be capable of signaling apoptosis. A full length DR5 receptor sequence in WO98/35986 (corresponding to US Patent 6,072,047) is reported to be a 440 amino acid polypeptide, and that amino acid sequence is provided in Fig. 7. The full length DR5 receptor sequence in WO98/51793 is reported to be a 411 amino acid polypeptide, and that amino acid sequence is provided in Fig. 6. 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.

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)).

"Humanized" forms of non-human (*e.g.*, murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity-determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and

capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and maximize 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 CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optimally 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); Reichmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992). The humanized antibody includes a PRIMATIZED™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

Antibodies are typically proteins or polypeptides which exhibit binding specificity to a specific antigen. Native antibodies are usually heterotetrameric glycoproteins, composed of two identical light (L) chains and two identical heavy (H) chains. Typically, each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between 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 and heavy chain variable domains [Chothia et al., J. Mol. Biol., 186:651-663 (1985); Novotny and Haber, Proc. Natl. Acad. Sci. USA, 82:4592-4596 (1985)]. The light chains of antibodies from any vertebrate species can be assigned to one of two clearly distinct types, called kappa and 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, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG-1, IgG-2, IgG-3, and IgG-4; IgA-1 and IgA-2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called alpha, delta, epsilon, gamma, and mu, respectively.

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

The term "variable" is used herein to describe certain portions of the variable domains which differ 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 usually evenly distributed through the variable domains of antibodies. It is typically concentrated in three segments called complementarity determining regions (CDRs) or hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of the variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen binding site of antibodies [see Kabat, E.A. et al., Sequences of Proteins of Immunological Interest, National Institutes of Health, Bethesda, MD (1987)]. 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 cellular toxicity.

The monoclonal antibodies herein include chimeric, hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-Apo-2L receptor antibody with a constant domain (*e.g.* "humanized" antibodies), or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (*e.g.*, Fab, $F(ab')_2$, and Fv), so long as they exhibit the desired biological activity or properties. See, *e.g.* U.S. Pat. No. 4,816,567 and Mage et al., in Monoclonal Antibody Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc.: New York, 1987).

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues. Human antibodies can be produced using various techniques known in the art. In one embodiment, the human antibody is selected from a

phage library, where that phage library expresses human antibodies (Vaughan *et al.* Nature Biotechnology, 14:309-314 (1996): Sheets *et al.* PNAS, *(USA)* 95:6157-6162 (1998)); Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks *et al.*, J. Mol. Biol., 222:581 (1991)). Human antibodies can also be made by introducing human immunoglobulin loci into transgenic animals, *e.g.*, mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Patent Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks *et al.*, Bio/Technology, 10: 779-783 (1992); Lonberg *et al.*, Nature, 368: 856-859 (1994); Morrison, Nature, 368:812-13 (1994); Fishwild *et al.*, Nature Biotechnology, 14: 845-51 (1996); Neuberger, Nature Biotechnology, 14: 826 (1996); Lonberg and Huszar, Intern. Rev. Immunol., 13:65-93 (1995). Alternatively, the human antibody may be prepared via immortalization of human B lymphocytes producing an antibody directed against a target antigen (such B lymphocytes may be recovered from an individual or may have been immunized *in vitro*). See, *e.g.*, Cole *et al.*, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985); Boerner *et al.*, J. Immunol., 147 (1):86-95 (1991); and US Pat No. 5,750,373.

The term "Fc region" is used to define the C-terminal region of an immunoglobulin heavy chain which may be generated by papain digestion of an intact antibody. The Fc region may be a native sequence Fc region or a variant Fc region. Although the boundaries of the Fc region of an immunoglobulin heavy chain might vary, the human IgG heavy chain Fc region is usually defined to stretch from an amino acid residue at about position Cys226, or from about position Pro230, to the carboxyl-terminus of the Fc region (using herein the numbering system according to Kabat et al., supra). The Fc region of an immunoglobulin generally comprises two constant domains, a CH2 domain and a CH3 domain, and optionally comprises a CH4 domain.

By "Fc region chain" herein is meant one of the two polypeptide chains of an Fc region.

The "CH2 domain" of a human IgG Fc region (also referred to as "Cγ2" domain) usually extends from an amino acid residue at about position 231 to an amino acid residue at about position 340. The CH2 domain is unique in that it is not closely paired with another domain. Rather, two N-linked branched carbohydrate chains are interposed between the two CH2 domains of an intact native IgG molecule. It has been speculated that the carbohydrate may provide a substitute for the domain-domain pairing and help stabilize the CH2 domain. Burton, *Molec.*

Immunol.22:161-206 (1985). The CH2 domain herein may be a native sequence CH2 domain or variant CH2 domain.

The "CH3 domain" comprises the stretch of residues C-terminal to a CH2 domain in an Fc region (*i.e.* from an amino acid residue at about position 341 to an amino acid residue at about position 447 of an IgG). The CH3 region herein may be a native sequence CH3 domain or a variant CH3 domain (*e.g.* a CH3 domain with an introduced "protroberance" in one chain thereof and a corresponding introduced "cavity" in the other chain thereof; see US Patent No. 5,821,333).

"Hinge region" is generally defined as stretching from about Glu216, or about Cys226, to about Pro230 of human IgG1 (Burton, *Molec. Immunol*.22:161-206 (1985)). Hinge regions of other IgG isotypes may be aligned with the IgG1 sequence by placing the first and last cysteine residues forming inter-heavy chain S-S bonds in the same positions. The hinge region herein may be a native sequence hinge region or a variant hinge region. The two polypeptide chains of a variant hinge region generally retain at least one cysteine residue per polypeptide chain, so that the two polypeptide chains of the variant hinge region can form a disulfide bond between the two chains. The preferred hinge region herein is a native sequence human hinge region, *e.g.* a native sequence human IgG1 hinge region.

A "functional Fc region" possesses at least one "effector function" of a native sequence Fc region. Exemplary "effector functions" include C1q binding; complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (*e.g.* B cell receptor; BCR), etc. Such effector functions generally require the Fc region to be combined with a binding domain (*e.g.* an antibody variable domain) and can be assessed using various assays known in the art for evaluating such antibody effector functions.

A "native sequence Fc region" comprises an amino acid sequence identical to the amino acid sequence of an Fc region found in nature. A "variant Fc region" comprises an amino acid sequence which differs from that of a native sequence Fc region by virtue of at least one amino acid modification. Preferably, the variant Fc region has at least one amino acid substitution compared to a native sequence Fc region or to the Fc region of a parent polypeptide, *e.g.* from about one to about ten amino acid substitutions, and preferably from about one to about five amino acid substitutions in a native sequence Fc region or in the Fc region of the parent polypeptide. The variant Fc region herein will preferably possess at least about 80% sequence identity with a native sequence Fc region and/or with an Fc region of a parent polypeptide, and most preferably at least about 90% sequence identity therewith, more preferably at least about 95% sequence identity therewith.

The terms "Fc receptor" and "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 FcγRIII 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 (reviewed in 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)).

An "affinity matured" antibody is one with one or more alterations in one or more CDRs thereof which result in an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). Preferred affinity matured antibodies will have nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks *et al.* Bio/Technology, 10:779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR and/or framework residues is described by: Barbas *et al.* Proc Nat. Acad. Sci, *USA* 91:3809-3813 (1994); Schier *et al.* Gene, 169:147-155 (1995); Yelton *et al.* J. Immunol., 155:1994-2004 (1995); Jackson *et al.*, J. Immunol., 154(7):3310-9 (1995); and Hawkins *et al*, J. Mol. Biol., 226:889-896 (1992).

The terms "agonist" and "agonistic" when used herein refer to or describe a molecule which is capable of, directly or indirectly, substantially inducing, promoting or enhancing biological activity or activation of a receptor for Apo-2 ligand. Optionally, an "agonist Apo-2L receptor antibody" is an antibody which has activity that mimics or is comparable to Apo-2 ligand. Preferably, the agonist is a molecule which is capable of inducing apoptosis in a mammalian cell, preferably, a mammalian cancer cell. Even more preferably, the agonist is an antibody directed to an Apo-2L receptor and said antibody has apoptotic activity which is equal to or greater than Apo-2L polypeptide. Optionally, the

agonist activity of such molecule can be determined by assaying the molecule in an assay to examine apoptosis of one or more cancer cells. It is contemplated that the agonist may be linked to one or more polymer molecules such as polyethylene glycol.

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

"Biologically active" or "biological activity" for the purposes herein means (a) having the ability to induce or stimulate apoptosis in at least one type of mammalian cell, such as a cancer cell or virally-infected cell or bacteria-infected cell, *in vivo* or *ex vivo*; (b) capable of raising an antibody, i.e., immunogenic; or (c) retaining the activity of a native or naturally-occurring Apo-2 ligand polypeptide.

"NK cells" as used herein refer to lymphocytes which typically have CD16 and/or and/or NCAM and/or CD56 molecules expressed as cell curface markers but which do not express CD3. The NK cells refer to cells present *in vivo* in a mammal or *in vitro* in the form of a purified population of cells.

"NK cell activating agent" as used herein refers to agents which are able to enhance or increase cytolytic activity of resting (or untreated) NK cells in mammalian cancer cells or virus-infected cells. Such agents include but are not limited to, agents which activate one or more Toll receptors, such as Granzyme A or Granzyme B, various interleukins, such as IL-2, IL-12 IL-15, and interferons such as IFN-alpha, IFN-beta, and agonist antibodies to activating receptors such as NKp30, NKp44, NKG2D.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell *in vitro* and/or *in vivo*. Thus, the growth inhibitory agent may be one which significantly reduces the percentage of cells 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, oncogenes, and antineoplastic drugs" by Murakami *et al.* (WB Saunders: Philadelphia, 1995), especially p. 13.

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 chemical compound useful in the treatment of conditions like cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine,trietylenephosphoramide,triethylenethiophosphaoramide 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 CBI-TMI); 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, ranimustine; antibiotics such as the enediyne antibiotics (*e.g.* calicheamicin, especially calicheamicin γ_1^I and calicheamicin θ^I_1, see, *e.g.*, Agnew Chem Intl. Ed. Engl., 33:183-186 (1994); dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromomophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, 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, 5-FU; 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; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; 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.* paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, NJ) and doxetaxel (TAXOTERE®, Rhône-Poulenc Rorer, Antony, France);

chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); 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 including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

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-alpha and -beta; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-alpha; platelet-growth factor; transforming growth factors (TGFs) such as TGF-alpha and TGF-beta; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-alpha, -beta 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-1alpha, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12; a tumor necrosis factor such as TNF-alpha or TNF-beta; 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.

"Treatment" or "therapy" refer to both therapeutic treatment and prophylactic or preventative measures.

The term "effective amount" refers to an amount of a drug effective to treat a disease or disorder in a mammal. In the case of cancer, the therapeutically effective amount of the drug may reduce the number of

cancer cells; reduce the tumor size; inhibit (*i.e.*, slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (*i.e.*, slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the disorder. To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. For cancer therapy, efficacy *in vivo* can, for example, be measured by assessing tumor burden or volume, the time to disease progression (TTP) and/or determining the response rates (RR).

"Mammal" for purposes of treatment or therapy refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, *etc.* Preferably, the mammal is human.

The terms "cancer", "cancerous", or "maligant" 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, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include colon cancer, colorectal cancer, rectal cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, Hodgkin's and non-Hodgkin's lymphoma, testicular cancer, myeloma, esophageal cancer, gastrointestinal cancer, renal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, glioma, liver cancer, bladder cancer, hepatoma, breast cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

II. Methods and Materials

A. METHODS

Generally, the methods of the invention for inducing apoptosis or cytotoxicity in mammalian cells comprise exposing the cells to Apo-2 ligand or an Apo-2L receptor agonist antibody and NK cells or NK cell activating agent(s). Exemplary conditions or disorders to be treated with the Apo-2 ligand or agonist antibody and NK cells or NK cell activating agent include benign or malignant cancer, as well as viral infections.

Methods of treating mammalian cells with NK cells or NK cell activating agent, in combination with their treatment with the Apo-2L receptor agonist(s), can have a number of advantages over the administration of these agents as a single therapy. In particular, as noted above, these methods can facilitate treatment modalities by identifying the optimal conditions for the combined administration of these agents. Consequently, by identifying methods to optimize an apoptotic response, medical practitioners may be able to dispense these agents in a more convenient and patient friendly format. Specifically,

employing methods which optimize an apoptotic response, medical practitioners may administer these agents in a single bolus rather than in multiple injections, administer lower concentrations of these agents or administer these agents for shorter periods of time.

In accordance with one embodiment of the invention, there is provided a method of inducing apoptosis in mammalian cells comprising exposing the cells to an effective amount of an Apo-2 ligand receptor agonist and NK cells or NK cell activating agent. In the methods, the Apo-2 ligand receptor agonist typically comprises Apo2L/TRAIL or anti-DR4 or DR5 receptor antibody. Additional embodiments of the invention include variations on these methods such as those that employ additional therapeutic modalities, such as exposing the cancer cells to one or more growth inhibitory agents or radiation.

B. MATERIALS

The Apo-2L which can be employed in the methods includes the Apo-2L polypeptides described in Pitti et al., supra, WO 97/25428, supra, and WO97/01633, supra (the polypeptides referred to as TRAIL). It is contemplated that various forms of Apo-2L may be used, such as the full length polypeptide as well as soluble forms of Apo-2L which comprise an extracellular domain (ECD) sequence. Examples of such soluble ECD sequences include polypeptides comprising amino acids 114-281, 95-281, 91-281 or 92-281 of the Apo-2L sequence shown in Figure 1A of Pitti et al., J. Biol. Chem., 271:12687-12690 (1996) and Fig. 4 herein. It is presently believed that the polypeptide comprising amino acids 92-281 is a naturally cleaved form of Apo-2L. Applicants have expressed human Apo-2L in CHO cells and found that the 92-281 polypeptide is the expressed form of Apo-2L. Modified forms of Apo-2L, such as the covalently modified forms described in WO 97/25428 are included. In particular, Apo-2L linked to a non-proteinaceous polymer such as polyethylene glycol is included for use in the present methods. The Apo-2L polypeptide can be made according to any of the methods described in WO 97/25428.

Variants of Apo-2 ligand having apoptotic activity which can be used in the methods include, for example, those identified by alanine scanning techniques. Particular substitutional variants comprise amino acids 91-281 of Figure 1A of Pitti et al., J. Biol. Chem., 271:12687-12690 (1996) in which at least one of the amino acids at positions 203, 218 or 269 are substituted by an alanine residue. Optionally, the Apo-2 ligand variants may include one or more of these three different site substitutions.

It is contemplated that a molecule which mimics the apoptotic activity of Apo-2L may alternatively be employed in the presently disclosed methods. Examples of such molecules include agonistic antibodies which can induce apoptosis in at least a comparable or like

manner to Apo-2L. In particular, these agonist antibodies would comprise antibodies which bind one or more of the receptors for Apo-2L. Preferably, the agonist antibody is directed to an Apo-2L receptor which includes a cytoplasmic death domain, such as DR4 or DR5. Even more preferably, the agonist antibody binds to such a receptor and binding can be determined, e.g., using FACS analysis or ELISA. Agonist antibodies directed to the receptor called DR5 (or Apo-2) have been prepared using fusion techniques such as described below. One of the DR5 or Apo-2 receptor agonist antibodies is referred to as 3F11.39.7 and has been deposited with ATCC as deposit no. HB-12456 on January 13, 1998. Other DR5 receptor antibodies include 3H3.14.5, deposited with ATCC. Agonist activity of the Apo-2L receptor antibodies can be determined using various methods for assaying for apoptotic activity, and optionally, apoptotic activity of such antibody can be determined by assaying the antibody, alone or in a cross-linked form using Fc immunoglobulin or complement in an assay to examine apoptosis of cells expressing an Apo-2L receptor such as DR4 or DR5.

Additionally, agonist antibodies directed to another Apo-2L receptor, called DR4, have also been prepared. One of the DR4 agonist antibodies is referred to as 4H6.17.8 and has been deposited with ATCC as deposit no. HB-12455 on January 13, 1998. Still further agonist DR4 antibodies include the antibodies 4E7.24.3, 1H5.25.9, 4G7.18.8, and 5G11.17.1 which have been deposited with ATCC. Agonist activity of the Apo-2L receptor antibodies can be determined using various methods for assaying for apoptotic activity, and optionally, apoptotic activity of such antibody can be determined by assaying the antibody, alone or in a cross-linked form using Fc immunoglobulin or complement.

Agonist antibodies contemplated by the invention include antibodies which bind a single Apo-2L receptor or more than one Apo-2L receptor. An antibody which binds more than one Apo-2L receptor can be characterized as an antibody that "cross-reacts" with two or more different antigens and capable of binding to each of the different antigens, *e.g.* as determined by ELISA or FACS. Optionally, an antibody which "specifically cross-reacts" with two or more different antigens is one which binds to a first antigen and further binds to a second different antigen, wherein the binding ability of the antibody for the second antigen at an antibody concentration of about 10μg/mL is from about 50% to about 100% (preferably from about 75% to about 100%) of the binding ability of the first antigen as determined in a capture ELISA. For example, the antibody may bind specifically to DR5 (the "first antigen") and specifically cross-react with another Apo-2L receptor such as DR4 (the "second antigen"), wherein the extent of binding of about 10μg/mL of the antibody to DR4 is about 50% to about 100% of the binding ability of the antibody for DR5 in a capture ELISA. Various cross-reactive antibodies

to Apo-2L receptors are described in further detail in International Patent application number PCT/US99/13197.

As described below, exemplary forms of such antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies.

1. Polyclonal Antibodies

The antibodies of the invention may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include a DR4 or DR5 polypeptide (or a DR4 or DR5 ECD) or a fusion protein thereof. It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation. The mammal can then be bled, and the serum assayed for antibody titer. If desired, the mammal can be boosted until the antibody titer increases or plateaus.

2. Monoclonal Antibodies

The antibodies of the invention may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, Nature, 256:495 (1975). In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized *in vitro*.

The immunizing agent will typically include a DR4 or DR5 polypeptide or a fusion protein thereof, such as a DR4 or DR5 ECD-IgG fusion protein.

Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell [Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, (1986) pp. 59-103]. Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually,

rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental 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 immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, California and the American Type Culture Collection, Manassas, Virginia. An example of such a murine myeloma cell line is P3X63AgU.1 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, Marcel Dekker, Inc., New York, (1987) pp. 51-63].

The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against the Apo-2L receptor. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). Such techniques and assays are known in the art. The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Pollard, Anal. Biochem., 107:220 (1980).

After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods [Goding, supra]. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium or RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in vivo* as ascites in a mammal.

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

The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be 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 of the invention 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 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. 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., supra] or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention, or can be substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody.

The antibodies of the invention include "cross-linked" antibodies. The term "cross-linked" as used herein refers to binding of at least two IgG molecules together to form one (or single) molecule. The Apo-2L receptor antibodies may be cross-linked using various linker molecules, optionally the DR4 antibodies are cross-linked using an anti-IgG molecule, complement, chemical modification or molecular engineering. It is appreciated by those skilled in the art that complement has a relatively high affinity to antibody molecules once the antibodies bind to cell surface membrane. Accordingly, it is believed that complement may be used as a cross-linking molecule to link two or more antibodies bound to cell surface membrane. Among the various murine Ig isotypes, IgM, IgG2a and IgG2b are known to fix complement.

The antibodies of the invention may optionally comprise dimeric antibodies, as well as multivalent forms of antibodies. Those skilled in the art may contruct such dimers or multivalent forms by techniques known in the art and using the anti-Apo-2L receptor antibodies herein.

The antibodies of the invention may also comprise monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

In vitro methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques

known in the art. For instance, digestion can be performed using papain. Examples of papain digestion are described in WO 94/29348 published 12/22/94 and U.S. Patent No. 4,342,566. Papain digestion of antibodies typically produces two identical antigen binding fragments, called Fab fragments, each with a single antigen binding site, and a residual Fc fragment. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen combining sites and is still capable of cross-linking antigen.

The Fab fragments produced in the antibody digestion also contain the constant domains of the light chain and the first constant domain (CH_1) 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 CH_1 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 a 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.

Single chain Fv fragments may also be produced, such as described in Iliades et al., FEBS Letters, 409:437-441 (1997). Coupling of such single chain fragments using various linkers is described in Kortt et al., Protein Engineering, 10:423-433 (1997).

In addition to the antibodies described above, it is contemplated that chimeric or hybrid antibodies may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate.

The Apo-2L receptor antibodies of the invention may further comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. 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 CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [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)].

Methods for humanizing non-human antibodies are well known in the art. Generally, 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 rodent CDRs or CDR 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 CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies. Sources of such import residues or import variable domains (or CDRs) include the deposited anti-Apo-2L receptor antibodies 4H6.17.8, 3F11.39.7, 4E7.24.3, 1H5.25.9, 4G7.18.8, 5G11.17.1, and 3H3.14.5.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important in order to reduce antigenicity. According to the "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 (FR) for the humanized antibody [Sims et al., J. Immunol., 151:2296-2308 (1993); Chothia and Lesk, J. Mol. Biol., 196:901-917 (1987)]. Another method uses a particular framework 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, 89:4285-4289 (1992); Presta et al., J. Immunol., 151:2623-2632 (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 consensus and import sequence so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding [see, WO 94/04679 published 3 March 1994].

Human monoclonal antibodies may be made via an adaptation of the hybridoma method first described by Kohler and Milstein by using human B lymphocytes as the fusion partner. Human B lymphocytes producing an antibody of interest may, for example, be isolated from a human individual, after obtaining informed consent. For instance, the individual may be producing antibodies against an autoantigen as occurs with certain disorders such as systemic lupus erythematosus (Shoenfeld *et al.* J. Clin. Invest., 70:205 (1982)), immune-mediated thrombocytopenic purpura (ITP) (Nugent *et al.* Blood, 70(1): 16-22 (1987)), or cancer. Alternatively, or additionally, lymphocytes may be immunized *in vitro*. For instance, one may expose isolated human periperal blood lymphocytes *in vitro* to a lysomotrophic agent (*e.g.* L-leucine-O-methyl ester, L-glutamic acid dimethly ester or L-leucyl-L-leucine-O-methyl ester) (US Patent No. 5,567,610, Borrebaeck *et al.*); and/or T-cell depleted human peripheral blood lymphocytes may be treated *in vitro* with adjuvants such as 8-mercaptoguanosine and cytokines (US Patent No. 5,229,275, Goroff *et al.*).

The B lymphocytes recovered from the subject or immunized *in vitro*, are then generally immortalized in order to generate a human monoclonal antibody. Techniques for immortalizing the B lymphocyte include, but are not limited to: (a) fusion of the human B lymphocyte with human, murine myelomas or mouse-human heteromyeloma cells; (b) viral transformation (*e.g.* with an Epstein-Barr virus; see Nugent *et al.*, *supra*, for example); (c) fusion with a lymphoblastoid cell line; or (d) fusion with lymphoma cells.

Lymphocytes may be 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. Suitable human myeloma and mouse-human heteromyeloma cell lines have been described (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).

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. 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 chromatography, gel electrophoresis, dialysis, or affinity chromatography.

Human antibodies may also be generated using a non-human host, such as a mouse, which is capable of producing human antibodies. As noted above, transgenic mice are now available 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); US Patent No. 5,591,669; US Patent No. 5,589,369; and US Patent No. 5,545,807. Human antibodies may also be prepared using SCID-hu mice (Duchosal *et al. Nature* 355:258-262 (1992)).

In another embodiment, the human antibody may be selected from a human antibody phage display library. The preparation of libraries of antibodies or fragments thereof is well known in the art and any of the known methods may be used to construct a family of transformation vectors

which may be introduced into host cells. Libraries of antibody light and heavy chains in phage (Huse *et al.*, *Science*, 246:1275 (1989)) or of fusion proteins in phage or phagemid can be prepared according to known procedures. See, for example, Vaughan *et al.*, *Nature Biotechnology* 14:309-314 (1996); Barbas *et al.*, *Proc. Natl. Acad. Sci., USA*, 88:7978-7982 (1991); Marks *et al.*, *J. Mol. Biol.*, 222:581-597 (1991); Hoogenboom and Winter, *J. Mol. Biol.*, 227:381-388 (1992); Barbas *et al.*, *Proc. Natl. Acad. Sci., USA*, 89:4457-4461 (1992); Griffiths *et al.*, *EMBO Journal*, 13:3245-3260 (1994); de Kruif *et al.*, *J. Mol. Biol.*, 248:97-105 (1995); WO 98/05344; WO 98/15833; WO 97/47314; WO 97/44491; WO 97/35196; WO 95/34648; US Patent No. 5,712,089; US Patent No. 5,702,892; US Patent No. 5,427,908; US Patent No. 5,403,484; US Patent No. 5,432,018; US Patent No. 5,270,170; WO 92/06176; WO 99/06587; US Patent No. 5,514,548; WO97/08320; and US Patent No. 5,702,892. The antigen of interest is panned against the phage library using procedures known in the field for selecting phage-antibodies which bind to the target antigen.

The Apo-2L receptor antibodies, as described herein, will optionally possess one or more desired biological activities or properties. Such antibodies may include but are not limited to chimeric, humanized, human, and affinity matured antibodies. As described above, the antibodies may be constructed or engineered using various techniques to achieve these desired activities or properties. In one embodiment, the Apo-2L receptor antibody will have a DR4 or DR5 receptor binding affinity of at least 10^5 M^{-1}, preferably at least in the range of 10^6 M^{-1} to 10^7 M^{-1}, more preferably, at least in the range of 10^8 M^{-1} to 10^{12} M^{-1} and even more preferably, at least in the range of 10^9 M^{-1} to 10^{12} M^{-1}. The binding affinity of the antibody can be determined without undue experimentation by testing the antibody in accordance with techniques known in the art, including Scatchard analysis (see Munson et al., supra).

In another embodiment, the Apo-2L receptor antibody of the invention may bind the same epitope on DR4 or DR5 to which Apo-2L binds, or bind an epitope on DR4 or DR5 which coincides or overlaps with the epitope on DR4 or DR5, respectively, to which Apo-2L binds. The antibody may also interact in such a way to create a steric conformation which prevents Apo-2 ligand binding to DR4 or DR5. The epitope binding property of the antibody of the present invention may be determined using techniques known in the art. For instance, the antibody may be tested in an *in vitro* assay, such as a competitive inhibition assay, to determine the ability of the antibody to block or inhibit binding of Apo-2L to DR4 or DR5. Optionally, the antibody may be tested in a competitive inhibition assay to determine the ability of, e.g., a DR4 antibody to inhibit binding of an Apo-2L polypeptide to a DR4-IgG construct or to a cell expressing DR4. Optionally, the antibody will be capable of

blocking or inhibiting binding of Apo-2L to the receptor by at least 50%, preferably by at least 75% and even more preferably by at least 90%, which may be determined, by way of example, in an *in vitro* competitive inhibition assay using a soluble form of Apo-2 ligand (TRAIL) and a DR4 ECD-IgG.

In a preferred embodiment, the antibody will comprise an agonist antibody having activity which mimics or is comparable to Apo-2 ligand (TRAIL). Preferably, such an agonistic DR4 or DR5 antibody will induce apoptosis in at least one type of cancer or tumor cell line or primary tumor. The apoptotic activity of an agonistic DR4 or DR5 antibody may be determined using known *in vitro* or *in vivo* assays. *In vitro*, apoptotic activity can be determined using known techniques such as Annexin V binding. *In vivo*, apoptotic activity may be determined, e.g., by measuring reduction in tumor burden or volume.

3. Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for an Apo-2L receptor, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit.

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have different specificities [Milstein and Cuello, Nature, 305:537-539 (1983)]. Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

Antibody variable domains with the desired binding specificities (antibody-antigen combining sites) can be 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. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986).

4. Heteroconjugate Antibodies

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells [U.S. Patent No. 4,676,980], and for treatment of HIV infection [WO 91/00360; WO 92/200373; EP 03089]. It is contemplated that the antibodies may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate and those disclosed, for example, in U.S. Patent No. 4,676,980.

5. Triabodies

Triabodies are also within the scope of the invention. Such antibodies are described for instance in Iliades et al., supra and Kortt et al., supra.

6. Other Modifications

Other modifications of the Apo-2L receptor antibodies are contemplated herein. The antibodies of the present invention may be modified by conjugating the antibody to a cytotoxic agent (like a toxin molecule) or 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. This technology is also referred to as "Antibody Dependent Enzyme Mediated Prodrug Therapy" (ADEPT).

The enzyme component of the immunoconjugate useful for ADEPT 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; caspases such as caspase-3; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as beta-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; beta-lactamase useful for converting drugs derivatized with beta-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 can be covalently bound to the antibodies by techniques well known in the art such as the use of heterobifunctional crosslinking reagents. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention 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).

Further antibody modifications are contemplated. 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. The antibody also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences*, 16th edition, Osol, A., Ed., (1980). 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 U.S. 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.

7. Recombinant Methods

The invention also provides isolated nucleic acids encoding the antibodies as disclosed herein, vectors and host cells comprising the nucleic acid, and recombinant techniques for the production of the antibody.

For recombinant production of the antibody, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the antibody is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the antibody). Many vectors are 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.

The methods herein include methods for the production of chimeric or recombinant anti-Apo-2L receptor antibodies which comprise the steps of providing a vector comprising a DNA sequence encoding an anti-Apo-2L receptor antibody light chain or heavy chain (or both a light chain and a heavy chain), transfecting or transforming a host cell with the vector, and culturing the host cell(s) under conditions sufficient to produce the recombinant anti-Apo-2L receptor antibody product.

(i) Signal sequence component

The anti-Apo-2L receptor antibody of this invention may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which is preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. The heterologous signal sequence selected preferably is one that is recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the native antibody signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native signal sequence may be substituted by, *e.g.*, the yeast invertase leader, α factor leader (including *Saccharomyces* and *Kluyveromyces* α-factor leaders), or acid phosphatase leader, the *C. albicans* glucoamylase leader, or the signal described in WO 90/13646. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available. The DNA for such precursor region is ligated in reading frame to DNA encoding the antibody.

(ii) Origin of replication component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

(iii) Selection gene component

Expression and cloning vectors may contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin, mycophenolic acid and hygromycin.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the antibody nucleic acid, such as DHFR, thymidine kinase, metallothionein-I and -II, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, *etc.*

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity.

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding the anti-Apo-2L receptor antibody, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, *e.g.*, kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp*1 gene present in the yeast plasmid YRp7 (Stinchcomb *et al.*, *Nature*, 282:39 (1979)). The *trp*1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1. Jones, *Genetics*, 85:12 (1977). The presence of the *trp*1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu*2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu*2 gene.

In addition, vectors derived from the 1.6 μm circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts. Alternatively, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis*. Van den Berg, *Bio/Technology*, 8:135 (1990). Stable multi-copy expression vectors for secretion of mature

recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed. Fleer *et al.*, *Bio/Technology*, 9:968-975 (1991).

(iv) Promoter component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the antibody nucleic acid. Promoters suitable for use with prokaryotic hosts include the *phoA* promoter, β-lactamase and lactose promoter systems, alkaline phosphatase, a tryptophan (trp) promoter system, and hybrid promoters such as the tac promoter. However, other known bacterial promoters are suitable. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the anti-Apo-2L receptor antibody.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CNCAAT region where N may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase or other glycolytic enzymes, such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

Anti-Apo-2L receptor antibody transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, *e.g.*, the actin promoter or an immunoglobulin promoter, from heat-shock

promoters, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978. See also Reyes *et al.*, *Nature* 297:598-601 (1982) on expression of human β-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus. Alternatively, the rous sarcoma virus long terminal repeat can be used as the promoter.

(v) Enhancer element component

Transcription of a DNA encoding the anti-Apo-2L receptor antibody of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, *Nature* 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the antibody-encoding sequence, but is preferably located at a site 5' from the promoter.

(vi) Transcription termination component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the multivalent antibody. One useful transcription termination component is the bovine growth hormone polyadenylation region. See WO94/11026 and the expression vector disclosed therein.

(vii) Selection and transformation of host cells

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include 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.* One optional *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for Apo-2L receptor antibody-encoding vectors. *Saccharomyces cerevisiae,* or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe; Kluyveromyces* hosts such as, *e.g., K. lactis, K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K . thermotolerans,* and *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa; Schwanniomyces* such as *Schwanniomyces occidentalis;* and filamentous fungi such as, *e.g., Neurospora, Penicillium, Tolypocladium,* and *Aspergillus* hosts such as *A. nidulans* and *A. niger.*

Suitable host cells for the expression of glycosylated antibody are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, *e.g.,* the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al., J. Gen Virol.* 36:59 (1977)); baby

hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR /(CHO, Urlaub *et al., Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al., Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; a human hepatoma line (Hep G2); and myeloma or lymphoma cells (*e.g.* Y0, J558L, P3 and NS0 cells) (see US Patent No. 5,807,715).

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

(viii) Culturing the host cells

The host cells used to produce the antibody of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham *et al., Meth. Enz.* 58:44 (1979), Barnes *et al., Anal. Biochem.*102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as culture media for the host cells. Any of these 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), nucleotides (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.

(ix) Purification

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter *et al., Bio/Technology* 10:163-167 (1992) describe a procedure for isolating

antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 minutes. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc region that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark *et al., J. Immunol. Meth.* 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human γ3 (Guss *et al., EMBO J.* 5:15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™ resin (J. T. Baker, Phillipsburg, NJ) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

NK cells can be obtained from a mammal using techniques known in the art. Optionally, the NK cells are human cells obtained and purified from a human donor. NK cells may be purified using materials that are readily and commercially available, including but not limited to, those described in the Examples below.

NK cell activating agents include various small molecules, cytokines and antibodies such as Toll receptor activating agents, IL-2, IL-12, IL-15, IFN-alpha, IFN-beta, and agonist antibodies to activating receptors such as NKp30, NKp44, NKG2D. Such agents are readily and commercially available.

C. FORMULATIONS

The Apo-2 ligand or Apo-2L receptor agonist antibody and NK cells or NK cell activating agent are preferably administered in a carrier. The molecules can be administered in a single carrier, or alternatively, can be included in separate carriers. Suitable carriers and their formulations are described in Remington's Pharmaceutical Sciences, 16th ed., 1980, Mack Publishing Co., edited by Osol et al. Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the carrier to render the formulation isotonic. Examples of the carrier include saline, Ringer's solution and dextrose solution. The pH of the solution is preferably from about 5 to about 8, and more preferably from about 7.4 to about 7.8. 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 concentration of agent being administered. The carrier may be in the form of a lyophilized formulation or aqueous solution.

Acceptable carriers, excipients, or stabilizers are preferably nontoxic to cells and/or recipients at the dosages and concentrations employed, and include buffers such as 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; chelating agents such as EDTA; 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).

The formulation may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Alternatively, or in addition, the composition may comprise a cytotoxic agent, cytokine or growth inhibitory agent. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The Apo-2L or agonist antibody and NK cells or NK cell activating agent may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-

(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980).

The formulations to be used for *in vivo* administration should be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, *e.g.* films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, 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. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods.

D. MODES OF ADMINISTRATION

The Apo-2L or Apo-2L receptor agonist antibody and NK cells or NK cell activating agent 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, intracerobrospinal, 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 for administering Apo-2 ligand or agonist antibody and NK cells or NK cell activating agent may be determined empirically, and making such determinations is within the skill in the art. It is presently believed that an effective dosage or amount of Apo-2 ligand used alone may range from about 1 μg/kg to about 100 *mg*/kg of body weight or more per day. An effective dosage or amount of NK cells or NK cell activating agent used alone may range from about 1 mg/m^2 to about 150 mg/m^2. 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). Those skilled in the art will understand that the dosage of Apo-2 ligand or agonist antibody and NK cells or NK cell activating agent that must be administered will vary depending on, for example, the

mammal which will receive the Apo-2 ligand or agonist antibody and NK cells or NK cell activating agent, the route of administration, and other drugs or therapies being administered to the mammal.

Depending on the type of cells and/or severity of the disease, about 1 μg/kg to 15 mg/kg (*e.g.* 0.1-20 mg/kg) of agonist antibody is an initial candidate dosage for administration, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful.

It is contemplated that one or more Apo-2L receptor agonists may be employed in the methods. For example, the skilled practitioner may employ Apo-2 ligand, DR4 agonist antibody, DR5 agonist antibody, or combinations thereof. Optionally, the Apo-2L receptor agonist antibody will comprise a cross-reactive antibody which binds to both DR4 and DR5.

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, other chemotherapies (or chemotherapeutic agents) and/or radiation therapy, immunoadjuvants, growth inhibitory agents, cytokines, and other non-Her-2 antibody-based therapies. Examples include interleukins (*e.g.*, IL-1, IL-2, IL-3, IL-6), leukemia inhibitory factor, interferons, TGF-beta, erythropoietin, thrombopoietin, and anti-VEGF antibody. Other agents known to induce apoptosis in mammalian cells may also be employed, and such agents include TNF-α, TNF-β (lymphotoxin-α), CD30 ligand, 4-1BB ligand, and Apo-1 ligand.

Additional chemotherapies contemplated by the invention include chemical substances or drugs which are known in the art and are commercially available, such as Adriamycin, Doxorubicin, 5-Fluorouracil, Cytosine arabinoside ("Ara-C"), Cyclophosphamide, Leucovorin, Thiotepa, Busulfan, Cytoxin, Taxol, Toxotere, Methotrexate, Cisplatin, Melphalan, Vinblastine, Bleomycin, Etoposide, Ifosfamide, Mitomycin C, Mitoxantrone, Vincreistine, Vinorelbine, Carboplatin, Teniposide, Daunomycin, Carminomycin, Aminopterin, Dactinomycin, Mitomycins, Esperamicins (see U.S. Pat. No. 4,675,187), Melphalan and other related nitrogen mustards. Also included are agents that act to regulate or inhibit hormone action on tumors such as tamoxifen and onapristone.

Preparation and dosing schedules for such chemotherapy 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 with the Apo-2L or agonist antibody

and/or NK cells or NK cell activating agent or may be given simultaneously therewith.

The chemotherapy is preferably administered in a carrier, such as those described above. The mode of administration of the chemotherapy may be the same as employed for the Apo-2 ligand or agonist antibody or NK cells or NK cell activating agent or it may be administered via a different mode.

Radiation therapy can be administered according to protocols commonly employed in the art and known to the skilled artisan. Such therapy may include cesium, iridium, iodine, or cobalt radiation. The radiation therapy may be whole body irradiation, or may be directed locally to a specific site or tissue in or on the body. Typically, radiation therapy is administered in pulses over a period of time from about 1 to about 2 weeks. The radiation therapy may, however, be administered over longer periods of time. Optionally, the radiation therapy may be administered as a single dose or as multiple, sequential doses.

Following administration of Apo-2 ligand or agonist antibody and NK cells or NK cell activating agent, 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, tumor mass may be observed physically, by biopsy or by standard x-ray imaging techniques.

III. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container and a label. Suitable containers include, for example, bottles, vials, syringes, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition 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). The active agents in the composition are the Apo-2 ligand or agonist antibody and NK cells or NK cell activating agent. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

The following examples are offered by way of illustration and not by way of limitation. The disclosures of all citations in the specification are expressly incorporated herein by reference.

EXAMPLES

Example 1

Effect of Toll Receptor Activators and NK cells on 4T1 cells

4T1 cells (a metastatic mammary carcinoma cell line derived from BALB/C mice, obtained from Fred Miller, Karmanos Cancer Institute) were assayed in an *in vitro* cytotoxicity assay to examine tumoricical effects of various Toll receptor ("TLR") activators and NK cells. The 4T1 target cells were cultured in IDMEM medium supplemented with 10% FCS, glutamine and penicillin/streptomycin, labeled with 100 mCi ^{51}Cr for 1 hour at 37°C and then washed three times with culture medium.

To prepare purified effector NK cells for the assay, PBMCs from healthy human donors were isolated by standard density-gradient centrifugation over Ficoll-Paque Plus (Pharmacia). CD3 positive cells were first depleted using anti-CD3 microbeads (obtained from Miltenyi), and then the NK cells were purified using anti-CD56 microbeads (Miltenyi). The purity of the NK cell preparation was 95% or higher as determined by staining with mouse anti-human CD56 PE and mouse anti-human CD14 PE conjugates (BD Biosciences). The isolated cells were cultured in RPMI 1640 supplemented with heat inactivated 10% FCS, penicillin, streptomycin, 10mM sodium pyruvate, 2mM L-glutamine and 10mM HEPES.

10^4 of the labeled 4T1 cells were added to varying numbers of purified NK cells (either untreated control NK cells or NK cells treated with specific agent) at ratios of 1:1, 3:1, 6:1, 12:1, 25:1, and 50:1, respectively. NK cells were treated for 18 hours with one of the following TLR activating agents: sBLP (1 μg/ml, Bachem); poly(I:C) (50 μg/ml, Pharmacia); E. coli 055:B5 LPS (phenol extracted, 1 μg/ml, Sigma); Flagellin (1 μg/ml, A. Gewirtz, Emory Univerity); R-848 (5 μg/ml, Invivogen); CpG oligonucleotides (2006 and 2216, 5 μM, Invivogen). The cytotoxicity assays were performed in round bottom 96 well plates, and the samples were incubated in duplicate wells for 4 hours at 37°C, 5% CO_2. RPMI 1640 supplemented with heat inactivated 10% FCS, penicillin, streptomycin, 10mM sodium pyruvate, 2mM L-glutamine and 10mM HEPES was used during incubation.

After the 4 hour incubation, supernatants were aspirated from the wells and examined for ^{51}Cr release using a Gamma counter. The percent specific lysis was calculated as 100X (experimental cpm-spontaneous

cpm)/(total cpm-spontaneous cpm). The data are representative of at least three experiments and <5% SD was observed.

The results are illustrated in Figure 1. Little to no lysis of the 4T1 cells was observed when treated with control (resting) NK cells, even at high effector to target (E:T) ratios, while the cells were efficiently lysed by NK cells activated with poly (I:C). In addition, NK cells treated with the TLR7 and TLR8 activator, R-848, also efficiently killed the 4T1 target cells. Under the particular assay conditions, neither BLP, LPS, Flagellin, nor the CpG oligonucleotides induced cytotoxic activity of the purified NK cells toward the 4T1 targets.

Example 2

Induction of Apo2L/TRAIL by NK cells

A microarray based gene expression analysis using high-density oligonucleotide arrays (GeneChip, Affymetrix) was performed using RNA from resting and poly(I:C)-treated NK cells (NK cells were obtained and purified from 4 healthy donors using the technique described in Example 1 above). The NK cells were treated with poly(I:C) (50 μg/ml, Pharmacia) overnight in RPMI 1640 supplemented with heat inactivated 10% FCS, penicillin, streptomycin, 10mM sodium pyruvate, 2mM L-glutamine and 10mM HEPES. RNA was isolated using a RNeasy kit (Qiagen), and treated with RNase-free DNase-I (Ambion) to ensure removal of contaminating DNA. NK cells were lysed in lysis buffer (50mM HEPES pH7.5, 150mM NaCl, 1.5mM $MgCl_2$, 1mM EGTA, 10% glycerol and 1% Triton X-100, supplemented with Complete Protease Inhibitor Cocktail (Roche).

Affymetrix *U133A and B* GeneChip probe arrays were used to identify differentially expressed transcripts. Initially, the genes were ranked according to concordance in the pairwise comparison, and the Mann-Whitney pairwise comparison test was used to calculate significance. Genes for which the concordance was 100% were chosen for further analysis. This approach was previously validated to identify differentially expressed genes by confirming with real time RT-PCR. To determine confidence intervals shown in Table 1, the average $\log_{10}$-signal was computed for each probe set of the genes of interest, or the treatment and control groups. The estimated $\log_{10}$ fold change was defined as the difference between these averages. A pooled estimate of the standard deviation about the two groups' averages (computed separately for each probe set) was used to compute a 95% confidence interval for the $\log_{10}$ fold change. The estimated $\log_{10}$ fold change and the two endpoints of its 95% confidence interval were exponentiated back to the original signal scale, providing an estimate of fold change and a corresponding 95% confidence interval.

Additional information about the U133 probe sets is available from the manufacturer, Affymetrix.

The induction of IL-6, IL-8 and IFN-gamma observed in this analysis confirms previous results reported in Schmidt et al., J. Immunol., 172:138-143 (2004) and validates this approach to analyze other genes regulated by TLR activation (Table 1).

Table 1

Probe Set	Gene	log10-FoldChange	SE(log10-FoldChange)	95% CI low-end for FoldChange	FoldChange	95% high-end for FoldChange
202688_at	Apo2L	0.99	0.06	6.77	9.67	13.82
202687_s_at	Apo2L	1.13	0.1	7.55	13.42	23.86
214329_x_at	Apo2L	0.77	0.13	2.87	5.85	11.94
207113_s_at	TNFα	0.43	0.11	1.43	2.68	5.04
211333_s_at	FasL	0.4	0.1	1.46	2.49	4.26
210865_at	FasL	0.37	0.07	1.61	2.37	3.48
202859_x_at	IL-8	0.61	0.21	1.25	4.07	13.27
211506_s_at	IL-8	0.53	0.19	1.19	3.42	9.83
210354_at	IFNγ	1.16	0.29	2.8	14.45	74.52
205207_at	IL-6	1.02	0.21	3.23	10.43	33.67
210072_at	CCL19	1.54	0.16	14.49	34.71	83.14
205476_at	CCL20	0.9	0.33	1.22	7.96	51.84
204606_at	CCL21	0.08	0.2	0.39	1.2	3.69
206337_at	CCR7	0.68	0.12	2.4	4.79	9.55
206785_s_at	NKG2A	-0.01	0.06	0.69	0.97	1.37
214574_x_at	NKp30	-0.6	0.23	0.07	0.25	0.95
214181_x_at	NKp30	-0.67	0.3	0.04	0.22	1.19
211582_x_at	NKp30	-0.72	0.3	0.03	0.19	1.03
211581_x_at	NKp30	-0.59	0.27	0.06	0.26	1.15
210629_x_at	NKp30	-0.42	0.11	0.21	0.38	0.69
210690_at	NKG2-D	-0.18	0.11	0.34	0.66	1.26
205821_at	NKG2-D	-0.07	0.04	0.7	0.86	1.05
205495_s_at	Granulysin	0.1	0.09	0.76	1.25	2.08
37145_at	Granulysin	0.07	0.08	0.77	1.18	1.82
214617_at	Perforin 1	0.12	0.05	0.96	1.3	1.77
205488_at	Granzyme A	-0.08	0.05	0.64	0.83	1.09
210164_at	Granzyme B	0.02	0.05	0.79	1.04	1.37
210321_at	Granzyme H	-0.24	0.11	0.31	0.58	1.08
207460_at	Granzyme M	-0.09	0.03	0.69	0.81	0.94

Table 1: Analysis of gene expression in poly(I:C) treatment of NK cells.

Fold change values and associated 95% confidence intervals (degree of freedom=6) were determined for genes of interest upon treatment with poly(I:C) relative to untreated NK cells. Data for each probe set present on the Affymetrix U133 chip series corresponding to the genes of interest is shown.

In the case of genes such as TNF-alpha, FasL, IL-8 and CCL20, the low end of the confidence interval for fold-change was below 2.0. This could be attributable to donor variability, principally due to larger variability of donor #1. Attention was focused on genes thought to be potentially important effectors of NK cell function such as NCRs, death-receptor ligands, and genes implicated in the perforin-dependent cytoxicity (Table 1). Poly(I:C) treatment of NK cells did not significantly alter the expression of the mRNAs encoding the membrane-perturbing proteins perforin and granulysin, the granzymes A, B, M, or of the cell surface activating receptors NKG2D and NKp44. Expression of granzyme H and NKp30 was decreased 3-5 fold. Applicants are not currently aware of any reports that granzyme H induces cell death, and

its physiological role remains to be fully understood [Edwards et al., J. Biol.Chem., 274:30468-30473 (1999)]. In contrast, poly(I:C) treament increased the expression of Apo2L/TRAIL almost 10 fold. A smaller, but reproducible increase in the expression of mRNA for FasL and TNF-alpha, was also observed following activation of TLR3 on NK cells.

In another experiment, the purified NK cells were treated for 18 hours in RPMI 1640 supplemented with heat inactivated 10% FCS, penicillin, streptomycin, 10mM sodium pyruvate, 2mM L-glutamine and 10mM HEPES with poly(I:C) (50μg/ml), R-848 (5μg/ml) or hIFN-alpha (1000 U/ml, Sigma) in the presence or absence of 10 μg/ml cycloheximide and Apo2L/TRAIL was measured in the extracted RNA. RT-PCR was performed and the amount of cDNA was quantitated by RT-QPCR analysis, normalized to RPL19, and fold induction over control is shown in Fig. 2A. Primers were designed as per instructions (Primer Express) and sequences are: hRPL19-probe: AGGTCTAAGACCAAGGAAGCACGCAA (SEQ ID NO:7); hRPL19-For: ATGTATCACAGCCTGTACCTG (SEQ ID NO:8); hRPL19-Rev: TTCTTGGTCTCTTCCTCCTTG (SEQ ID NO:9); hApo2L-probe: CCCAATGACGAAGAGAGTATGAACAGCCC (SEQ ID NO:10); hApo2L-For: TCCAAAAGTGGCATTGCTTG (SEQ ID NO:11); hApo2L-Rev: CTGACGGAGTTGCCACTTGA (SEQ ID NO:12). cDNA was analyzed for expression of RPL19 and Apo2L/TRAIL by RT-QPCR (Applied Biosystems) as described in Zarember et al., J. Immunol., 168(2):554-61 (2002). Fold increase values are expressed as arbitrary units, relative to the calibrator gene RPL19.

The increased expression of Apo2L/TRAIL is likely to be important because Apo2L/TRAIL can induce apoptosis in a variety of cancer cell lines and 4T1 cells express DR4 receptor (data not shown). Quantitative RT-PCR analysis showed that poly(I:C) and R-848 treatments increased Apo2L/TRAIL mRNA 26 and 25 fold, respectively (see Fig. 2 A). In addition, a similar increase in Apo2L/TRAIL mRNA was observed in NK cells treated with IFN-alpha, confirming previous results reported in Biron, et al., Semin. Immunol., 10:383-390 (1998). The induction of Apo2L/TRAIL message in these stimulated cells was not blocked by the protein synthesis inhibitor cycloheximide, indicating that the elevation in Apo2L/TRAIL message is a primary effect of TLR stimulation on NK cells.

Resting and poly(I:C) stimulated NK cell lysates or their culture supernatants were assessed for the presence of Apo2L/TRAIL protein by quantitative ELISA (kit, BD Biosciences). To confirm Apo2L/TRAIL protein expression was increased following activation of NK cells with poly(I:C) or R848, an ELISA was performed. While Apo2L/TRAIL protein was not detectable in untreated cells or their supernatant, Apo2L/TRAIL protein was present in a cell-associated form in cells stimulated with poly(I:C) (Fig. 2 B). No significant levels of Apo2L/TRAIL were found to be released into supernatants from stimulated cells.

Cell surface Apo2L/TRAIL on purified NK cells treated with poly(I:C), R-848 or hIL-2 (30 ng/ml, BD Biosciences) was also analyzed by cytometry. Isotype control (IgG2a, Sigma) and anti-Apo2L/TRAIL antibody (5C2) were labeled with Alexa 488 (Molecular Probes). 5 x 10^5 cells were incubated with 2 μg of human IgG to block FcRs and 1 μg of the directly labeled mAbs at 4°C for 45 minutes, followed by 2 washes. Stained cells were analyzed using a FACScan cytometer (Becton Dickenson) and Cellquest software.

The results are illustrated in Fig. 2C. The solid line corresponds to isotype control and lighter line to the Apo2L/TRAIL staining. Results shown are a representative donor (out of three or more donors) in all three panels. R-848 or poly(I:C) treatment of cells resulted in elevated levels of membrane bound Apo2L/TRAIL protein using flow cytometry (Fig. 2 C). While Apo2L/TRAIL was not detected on the surface of untreated NK cells, treatment with poly(I:C) or R-848 resulted in detectable cell surface expression of Apo2L/TRAIL. As a positive control, IL-2 treatment of NK cells also increases the expression of cell surface Apo2L/TRAIL [Zamai et al., J. Exp. Med., 188:2375-2380 (1998)].

Example 3

Effects of anti-Apo2L Abs in blocking cytolytic activity of NK cells

Experiments were conducted which demonstrated that Apo2L/TRAIL is responsible for the enhanced activity of NK cells treated with TLR agonists. A cytotoxicity assay was performed as described in Example 1, except for the following modifications. Purified NK cells were treated with poly(I:C) or R-848 overnight and incubated with 4T1 cells. The role of TNF-alpha, FasL, and Apo2L/TRAIL was evaluated by preincubation of the NK cells with neutralizing antibodies to the respective ligands. The following neutralizing antibodies were added at final concentration of 2 μg/ml: anti-Fas-L (mIgG2b, R&D Systems), anti-TNF-alpha (mIgG1, Genentech), and anti-Apo2L/TRAIL (5C2, mIgG2a, ATCC HB-12258 and 1D1, mIgG2b; ATCC HB-12257). Isotype control antibodies were obtained from BD Biosciences. Isotype-matched mAbs were used as controls; these antibodies did not alter the lytic activity of resting or activated NK cells.

Utilizing a neutralizing antibody to Apo2L/TRAIL, the cytotoxic activity of NK cells activated with either R-848 or poly(I:C) was abolished, while antibodies to either TNF-alpha or FasL had no effect (Fig. 3 A). The cytotoxic activity of TLR-stimulated NK cells towards 4T1 cells was also blocked using DR5-Fc fusion protein, a recombinant form of the extracellular domain of DR5 that binds Apo2L/TRAIL and thereby neutralizes its function (data not shown).

Purified NK cells were treated with poly(I:C) or R-848 and incubated with B16BL10 melanoma cells (ATCC) at the indicated ratios. Purified NK cells stimulated with R-848 were incubated with HCT116 target

cells (ATCC) at the indicated ratios. The role of Apo2L/TRAIL in lysis assays in panels B and C was determined by preincubation with neutralizing (5C2) and non-neutralizing (1D1) anti-Apo2L/TRAIL mAbs. The data has been confirmed with NK cells from at least 3 donors and less that 5% SD was observed.

Unstimulated NK cells were not cytotoxic towards B16BL6 cells and exhibited limited activity on HCT116 cells at high E/T ratios (Fig. 3 B, C). In contrast, poly(I:C) or R-848 stimulated NK cells efficiently killed B16BL6 cells and neutralization of Apo2L/TRAIL completely blocked this activity (Fig. 3 B). Interestingly, while poly(I:C) (data not shown) or R-848 stimulated NK cells also displayed increased cytoxicity towards HCT116 cells, this activity was only partially blocked by neutralization of Apo2L/TRAIL activity. This suggests that TLR activation of NK cells also leads to induction of Apo2L/TRAIL-independent cytotoxic pathways which may be target cell dependent. These data demonstrate that the induction of Apo2L/TRAIL is an important step in the TLR mediated stimulation of NK cells cytotoxic activity against tumor cells.

Induction of Apo2L/TRAIL subsequent to TLR activation has previously been observed in other innate immune cells such as monocytes and DCs. However in those cases, the enhanced expression of Apo2L/TRAIL was a secondary effect. Specifically, stimulation of purified DCs by poly(I:C) purportedly lead to the secretion of IFN-beta which then induced Apo2L/TRAIL expression [Vidalain et al., J. Immunol., 167:3765-3772 (2001)]. Similar secondary effects were observed upon treatment of PBMCs with CpG oligodeoxynucleotide, wherein IFN-alpha secreted by plasmacytoid DCs lead to enhanced Apo2L/TRAIL expression by monocytes [Kemp et al., J. Immunol., 171:212-218 (2003)]. Consistent with this notion, studies involving the use of Apo2L/TRAIL deficient mice or the neutralization of Apo2L/TRAIL activity, have revealed a role for Apo2L/TRAIL expressing NK cells in controlling tumor metastasis [Cretney et al., J. Immunol., 168:1356-1361 (2002); Takeda et al., J. Exp. Med., 195:161-169 (2002)].

Example 4

Effects of combining Apo2L/TRAIL and activated NK cells on tumor cells

Utilizing a cytotoxicity assay as described in Example 1, recombinant Apo2L/TRAIL (amino acids 114-281, prepared as described in Ashkenazi et al., J. Clin. Invest., 104:155-162 (1999), was added to selected wells at a 200ng/ml concentration. For assessment of resistance of tumor cells, Apo2L/TRAIL concentrations up to 2 μg/ml were also used. The 4T1 cells were treated with a range of concentrations of the Apo2L/TRAIL protein but were resistant to lysis.

Experiments were also conducted to determine whether resting NK cells could be activated by the addition of the Apo2L/TRAIL protein. Resting NK cells (purified as described in Example 1) were incubated with the 4T1 target cells and increasing concentrations of the Apo2L/TRAIL protein. A dose dependent lysis of 4T1 cells was observed indicating that Apo2L/TRAIL can cooperate with resting NK cells. Some increase was seen in the case of activated NK cells upon addition of Apo2L/TRAIL, possibly due to sufficient amount of Apo2L/TRAIL already expressed by activated NK cells.

These results were also observed with mouse tumor cell lines, breast epithelial C57MG and melanoma origin B16BL10, wherein addition of Apo2L/TRAIL to resting NK cells increased the lysis observed (no significant increase was observed in the case of activated NK cells). Similar results were also observed wherein HCT116 cells were treated with 5 ng/ml Apo2L/TRAIL or resting NK cells, and the combination of both. The concentration of 5ng/ml of Apo2L/TRAIL was chosen since at this concentration no lysis of HCT116 cells was observed. The addition of Apo2L/TRAIL to limiting ratios of resting NK cells results in an enhanced cytotoxic effect. Therefore, combined treatment with Apo2L/TRAIL and resting NK cells may result in lysis of cancer cells that are otherwise resistant to death induced by Apo2L/TRAIL alone, or alternatively, the sensitivity of the cancer cells to Apo2L/TRAIL may be enhanced.

EXAMPLE 5

Effects of combining Apo2L/TRAIL and NK cells on tumor cells

Further assays were conducted to demonstrate or confirm the effects of Apo-2L protein and NK cells on cancer cells.

Techniques and materials:

NK cells were isolated from peripheral blood of healthy donors and NK cells were purified as described above. The NK cells were cultured in RPMI-1640 medium supplemented with heat inactivated FCS, streptomycin, penicillin, 10mM sodium pyruvate, 2mM L-glutamine and 10mM HEPES, pH 7.4. Activated NK cells were obtained by treatment for 16 hours with 5µg/ml R848 (Invivogen, dissolved at 1mg/ml in water; an activator of TLR7 and TLR8), IL-2, IL-12 and IL-15. 4T1 cells (BALB/c derived mammary carcinoma; obtained from Fred Miller, Karmanos Cancer Institute) and B16 melanoma (ATCC) cells were maintained in IDMEM and DMEM, respectively, supplemented with 10% FCS, glutamine, streptomycin and penicillin. PI9 was subcloned into pRKN-Flag, Cla and AscI sites, resulting in c-terminal tagged form of PI9. Stable cell lines of 4T1 or B16 cells were obtained following transfection with pRKN-PI9 Flag or empty vector using Polyfect. Clones were selected in medium supplemented with Geneticin (Gibco) (at 1

and 2mg/ml for 4T1 and B16 cells, respectively). Expression of PI9 was detected by anti-Flag antibody (M2 clone, Sigma).

For the cytotoxicity assays, target cells were labeled with 100mCi ^{51}Cr for 1 hour at 37°C and then washed 3 times before use. 10^4 target cells were added to varying numbers of effector cells at the indicated ratios and incubated in duplicate wells for 4 hours at 37°C, 5% CO_2, and supernatants were used to determine ^{51}Cr released using a Gamma counter. The cytotoxicity assays were performed in round bottom 96 well plates and the percent specific lysis was calculated as 100X (experimental cpm-spontaneous cpm)/(total cpm-spontaneous cpm). The role of Apo2L/TRAIL expressed by NK cells in the lysis of target cells was evaluated using anti-Apo2L/TRAIL mAbs (5C2 and 1D1, Genentech, Inc.). Isotype control antibodies were obtained from BD Biosciences. These control antibodies did not alter the lytic activity of NK cells.

In the protein analysis assays, rabbit anti- mPARP (Asp-214), caspase 3 (8G10) and cleaved active caspase-3 (Asp-175 5A1) antibodies were used as recommended by the manufacturer (Cell Signaling Technology) for immunoblotting analysis. Flag epitope antibody (M2, Sigma) was utilized for detection of Flag epitope-tagged PI9.

Total cell extracts were prepared from 4T1 cells using lysis buffer (0.1M Tris pH 8.0, 500mM NaCl, 2mM EDTA, 1% Triton X-100, 10% glycerol, supplemented with Complete Protease Inhibitor Cocktail, Roche), and the lysates were clarified by centrifugation at 16,000 rpm for 10 minutes at 4°C. Lysates were fractionated by SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were incubated with indicated antibodies followed by incubation with HRP-conjugated secondary antibodies (goat anti-rabbit, Cell Signaling Technology; and goat anti-mouse, Jackson Immunoresearch). Immune complexes were detected using enhanced chemiluminesence (Amersham).

Assay results:

The assay results are illustrated in Figs. 8-10.

The 4T1 and B16 cell lines were lysed by activated NK cells in an Apo2L/TRAIL dependent manner, while treatment of these cells with a range of concentrations of Apo2L/TRAIL protein revealed certain resistance to its apoptotic effects. Similar concentrations of Apo2L/TRAIL effectively mediated release of ^{51}Cr by HCT-116 or SKMES-1 cells, as previously reported.

Since NK cells possess cytotoxic granules that contain perforin dependent target cell killing machinery, the role of perforin and other cytotoxic granule contents in lysis of 4T1 cells was examined. The treatment of activated NK cells with a cell permeable peptide substrate inhibitor of granzyme B ("GraB") led to a reduction in lysis of 4T1 cells. In contrast, treatment with a general caspase inhibitor peptide (data not shown) did not block the release of Cr indicating specificity

of the GraB inhibitor and that membrane permeabilization is an event proximal to activation of caspases. Concanamycin A, which blocks maturation of perforin, also blocked the cytotoxic activity of NK cells. The delivery of the cytotoxic granules towards the contact zone with target cells is mediated by PI3K signaling, and inhibition of PI3K results in lack of movement of cytotoxic granules. The treatment of activated NK cells with wortmannin, an inhibitor of PI3K, also blocked the lytic activity, while the addition of other kinase inhibitors did not affect NK cell activity. These results collectively demonstrate a role of perforin-dependent pathways in lysis of 4T1 cells by activated NK cells.

4T1 cells were treated with Apo2L/TRAIL or activated NK cells, and were assessed for caspase-3 activation and production of cleaved the DNA repair enzyme, poly(ADP-ribosyl) polymerase ("PARP"), a substrate of caspase-3. Apo2L/TRAIL treatment did not result in cleavage of pro-caspase-3 and PARP, but activated NK cells possessed potent caspase-3 activation capability and PARP cleavage ability in 4T1 cells. Accordingly, the treatment of the tumor cells with activated NK cells resulted in the production of active forms of caspase-3 and PARP, events that are hallmarks of apoptotic cell death.

Since both cell surface Apo2L/TRAIL and perforin granules are essential for lysis of 4T1 cells, assays were conducted to examine if resting NK cells could be "armed" by the addition of soluble Apo-2L/TRAIL protein. There is precedence for membrane bound and soluble forms of TNF family members to display differential behavior upon application to the target cell. With the aim of corroborating the ^{51}Cr release assays, the clonogenic potential of 4T1 cells was evaluated by treatment with soluble Apo2L/TRAIL and resting NK cells. As seen in Cr release studies, the 4T1 cells treated individually with soluble Apo2L/TRAIL or resting NK cells at a ratio of 25 to 1 were not affected in terms of clonogenic capacity, however, simultaneous treatment of soluble Apo2L/TRAIL and NK cells resulted in a dramatic reduction in clones of 4T1 cells observed after 5 days. Similar reduction in the number of clones was observed in case of 4T1 cells incubated with activated NK cells. In the case of activated NK cells, the lysis of 4T1 cells was again strictly dependent on Apo2L/TRAIL activity since addition of 5C2 neutralizing antibody resulted in protection of 4T1 cell viability.

In the assays examining the effects on HCT116 cells, the treatment with soluble Apo2L/TRAIL and limiting ratios of resting NK cells resulted in an enhanced cytotoxic effect. Therefore, it is believed that the addition of Apo2L/TRAIL and resting NK cells can result in lysis of tumor cell lines that are otherwise resistant to death induced by Apo2L/TRAIL alone, or that the tumor cell sensitivity to Apo2L/TRAIL can be increased, as observed in the case of HCT116 cells.

Treatment of 4T1 cells with activated NK cells resulted in activation of caspase-3 and PARP. 4T1 cells were also treated with resting NK cells or soluble Apo2L/TRAIL, or combinations thereof. Treatment of 4T1 cells with resting NK cells did not result in the cleavage of PARP, however the combined treatment of resting NK cells along with soluble Apo2L/TRAIL resulted in the production of cleaved PARP, demonstrating the combined effects of resting NK cells and soluble Apo2L/TRAIL did result in apoptosis (consistent with observations in the above-described clonogenic and chromium release assays). It is believed that Granzyme B may be an important NK cell component in this synergistic or cooperative effect.

Deposit of Material

The following materials have been deposited with the American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110-2209, USA (ATCC):

Material	ATCC Dep. No.	Deposit Date
4H6.17.8	HB-12455	Jan. 13, 1998
3F11.39.7	HB-12456	Jan. 13, 1998
4E7.24.3	HB-12454	Jan. 13, 1998
1H5.25.9	HB-12695	April 1, 1999
4G7.18.8	PTA-99	May 21, 1999
5G11.17.1	HB-12694	April 1, 1999
3H3.14.5	HB-12534	June 2, 1998

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC '122 and the Commissioner's rules pursuant thereto (including 37 CFR '1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if a culture of the materials on deposit should die or be lost or destroyed when cultivated under suitable conditions, the materials will be promptly replaced on notification with another of the same. Availability of the deposited material is not to be construed as a license to practice the

invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written description is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the example presented herein. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

WHAT IS CLAIMED:

1. A method of enhancing apoptosis or cytotoxicity in mammalian cells comprising exposing mammalian cells to an effective amount of Apo-2 ligand receptor agonist and NK cells or NK cell activating agent(s).

2. The method of claim 1 wherein said Apo-2 ligand receptor agonist comprises Apo-2 ligand polypeptide.

3. The method of claim 1 wherein said mammalian cells are cancer cells.

4. The method of claim 1 wherein the mammalian cells are virally-infected or bacteria-infected cells.

5. The method of claim 2 wherein said Apo-2 ligand polypeptide comprises amino acids 39 to 281 of Figure 4 or a biologically active fragment thereof.

6. The method of claim 5 wherein said Apo-2 ligand polypeptide comprises amino acids 114 to 281 of Figure 4.

7. The method of claim 5 wherein said Apo-2 ligand polypeptide is linked to one or more polyethylene glycol (PEG) molecules.

8. The method of claim 1 wherein said Apo-2 ligand receptor agonist is an agonistic anti-Apo-2 ligand receptor antibody.

9. The method of claim 8 wherein said agonistic antibody comprises an anti-DR4 antibody.

10. The method of claim 8 wherein said agonistic antibody comprises an anti-DR5 antibody.

11. The method of claim 9 wherein said anti-DR4 antibody is a chimeric, humanized or human antibody.

12. The method of claim 10 wherein said anti-DR5 antibody is a chimeric, humanized or human antibody.

13. The method of claim 1 wherein said NK cells are purified NK cells from a donor mammal.

14. The method of claim 1 wherein said NK cell activating agent is selected from the group consisting of Toll receptor activating agents, IL-2, IL-12, IL-15, IFN-alpha, and IFN-beta.

15. The method of claim 1 wherein said NK cell activating agent is selected from the group consisting of agonist antibodies to activating receptors such as NKp30, NKp44, NKG2D.